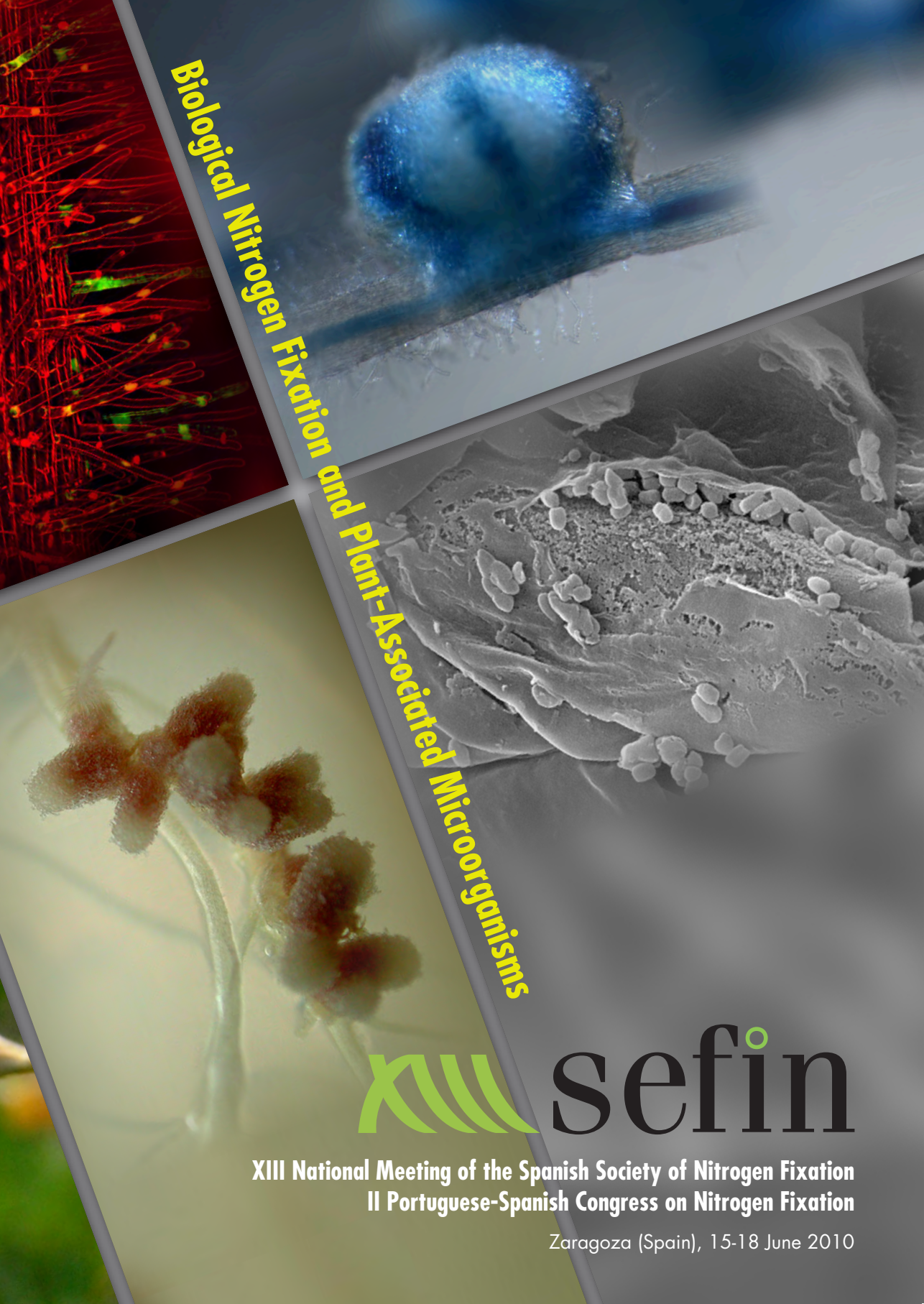

Biological Nitrogen Fixation and Plant-Associated Microorganisms

sefin

XIII National Meeting of the Spanish Society of Nitrogen Fixation
II Portuguese-Spanish Congress on Nitrogen Fixation

Zaragoza (Spain), 15-18 June 2010

Credits for photographs

1 *(front cover, upper left)* Production of hydrogen peroxide (green fluorescence) in root hairs of white clover after inoculation with rhizobia (Marta Robledo, Universidad de Salamanca).

2 *(front cover, upper right)* Nodule of *Lotus japonicus* showing promoter activity (dark blue staining) of a *nonsymbiotic hemoglobin* gene in vascular bundles (Alejandro Tovar-Méndez, Estación Experimental de Aula Dei, CSIC, Zaragoza).

3 *(front cover, lower left)* Pea nodules grown on agar medium (María Sánchez-Contreras, University of Bath, UK).

4 *(front cover, lower right)* Colonization of alfalfa roots by the biocontrol strain *Pseudomonas fluorescens* F113 (Marta Martín, Universidad Autónoma de Madrid).

5 *(back cover, upper)* Plants of *Lupinus hispanicus* inoculated with endosymbiotic bacteria isolated from *L. hispanicus* (center) or from *L. mariae-josephi* (outside circle) wild plants (Tomás Ruiz-Argüeso, Universidad Politécnica de Madrid).

6 *(back cover, lower)* Flowers and fruits of *Lotus corniculatus* (Niels Sandal, University of Aarhus, Denmark).

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Biological Nitrogen Fixation and Plant-Associated Microorganisms

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XIII National Meeting of the Spanish Society of Nitrogen Fixation
II Portuguese-Spanish Congress on Nitrogen Fixation

Zaragoza (Spain), 15-18 June 2010

Preface

This book contains the contributions presented at the XIII National Meeting of the Spanish Society of Nitrogen Fixation (SEFIN) and II Portuguese-Spanish Congress on Nitrogen Fixation, held in Zaragoza (Spain) from June 15-18, 2010. Contributions include 2 keynote lectures, 11 session conferences, 17 oral communications, and 82 posters. All the authors work in the field of biological nitrogen fixation and/or with plant-associated microorganisms (hence the title of the book), and their areas of expertise include ecology, plant physiology, agronomy, soil biology, microbiology, organic chemistry, biochemistry, and molecular and cell biology. The contributors came mostly from Spain (89) but there were also participants from Portugal and other European countries (10), as well as from Mexico and South America (10) and from Japan and the USA (3). The organizers and the SEFIN have encouraged the participation of predoctoral fellows (27%), in addition to staff and postdoctoral scientists (73%), and we can testify to the success of this initiative.

According to the instructions provided to the authors, all contributions have a title and short summary in English, whereas the rest of the text has been written optionally in English or Spanish. On behalf of the congress organizers, I would like to thank all the participants and members of the advisory and scientific committees for their invaluable help. We also hope you will find the information included in the book useful for future work, and trust that the book will provide you with a perspective of the highly varied, and yet complementary, aspects within the field of biological nitrogen fixation and other related beneficial processes for agriculture and the environment, such as the association of plants with arbuscular mycorrhizal fungi or with plant growth promoting rhizobacteria.

I would like also to take this opportunity to express our gratitude to our sponsors: Ministry of Science and Innovation (MICINN), National Institute of Agricultural and Food Research and Technology (INIA), Spanish Council for Scientific Research (CSIC), SEFIN, University of Zaragoza, Government of Aragón, Ibercaja, Bio-Rad, and ResBioAgro. Without their generous support this congress would not have been possible.

Manuel Becana
June 2010, Zaragoza

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Programme

The congress programme includes the participation of 2 plenary speakers, 10 session speakers, and 17 oral communications. Also, time is available on the last day of the congress for a round table and a general discussion of most relevant (oral or poster) contributions.

Tuesday, 15 June 2010

15:30-16:30 Registration at Paraninfo, University of Zaragoza

16:30-17:30 **Opening ceremony** (Paraninfo)

17:30-18:30 **Keynote lecture**
KL-1. **Ray Dixon.** *John Innes Centre, Norwich, UK*. Transcriptional regulation of nitrogen fixation and nitric oxide metabolism by bacterial enhancer binding proteins.

18:30-19:00 Coffee break

19:00-20:30 ***Session 1***
Ecology, diversity, and evolution of diazotrophic microorganisms
Coordinators: M. León Barrios, E. Martínez-Molina, J. Sanjuán

Session lectures

19:00-19:30 S1-L1. **Euan K. James.** *Scottish Crop Research Institute, Invergowrie, UK*. Beta-rhizobia: where do they come from and what do they nodulate?

19:30-20:00 S1-L2. **Manuel Fernández-López.** *Estación Experimental del Zaidín, Granada, Spain*. Forest rhizospheric ecosystems: prokaryotic diversity *versus* diazotrophy.

Oral communications

20:00-20:15 S1-01. **Beatriz Ruiz-Díez.** *Centro de Ciencias Medioambientales, CSIC, Madrid, Spain*. Genetic and phenotypic characterization of rhizobia from legumes grown in soils with high mercury contamination.

20:15-20:30 S1-02. **Juan Sanjuán.** *Estación Experimental del Zaidín, CSIC, Granada, Spain*. *Mesorhizobium loti* is an infrequent symbiont of *Lotus* spp.

20:30-22:00 Welcome cocktail (Gran Hotel)

Wednesday, 16 June 2010

8:30-9:00 Registration (continuation) at Gran Hotel

9:00-11:30 ***Session 2***

Genetics, genomics, and proteomics of diazotrophic microorganisms and associated plants

Coordinators: M.A. Caviedes, J. Imperial, J.E. Ruiz-Sainz

Session lectures

9:00-09:30 S2-L1. **Philippe Normand.** *Ecologie Microbienne, Université Lyon 1, Villeurbanne, France*. Genomic analysis of the actinorhizal symbiosis partners.

9:30-10:00 S2-L2. **Georgina Hernández.** *Centro de Ciencias Genómicas, Cuernavaca, Mexico*. Common bean-*Rhizobium* symbiosis: functional genomics of legume responses to abiotic stress.

Oral communications

10:00-10:15 S2-01. **José A. Hernández.** *Midwestern University, Glendale, USA*. How to bring a sensitive metal cluster to work: the role of the metallochaperone NafY from *Azotobacter vinelandii* in the biosynthesis of nitrogenase.

10:15-10:30 S2-02. **José I. Jiménez-Zurdo.** *Estación Experimental del Zaidín, CSIC, Granada, Spain*. Impact of the *Sinorhizobium meliloti* RNA chaperone Hfq in transport, metabolism and symbiosis.

10:30-10:45 S2-03. **Joaquina Nogales.** *Estación Experimental del Zaidín, CSIC, Granada, Spain*. Transcriptome profiling of a *Sinorhizobium meliloti fadD* mutant reveals the role of rhizobactin 1021 biosynthesis and regulation genes in the control of swarming.

10:45-11:00 S2-04. **Miguel Redondo-Nieto.** *Universidad Autónoma de Madrid, Spain*. Genomic sequence of *Pseudomonas fluorescens* F113. Differences with other *P. fluorescens*.

11:00-11:30 **Poster presentation (sessions 1 y 2)**

11:30-12:00 Coffee break

12:00-14:15 ***Session 3***

Plant-microorganism interactions

Coordinators: C. Azcón-Aguilar, J.A. Lucas, J. Palacios

Session lectures

12:00-12:30 S3-L1. **Federico Sánchez.** *Instituto de Biotecnología, Cuernavaca, Mexico*. Control of nodule development and programmed cell death in common bean.

12:30-13:00 S3-L2. **Peter Mergaert.** *Institut des Sciences du Végétale, Gif-sur-Yvette, France*. Role of the secretory pathway and antimicrobial peptides in the accommodation and differentiation of endosymbiotic *Rhizobium* bacteria in *Medicago* nodules.

Oral communications

13:00-13:15 S3-01. **Carmen Quinto.** *Instituto de Biotecnología, Cuernavaca, Mexico*. Deciphering the role of NADPH oxidases in bean roots after rhizobial infection.

13:15-13:30 S3-02. **Isidro Abreu.** *Universidad Autónoma de Madrid, Spain*. Infection thread development requires borate dependent *Rhizobium* polysaccharide capsule production and formation of a legume-AGP extensin-Rhamnogalacturonan II complex.

13:30-13:45 S3-03. **Emma Barahona.** *Universidad Autónoma de Madrid, Spain*. *Pseudomonas fluorescens* F113 can produce a second flagellum important for rhizosphere colonization

13:45-14:00 S3-04. **Aaron Cabrera-Asensio.** *Universidad Pública de Navarra, Pamplona, Spain*. Identification and characterization of a cambialistic superoxide dismutase from bacteroids in the cytosol of pea nodules.

14:00-14:15 **Poster presentation (session 3)**

14:15-16:00 Lunch

16:00-17:00 First Award Lecture "Antonio J. Palomares". **Raúl Rivas.** *Departamento de Microbiología y Genética, Universidad de Salamanca, Spain*. Taxonomic perspective of bacteria involved in legume symbiotic nitrogen fixation: from a free-living soil bacterium to root nodulation.

17:30-20:30 City tour (meeting point at the City Hall)

21:00 Gala dinner (Gran Hotel)

Thursday, 17 June 2010

9:00-11:45 ***Session 4***

Physiology and metabolism

Coordinators: M.J. Delgado, M. Fernández-Pascual, C. Lluch

Session lectures

9:00-9:30 S4-L1. **Toshiki Uchiumi.** *Kagoshima University, Kagoshima, Japan*. Nitric oxide and lipopolysaccharides in the *Rhizobium*-legume symbiosis.

9:30-10:00 S4-L2. **O. Mario Aguilar.** *Instituto de Bioquímica y Biología Molecular, Universidad Nacional de La Plata, Argentina*. Host genes involved in nodulation preference in common bean - *Rhizobium etli* symbiosis.

Oral communications

10:00-10:15 S4-01. **Marta Albareda.** *Universidad Politécnica de Madrid, Spain*. Differential roles of HypC and HupF proteins for hydrogenase synthesis in *Rhizobium leguminosarum*.

10:15-10:30 S4-02. **Francisco J. Palma.** *Universidad de Granada, Spain*. Role of polyols in the response of alfalfa to salt stress.

10:30-10:45 S4-03. **Cristina Sánchez.** *Estación Experimental del Zaidín, CSIC, Granada, Spain*. The role of *Bradyrhizobium japonicum nirB* gene in nitric oxide detoxification in soybean nodules.

10:45-11:00 S4-04. **Christiana E. Staudinger.** *University of Vienna, Austria*. Evidence for a differential nitrogen nutrition regulated drought stress response mechanism observed in *Medicago truncatula*.

11:00-11:30 Coffee break

11:30-11:45 **Poster presentation (session 4)**

11:45-14:00 **Poster viewing**

14:00-16:00 Lunch

16:00-18:30 ***Session 5***

Applications in agriculture and environment

Coordinators: B. Rodelas, M. Sánchez-Díaz, F. Temprano

Session lectures

16:00-16:30 S5-L1. **Jesús Caballero-Mellado.** *Centro de Ciencias Genómicas, Cuernavaca, Mexico*. Plant growth promotion, biological control, and bioremediation mechanisms in novel plant-associated nitrogen-fixing *Burkholderia* species.

16:30-17:00 S5-L2. **José Olivares.** *Estación Experimental del Zaidín, Granada, Spain*. Nitrogen fixation and climate change.

Oral communications

17:00-17:15 S5-01. **Gorka Erice.** *Universidad de Navarra, Pamplona, Spain*. Climate change affects taproot reserves of nodulated alfalfa.

17:15-17:30 S5-02. **Maximino Manzanera.** *Universidad de Granada, Spain*. Plant Growth Promoting Rhizobacteria for protection against drought.

17:30-18:00 Coffee break

18:00-18:15 S5-03. **Paula García-Fraile.** *Universidad de Salamanca, Spain*. Root colonization and growth promotion of tomato and pepper seedlings by *Rhizobium leguminosarum* isolated from peat.

18:15-18:30 **Poster presentation (session 5)**

18:30-19:30 **Poster viewing**

Friday, 18 June 2010

9:00-10:30	***General discussion and conclusions*** Coordinators: C. Arrese-Igor, J.M. Barea, I. Bonilla, M. Igual, J.J. Pueyo, T. Ruiz-Argüeso
10:30-11:30	**Keynote lecture** KL-2. **Carroll P. Vance**. *Department of Agronomy and Plant Genetics, University of Minnesota, St. Paul, USA*. Legume genomics: promise *versus* reality.
11:30-12:00	Coffee break
12:00-13:00	**General assembly of SEFIN**
13:00-13:30	Closing ceremony
13:30-15:30	Farewell cocktail (Gran Hotel)

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Session 4
Physiology and metabolism

Session 5

Applications in agriculture and environment

Keynote lectures

Transcriptional regulation of nitrogen fixation and nitric oxide metabolism by bacterial enhancer binding proteins

Dixon R*

*Department of Molecular Microbiology, John Innes Centre, Norwich NR4 7UH, UK. *ray.dixon@bbsrc.ac.uk*

Summary
Prokaryotic enhancer binding proteins (EBPs) represent a family of specialised activator proteins that interact with RNA polymerase (RNAP) containing the sigma factor σ^{54}, to activate transcription from upstream sites via DNA looping. These proteins interact with enhancer-like upstream activator sequences via a C-terminal DNA binding domain and contain a conserved central domain belonging to the AAA+ family that couples ATP hydrolysis to the activation of transcription by σ^{54}-RNAP. The activity of EBPs is highly regulated in response to environmental cues through amino-terminal regulatory modules and in some cases by interactions with other regulatory proteins. In this talk, I will describe how two different EBPs, NifA and NorR, are controlled by environmental signals in order to regulate nitrogen fixation and nitric oxide detoxification respectively.

The NifL - NifA system

In *Azotobacter vinelandii*, transcriptional activation by the NifA EBP is tightly regulated by protein-protein interactions with its partner, NifL, to integrate signals of redox, fixed nitrogen and carbon status to stringently control nitrogen fixation. Signal communication between NifL and NifA is modulated by ligand binding, redox changes and interaction with the signal transduction protein GlnK (Dixon & Kahn, 2004). The ligand 2-oxoglutarate, a key metabolic signal of the carbon status that binds to the GAF domain of NifA, plays a major role in antagonising the inhibitory influence of NifL under conditions appropriate for nitrogen fixation (Little *et al.*, 2000; Little & Dixon, 2003; Martinez-Argudo *et al.*, 2004b). We have evidence that redox and fixed nitrogen signals elicit conformational changes in NifL that enable interaction with the 2-oxoglutarate bound form of NifA (Martinez-Argudo *et al.*, 2004a; Little *et al.*, 2007). Our results suggest that redox signal relay within NifL is conveyed by changes in the quaternary structure of the amino-terminal PAS domains (Slavny *et al.*, 2010).

The NorR protein

The recently identified transcriptional activator NorR, appears to sense nitric oxide (NO) as its sole regulatory function and activates expression of genes required for NO detoxification in *Escherichia coli*. NorR possesses an amino-terminal GAF domain, which contains a mononuclear non-haem iron centre that binds a single molecule of NO (D'Autreaux *et al.*, 2005). The interaction of the GAF domain with NO relieves intramolecular repression of the activation domain (AAA+) of NorR and leads to ATP hydrolysis, which promotes transcriptional activation by σ54 RNAP holoenzyme (Tucker *et al.*, 2010). Our evidence suggests that in the absence of NO, the GAF domain represses the activity of the AAA+ domain by interacting with surface loops that contact σ^{54} during the ATP hydrolysis cycle. Thus in the uninduced state, the GAF domain of NorR, prevents the activator from making productive interactions with the σ^{54} RNAP holoenzyme.

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Legume genomics: promise *versus* reality

Vance CP*

*U.S.D.A. Agricultural Research Service, Department of Agronomy and Plant Genetics, University of Minnesota, St Paul, MN 55108, USA. *Carroll.Vance@ARS.USDA.GOV*

Summary

Discoveries through legume molecular biology and genomics have laid the foundation for understanding root nodule developmental biology, nodule nitrogen (N) and carbon (C) metabolism, and signal transduction. These advances have shown how the interaction between the legume plant and the rhizobial partner is a complex dance involving numerous partners. However, little of this fundamental information has been translated into improved N_2 fixation and plant improvement. High throughput RNA and DNA sequencing coupled with development of recombinant inbred lines (RILs), near inbred lines (NILs), and mapping of quantitative trait loci (QTLS) will allow the identification of traits that are most amenable to improving legume N_2 fixation and yield.

Introduction

Legume root nodules, the specialized organs in which symbiotic N_2 fixation (SNF) occurs, are structurally and metabolically complex organs. Their development and function depends upon coordinated gene expression between the host plant and rhizobial partner. Depending upon the symbiosis, nodule growth can be either determinate or indeterminate giving rise to elongate, corraloid, or spherical nodules, respectively. Within nodule cells the bacterial partner is enclosed by a host plant derived membrane termed the symbiosome membrane. The symbiosome membrane separates the host plant cytosol from the bacterial partner but yet allows the selective transport of molecules between the two organisms. Root nodules also differ in principal N assimilation and transport products. Legume species with indeterminate root nodules, such as *Medicago truncatula* and pea (*Pisum sativum*), generally assimilate and transport symbiotically fixed N as amides. By contrast, most legume species having determinate nodules, such as common bean (*Phaseolus vulgaris*) and soybean (*Glycine max*), assimilate and transport N as ureides. Adding to the complexity is that most metabolic processes in nodules related to SNF take place at a reduced oxygen concentration of 10 μM which is needed to protect the rhizobial enzyme nitrogenase from inactivation. Basically, N_2-fixing symbiotic root nodules of legumes are model factories for C and N metabolism housed in an oxygen limited structure which is adapted for rapid transport of small molecules. Although nodules comprise less than 5% of legume total biomass, they can catalyze the reduction of more than 100 kg N hectare each year. Globally fixing 45-60 Tg N_2 each year is astounding because only a few kgs of rhizobial nitrogenase are involved in catalysis. Although root nodules comprise only a small proportion of plant weight, they consume between 13-28% of legume total photosynthate. It is therefore strikingly apparent that integration of metabolism, transport of nutrients, and protection of the rhizobial partner from oxygen create a labyrinth of biochemical and genetic interactions.

Nodule molecular biology

Until the mid-1980s, research on legume plant aspects of SNF was mainly observational and descriptive focusing on: root nodule development; impact of abiotic stress on root nodule development and function; physiology and biochemistry of the process; and plant selection for

traits related to SNF. Plant research on SNF from the mid-1980s to 2000 took on a molecular flavor with the characterization of numerous plant genes expressed during the nodulation process. Genes having expression primarily in nodules were referred to as nodulins and categorized as either early or late nodulins depending upon when in the nodulation process they were first detected. Genes characterized as nodulins generally encoded proteins that were involved in metabolism, membrane function, and cell wall formation. In the pre-genomics era published studies usually considered the characterization, expression, and localization of a single individual gene.

Realizing that a fundamental understanding of the legume plant contribution to SNF would require tractable genetic species and a whole genome systems approach, *Medicago truncatula* and *Lotus japonicus* were proposed as model species to investigate the genomics of symbiosis. Both species are diploid, self-compatible, transformable, and have small genomes. Acceptance of these species as model systems moved research on plant regulation of SNF into the genomic era and have defined the underpinnings of root nodule signal transduction. More recently, the sequencing of the soybean genome and soybean transformation have made this species an attractive system for genomic studies even though it has a large genome and is polyploid. Between 2000 and 2009, more than 600 scientific papers were published on legume genomics and genetics, many of which relate to SNF root nodule development and function. Through a combination of whole genome sequencing, comparative gene order and structure, expressed sequence tag (EST) discovery, proteomics, metabolomics, forward and reverse genetics, and bioinformatics, our knowledge and understanding of the plant contribution to SNF has skyrocketed.

The way forward

Because genome sequencing, transcript profiling, proteome analysis, metabolite profiling, mutant analysis, and comparative genomics have progressed at a logarithmic pace, we know more about the plant genes involved in SNF than could have been imagined a decade ago. However, we have only scratched the surface of developing a systems understanding of the process. In addition, little SNF genomics information has been translated into improvement of legume crops. We are currently challenged in integrating genomics information into coherent molecular biochemical models that define how to improve SNF. A long-standing goal of plant genomics, beyond a fundamental understanding of gene action and control, has been to use the information gleaned through genomic research into crop yield and quality gains. Although a wide array of single gene traits have been improved through molecular approaches, that list is much smaller for quantitative trait loci (QTLs) such as SNF. However, in recent years combined genomic approaches have revealed that some traits thought to be controlled by QTLs are defined predominantly by single genes. For example, sugar yield in tomatoes, grain productivity in rice, and carotenoids in maize. Definition of these complex traits required collaborations and integration between geneticists, biochemists, physiologists, bioinformaticians, agronomists, and plant breeders. The gen-, prote-, metabol-, transcript-omics disciplines are large and multidisciplinary and will give illuminating insights into legume crop production and SNF. To achieve this perspicacity and improve human well-being through food security will require exceptional communication as well as exceptional science.

Session 1

Ecology, diversity, and evolution of diazotrophic microorganisms

Beta-rhizobia: where do they come from and what do they nodulate?

James EK*

*EPI Division, Scottish Crop Research Institute, Invergowrie, Dundee DD2 5DA, UK. *euan.james@scri.ac.uk*

Summary

Nodulation in legumes occurs not only in symbiosis with members of the Class Alphaproteobacteria (including *Rhizobium*), but also with several members of the Betaproteobacteria. This has been most conclusively established in the large legume genus *Mimosa* (sub-family Mimosoideae), which contains >500 species, most of which are native to South and Central America. Indeed, several *Burkholderia* species/strains have been isolated from native and invasive *Mimosa* spp. in the tropics, but most have been isolated from species in the seasonally dry Cerrado and Caatinga biomes of Brazil, which are the main centres of diversity of *Mimosa*, thus suggesting long-standing (*c.* 50 mya) co-evolution of *Mimosa* with *Burkholderia*. *Cupriavidus taiwanensis* (a relative of *Burkholderia* previously named *Ralstonia taiwanensis*) has also been frequently isolated from non-native invasive *Mimosa* species in Asia (especially *M. diplotricha* and *M. pudica*), but although it can be locally common as a symbiont when soil conditions are suitable it can be easiliy out-competed by *Burkholderia* strains for nodulation of *Mimosa* if pH and/or soil %N are reduced. Some *Mimosa*-nodulating *Burkholderia* strains (eg. *B. phymatum* STM815) are capable of effectively nodulating other Mimosoid legumes, such as those in the Piptadenia Group in the tribe Mimosae, as can some *Acacia* (s.s.) species. A separate group of *Burkholderia* strains related to the South African strain *B. tuberum* STM678 have very different nodulation genes from the *Mimosa*-nodulating Beta-rhizoba, and hence also have a very different host range. These *B. tuberum*-type symbionts cannot nodulate *Mimosa* or any Mimosoids, but instead can nodulate legumes in some tribes in the sub-family Papilionoideae, most notably those in the South African tribe Podalyreae, such as *Cyclopia*, which are native to the Cape region.

What are Beta-rhizobia?

Until recently, "rhizobia" were considered to consist of a limited number of genera in the family Rhizobiales in the Alpha-Proteobacterieaceae, but a considerable body of evidence has accumulated to show that legumes are not nodulated exclusively by members of the Rhizobiaceae in the α-Proteobacteria class, but may also form effective nodules with members of the β-Proteobacteria class, the so-called "Beta-rhizobia". All Beta-rhizobia that have been confirmed to nodulate legumes are in the genera *Burkholderia* or *Cupriavidus*. *Burkholderia* is a large genus comprising many species. It includes plant and animal pathogens, but most of these are in the "*B. cepacia* complex", which is phylogenetically distinct from the "environmental" *Burkholderias*, which include most diazotrophic species and all the confirmed legume-nodulating strains (Bontemps *et al.,* 2010). The closely-related genus *Cupriavidus* is much smaller, and contains only one confirmed diazotrophic/nodulating species, *C. taiwanensis*, although other nodulating species may yet be described (Elliott *et al.,* 2007a, 2009).

A particular affinity for *Mimosa*

First reports of Beta-rhizobia came from *C. taiwanensis* strains isolated from the invasive weed *Mimosa pudica* in Taiwan, which were subsequently shown to be able to nodulate a range of *Mimosa* spp. (Elliott *et al.,* 2007a). In parallel, *Burkholderia* strains from South America and Taiwan were also shown to be able to nodulate several *Mimosa* spp. (Elliott *et al.,* 2007a, 2009). However, although it appeared from this limited number of strains that Beta-rhizobia were genuinely symbiotic, particularly with *Mimosa*, the low number of hosts from which they had been isolated meant that the diversity of beta-proteobacteria known to fix nitrogen in legume root nodules was still very limited compared to that of alpha-

proteobacterial symbionts and, moreover, little was known about their ability to fix N_2 in their native environments. Therefore, a survey of nodulation in the genus *Mimosa* was undertaken in major centres of diversity of the genus: the Cerrado, a savannah region in central Brazil which is typified by acidic and Al-rich soils, and the Caatinga, a semi-arid region in the North East of Brazil with acidic, Fe-rich soils. Nodules were collected from 65 of the 68 *Mimosa* spp. found, and 45 of the species, including all endemics, were newly reported as nodulating (dos Reis Junior *et al.,* 2010). All but two of the 144 confirmed symbionts obtained from these were *Burkholderia*, and they could be divided into seven clades defined by their 16S rRNA and *recA* gene sequences, and these represented distinct and divergent species complexes. Their symbiosis-related genes (*nodC, nifH*) formed deep *Burkholderia*-specific clades within their respective phylogenies, and the same groups were identified as for 16S and *recA*, implying that these genes have diverged within *Burkholderia* without substantial horizontal gene transfer between species complexes (Bontemps *et al.,* 2010). In parallel, the endosymbiotic bacteria in nodules from 47 species were identified *in situ* as *Burkholderia* using an antibody against *B. phymatum* STM815 (Figure, **A**), and both the $\delta^{15}N$ technique and *in situ* immunolocalisation with an antibody against nitrogenase (Figure, **B**) gave clear evidence for effective symbiotic N_2 fixation by most of the species examined (dos Reis Junior *et al.,* 2010).

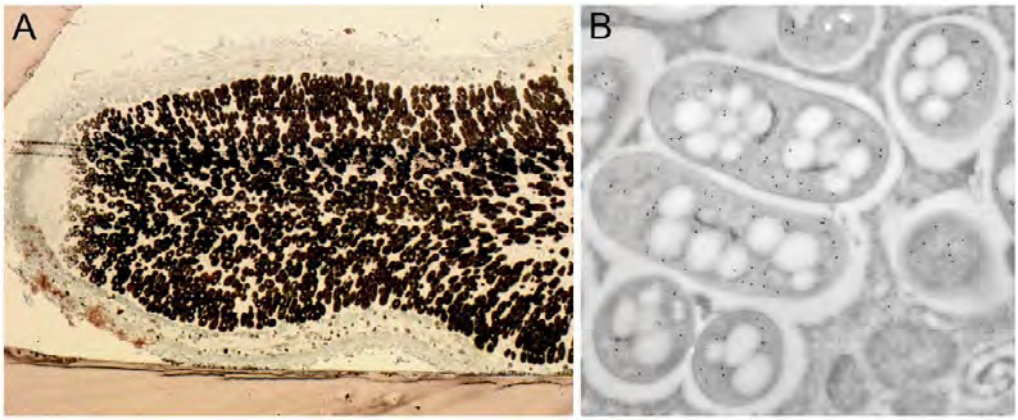

Figure. A. Light micrograph of a *Mimosa* nodule from the Brazilian Cerrado that has been immunogold labeled with an antibody against *Burkholderia phymatum*. **B.** Transmission electron micrograph of bacteroids from a *M. caesalpiniifolia* nodule that has been immunogold labeled with an antibody against nitrogenase.

Can they compete with *Rhizobium* and other alpha-rhizobia for nodulation?

Competition studies have been performed between *Mimosa*-nodulating α-proteobacteria (*R. etli* TJ167, *R. tropici* NGR181 and UPRM8021) and two of the β-rhizobial symbionts (*B. mimosarum* PAS44 and *C. taiwanensis* LMG19424) for nodulation of these invasive *Mimosa* species (Elliott *et al.,* 2009). Under all conditions, *B. mimosarum* out-competed *C. taiwanensis* and all three α-proteobacteria to the point of exclusion, but the competitive domination of *B. mimosarum* over *C. taiwanensis* was reduced in the presence of nitrate for all three plant hosts, with the largest significant effect on *M. pudica*, in which *C. taiwanensis* formed 57% of the nodules in the presence of 0.5 mM KNO_3. These data show that in low nutrient acidic soils *Burkholderia* will most likely be the preferred symbionts of endemic *Mimosa* spp. (and possibly other legumes that have evolved to live in low N, acidic soils). However, other rhizobia (alpha and beta-) can form symbioses with invasive *Mimosa* species, such as *M. pudica*, which are adapted to a wider range of soil types, but only if soil N and pH

What else can they nodulate?

Burkholderia phymatum STM815 and *C. taiwanensis* LMG19424, which have previously been shown to effectively nodulate several species in the large genus *Mimosa* (Elliott *et al.*, 2007a; dos Reis Junior *et al.,* 2010) were tested for their ability to nodulate 68 species from a diverse set of lineages scattered across the legume sub-family Mimosoideae. *Cupriavidus taiwanensis* could nodulate only one species effectively (*Leucaena multicapitula*), whereas *B. phymatum* effectively nodulated 24 species, particularly those closely related to *Mimosa* in the "Piptadenia Group", as well as several *Acacia* spp. in the sub-genus *Acacia*, which is phylogenetically close to the Mimoseae. It is concluded that *C. taiwanensis* LMG19424 can be a highly promiscuous and opportunistic colonizer of mimosoid legumes, but is rarely capable of forming a symbiotic relationship outside the genus *Mimosa*, whereas *B. phymatum* STM815 can form symbioses with a range of disparate mimosoid legumes, thus inviting comparisons with other broad host range rhizobia, such as *Sinorhizobium* sp. NGR234. In parallel, *Burkholderia tuberum* STM678, a nodulator of *Cyclopia* with very different nod genes to the other Beta-rhizobia (Elliott *et al.,* 2007b), was tested for its ability to nodulate a range of legumes in the papilionoid tribe Podalyriae; it effectively nodulated three species of *Podalyria* and one species of *Virgilia*, but failed to nodulate any members of the sub-family mimosoideae.

Concluding remarks and further work

(1) Beta-rhizobia are the preferred symbionts of *Mimosa* and have co-evolved with them over a very long period (*c.* 50 mya).

(2) They are highly effective as symbionts, with *Burkholderia* strains being the most competitive for nodulation of *Mimosa* under N-limited conditions.

(3) Although they are particularly associated with *Mimosa* and some related genera, they can also nodulate some Papilionoid legumes, so what are the genetical, taxonomic and geographical factors underlying the ability (or preference) of particular legumes to nodulate with Beta-rhizobia?

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Forest rhizospheric ecosystems: prokaryotic diversity *versus* diazotrophy

Fernández-López M*, Fernández-González AJ, Cobo-Díaz JF, Villadas PJ, Toro N

*Grupo de Ecología Genética de la Rizosfera, Departamento de Microbiología del Suelo y Sistemas Simbióticos, Estación Experimental del Zaidín, CSIC, Apartado 419, 18080 Granada, Spain. *manuel.fernandez@eez.csic.es*

Summary

The study of the prokaryotic diversity is under a revolutionary change. From the classical approach of bacterial morphology through the construction of gene libraries, the concept of 'prokaryotic diversity' could be currently included in the term "metagenome". This term is used to describe technologies and approaches in which the sequence and function of genes from ecosystems are obtained, without the cultivation of the microorganisms. This pool of sequenced genes also include those genes with taxonomic and phylogenetic value, as it is the case of ribosomal genes. The arrival of next-generation sequencing (NGS) technologies has allowed the majority of research groups to develop their own sequencing projects: the 'democratization' of the sequencing technologies. Nowadays the in-depth study of complex ecosystems is an affordable work, since it is possible to get thousands of reads in a short period of time and at relatively low cost. In this way, we are studying the rhizosphere of *Quercus* trees with different needs for water, the holm-oak (*Q. ilex ballota*) and the melojo oak (*Q. pyrenaica*). In the Mediterranean basin the predicted effects of climate change (increased temperature and decreased rainfall) will generate the migratory movement, latitudinal and/or altitudinal, of plant species. The study of the prokaryotic communities could reflect or predict this phenomenon.

Históricamente los procariotas se han clasificado en función de aspectos tales como la estructura de la célula, su metabolismo o las diferencias de sus componentes celulares. En los años 70 se introdujo la hibridación ADN-ADN como criterio para diferenciar entre especies, y sólo aquellos aislados con una homología de ADN superior al 70% se consideraron pertenecientes a la misma especie. Posteriormente los avances en técnicas moleculares como la clonación (años 80) o la reacción en cadena de la polimerasa (PCR, años 90) permitieron la introducción de otros marcadores, siendo el paradigma de los mismos el gen 16S *rRNA*. La introducción de estas técnicas moleculares permitió realizar estimaciones realistas sobre la diversidad de microorganismos presentes en el suelo. Constituyendo ya un tópico citar lo mucho que desconocemos, dejando más claro aún lo escaso de nuestro conocimiento en este campo. Así se ha citado ampliamente que sólo hemos sido capaces de cultivar el 1% del total de microorganismos; o que en 1 gramo de suelo podemos encontrar entre 10^3 y 10^4 especies diferentes, representando una abundancia total de 10^9 procariotas por gramo de suelo (Gans *et al.,*2005; Torsvik *et al.,* 1990).

A comienzos de esta década se realizó un gran esfuerzo para obtener la secuencia completa de genomas procarióticos, dando lugar a una época de desarrollos genómicos. Así el concepto de diversidad microbiana se ha visto enriquecido por otros nuevos como el de pan-genoma, core-genome (genoma compartido) o el de genes accesorios. La comparación de los genomas de cepas de una misma especie ha mostrado que pueden compartir tan sólo el 40% de sus genes (core-genome). De gran importancia para el estudio de la diversidad procariótica de distintos ecosistemas fue el desarrollo del concepto de metagenoma, aplicado al conjunto total de genes presente en un ambiente determinado (Rondon *et al.,* 2000). Además, el desarrollo vertiginoso de nuevas técnicas de secuenciación masiva o ultrasecuenciación, la llamada next-generation sequencing (NGS) technology, nos está llevando a una nueva era: la post-genómica (Medini *et al.,* 2008). Estos nuevos métodos de secuenciación basados en distintas técnicas y puestos a punto por diferentes compañías, tienen en común su bajo coste y su alto rendimiento: el gran número de lecturas que ofrecen en un periodo corto de tiempo.

Así la pirosecuenciación 454 Titanium de Roche permite obtener 1 millón de lecturas de 400 bp, la secuenciación por síntesis SOLEXA de Ilumina nos ofrece 30 millones de lecturas de 50 bp o la secuenciación por ligación SOLID rinde 171 millones de lecturas de 35 bp a 50 bp (MacLean *et al.,* 2009). La aplicación de NGS lleva aparejado el uso de herramientas bioinformáticas y estadísticas que nos permitan manejar la enorme cantidad de información que generan y que al mismo tiempo nos den garantía de las conclusiones alcanzadas. Estos nuevos avances, junto con la aproximación metagenómica hacen que hoy en día podamos abordar estudios como el de la distribución ambiental de los taxones procarióticos (Tamanes *et al.,* 2010) en cualquier ecosistema del planeta. En este último trabajo, sobre 2.281 muestreos para estimar la diversidad procariótica, 732 se llevaron a cabo en ecosistemas terrestres, de los que 584 corresponden a suelo y de éstos, 63 muestreos se realizaron en suelos de bosques, habiéndose obtenido un total de 7880 secuencias del gen 16S *rRNA*.

Donde las condiciones ambientales lo permiten, los bosques son la etapa clímax en la sucesión de un ecosistema, presentando al mismo tiempo la menor diversidad de especies y la mayor especialización de las mismas. Esto hace que pequeños cambios ambientales puedan tener un gran impacto sobre estas formaciones. Si consideramos un escenario de cambio climático como el que se prevé para la zona mediterránea, con una mayor temperatura y una menor precipitación, es claro que las especies vegetales no adaptadas a estas nuevas condiciones tendrán que migrar en latitud y/o altitud. A su vez las interacciones que se establecen con los microorganismos de su rizosfera también se verán afectadas. Se ha descrito que una mayor concentración de CO_2 en la atmósfera puede resultar en una mayor incorporación del mismo por parte de las plantas, pero al mismo tiempo se incrementa también el CO_2 que se libera de nuevo a la atmósfera desde la rizosfera (Carney *et al.,* 2007). A su vez, esta mayor concentración de CO_2 atmosférico, hace que se modifiquen las poblaciones de microorganismos del suelo, detectándose estas variaciones incluso al nivel de phylum (Castro *et al.,* 2010). Los microorganismos desempeñan un papel fundamental en el cierre de los ciclos biogeoquímicos de los elementos, contribuyendo al mantenimiento de los flujos de materia y energía en los ecosistemas. Y además pueden tener unos efectos beneficiosos sobre las plantas promoviendo su desarrollo o protegiéndolas frente al ataque de patógenos. Por ello las modificaciones en la composición de las comunidades microbianas pueden tener en efecto nefasto sobre el ecosistema en su conjunto. Uno de los elementos más importante para el desarrollo de los vegetales es el Nitrógeno (N), ya que se ha descrito que, junto con el agua, es el factor que más comúnmente limita el desarrollo de las plantas. Así las comunidades de microorganismos procarióticos diazotrofos son esenciales para la incorporación de este elemento al ecosistema y así compensar las pérdidas por desnitrificación, lixiviación,…

En nuestro grupo de investigación estamos trabajando tanto en re-vegetación de zonas boscosas degradadas (incendios) como en la recuperación de las formaciones autóctonas tipo encinares y robledales (*Quercus ilex ballota* y *Q. pyrenaica*) en un contexto de cambio climático. Así, se ha estudiado la diversidad procariótica de la rizosfera del roble melojo, o rebollo, a diferentes altitudes en la cara Sur de Sierra Nevada, bajo la influencia de la humedad del mar, y al mismo tiempo la de los encinares de la misma zona que sufrieron un incendio forestal en septiembre de 2005. En concreto las zonas de estudio han sido el robledal de Cañar a 1.823 m. y 1.482 m de altitud (CNA y CNB, respectivamente) y la zona de incendio del Cortijo de Tello en Lanjarón a 1.566 m. (encinas quemadas) y 1.790 m. (encinas no quemadas, LJQ y LJN respectivamente). Esta aproximación nos debe de mostrar si hay diferencias en la rizosfera de roble a distintas altitudes que nos permitan identificar bioindicadores procarióticos para seguimiento de cambio climático, y de igual manera si es posible detectar la transición robledal-encinar. La determinación de la diversidad procariótica

se ha basado en la aplicación de distintas técnicas: por una parte la construcción de genotecas, 3 por cada zona de estudio, del gen 16S *rRNA*, y posterior secuenciación completa de 70 clones de cada zona y por otra parte la secuenciación de 50 clones de cada zona para el estudio de la diversidad de fijación de N mediante la amplificación del gen *nif*H. La segunda aproximación ha consistido en la utilización de técnicas NGS para la pirosecuenciación tanto de amplicones como del ADN ambiental total obtenido en cada una de las localizaciones.

Los resultados obtenidos se han analizado con distintos programas informáticos, y comparando con las bases de datos del Ribosomal Database Project II, versión 10 y con las de NCBI. Estos resultados nos indican que los Phylum más abundantes son, por orden, Proteobacteria, Actinobacteria, Bacteroidetes, Planctomycetes y Acidobacteria, aunque hay diferencias significativas respecto a la abundancia de cada uno de ellos en cada zona. Las diferencias que se observan son tanto a nivel de phylum como a nivel de orden o familia. Dada la profundidad del análisis metagenómico (en torno a 12.000 lecturas de amplicones de cada localización) podemos determinar la existencia de un mínimo de 2.500 OTUs por zona y que las diferencias se manifiestan más a nivel cuantitativo que cualitativo, no existiendo especies propias de una zona particular. El detalle y las implicaciones de estos resultados se discutirá en la presentación.

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Taxonomic perspective of bacteria involved in legume symbiotic nitrogen fixation: from a free-living soil bacterium to root nodulation

Rivas R*

*Departamento de Microbiología y Genética, Universidad de Salamanca, 37007 Salamanca, Spain. *raulrg@usal.es*

Summary

The microorganisms able to establish nitrogen-fixing symbiosis with legumes were discovered in the 19th century. Over the years, the term 'rhizobia' has become widely used for all the bacteria that are capable of nodulation and nitrogen fixation in association with legumes. In spite of the taxonomic deficiencies existent in the group of rhizobia, its taxonomic situation began to change in the 80's, and rhizobia taxonomy has changed considerably over the last 30 years. The analysis of different phylogenetic markers, from the 16S rRNA gene to the ITS and housekeeping genes, has allowed a breakthrough in rhizobial taxonomy as it is the description of species unable to nodulate legumes. Recently, several non-rhizobial species belonging to alpha and beta subgroup of Proteobacteria have been identified as nitrogen-fixing legume symbionts.

Los microorganismos capaces de establecer simbiosis fijadoras de nitrógeno con leguminosas fueron descritos a finales del siglo XIX por Frank, que comprobó la nodulación de *Pisum* y *Vicia* por una especie de bacteria a la que denominó *Rhizobium leguminosarum*. Esta simbiosis, se ha venido estudiando desde hace más de 100 años como un claro ejemplo de las asociaciones mutualistas, siendo el resultado de millones de años de co-evolución de un proceso complejo y exquisitamente regulado que desempeña un papel crucial, ya que ofrece la capacidad de convertir nitrógeno molecular atmosférico en formas utilizables por la planta, en un proceso denominado fijación biológica de nitrógeno. Desde el momento de la notificación de esta relación simbiótica, el término rhizobia y en ocasiones *Rhizobium* ha sido el nombre general que se ha dado al grupo filogenéticamente diferente de bacterias del suelo que son capaces de inducir la formación de nódulos en las raíces o tallos de las leguminosas y llevar a cabo la fijación de nitrógeno. En realidad, actualmente los rizobia son un grupo de bacterias muy diversas desde el punto de vista taxonómico, metabólico y genético debido a que en los últimos años el número de especies descritas se ha incrementado rápidamente y la clasificación se ha vuelto más compleja.

Al principio, la descripción de las nuevas especies de rhizobia se basaba principalmente en la especie de leguminosa que nodulaba la bacteria, lo que condujo a la definición de los grupos de nodulación cruzada, basados en la especificidad de nodulación después de realizar experimentos de infectividad en varias leguminosas. La descripción de estas especies se registró en el Manual de Bergey que ha jugado un papel fundamental en la taxonomía de los rhizobia, ya que registró la historia de las diversas especies de estos microorganismos que iban apareciendo desde el principio de la Bacteriología hasta 1980 en que fueron oficialmente validadas en la revista International Journal of Systematic Bacteriology (IJSB) e incluidas en las listas por Skerman *et al*. (1980). El empleo de las especies simbióticas cómo criterio de clasificación obstaculizó el desarrollo de la taxonomía de los rhizobia durante décadas ya que, en este momento sabemos que muchos factores determinantes para la simbiosis están localizados en plásmidos cuya naturaleza autoconjugativa los inhabilita cómo herramientas taxonómicas. En la década de 1980 la situación comenzó a cambiar al aplicar un mayor número de criterios fenotípicos y moleculares que revolucionaron la clasificación, dando

lugar a la descripción de nuevos taxones cómo *Bradyrhizobium, Sinorhizobium Phyllobacterium* y *Azorhizobium*. Aunque en el año 1984, Woese propuso la clasificación de las bacterias sobre la base del gen ribosómico 16S que permitía una clasificación filogenética de las bacterias con independencia de sus características fenotípicas o simbióticas y que incluía a los rhizobia dentro de la subdivisión alfa de Proteobacteria, no fue hasta 1991 cuando la secuencia de este gen se incluyó cómo criterio esencial, junto con la hibridación ADN-ADN, en la descripción de nuevas especies de rhizobia. Algunos años más tarde, el análisis de perfiles de LMW RNA de los miembros de la familia *Rhizobiaceae* apoyó la separación de los géneros *Mesorhizobium*, *Rhizobium* y *Bradyrhizobium* (Velázquez *et al*., 1998).

A pesar que, en la actualidad la clasificación de las bacterias está basada en el gen ribosómico 16S, y de la importancia que ha supuesto la aplicación de este gen en la taxonomía de los rhizobia, apoyando la descripción de especies sin capacidad noduladora cómo *Bradyrhizobium betae* (Rivas *et al*., 2004), lo cierto es que en ocasiones presenta ciertas limitaciones para diferenciar entre especies. Por esta razón, en los últimos años se ha propuesto para la identificación y análisis filogenético en determinados grupos de rhizobia, la utilización de algunos genes (*housekeeping genes*) esenciales en la viabilidad de la célula (Rivas *et al*., 2009). Del mismo modo, el espacio intergénico situado entre el gen ribosómico 16S y el gen ribosómico 23S (ITS), ha demostrado ser de utilidad en la taxonomía de algunos grupos de rhizobia (Kwon *et al*., 2005). A los problemas ocasionados por la falta de un criterio consensuado a lo largo de la historia de la taxonomía de los rhizobia debemos unir el hecho real, de que en ocasiones no se han tenido en cuenta las normas generales de la nomenclatura, fomentando situaciones indeseables dentro de la familia *Rhizobiaceae* (Judicial Commission of the International Committee on Systematics of Prokaryotes, 2008).

Por último, cabe señalar que durante más de un siglo se pensó que los rhizobia eran las únicas bacterias capaces de originar nódulos en raíces de leguminosas. Sin embargo, en los últimos ocho años, se ha documentado la formación de nódulos y la fijación de nitrógeno en raíces de leguminosas por diversos géneros de bacterias tales como *Methylobacterium, Blastobacter, Devosia, Ochrobactrum, Phyllobacterium, Burkholderia* y *Cupriavidus,* pertenecientes a las subdivisiones alfa y beta de Proteobacteria y filogenéticamente alejados de los habituales rhizobia. Esta situación reafirma que es un error común en la taxonomía de los rhizobia el uso de los síntomas en la planta cómo criterio para la clasificación e identificación de los aislados, y que los genes simbióticos pueden ser útiles para estudios de biogeografía o para definir biovares dentro de las especies de rhizobia, pero no como marcadores taxonómicos ya que los resultados obtenidos demuestran la existencia de eventos de transferencia horizontal genética de los elementos simbióticos, probablemente ocasionados cómo consecuencia de una selección natural a los cambios adaptativos pudiendo darse el caso de que un patógeno vegetal se convierta en un simbionte de leguminosas (Marchetti *et al*., 2010).

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Genetic and phenotypic characterization of rhizobia from legumes grown in soils with high mercury contamination

Ruiz-Díez B[1*], Fajardo S[1], Quiñones MA[1], López MA[2], Higueras PL[1], de Felipe MR[1], Fernández-Pascual M[1]

*[1]Departamento de Fisiología y Ecología Vegetal, Centro de Ciencias Medioambientales, CSIC, 28006 Madrid, Spain; [2]Departamento de Ingeniería Geológica y Minera. Escuela Universitaria Politécnica de Almadén, Universidad de Castilla-La Mancha, 13400 Almadén, Ciudad Real, Spain.*beatriz.ruiz@ccma.csic.es*

Summary

In order to study the biodiversity of rhizobia in mercury (Hg) contaminated areas, soil samples and native legumes were collected from 3 zones: San Quintín, Almadenejos and Las Cuevas (Ciudad Real, Spain). We have identified 19 strains of rhizobia from 5 different plant species able to survive in areas contaminated with Hg. The phenotypic characterization has allowed us to classify the bacteria in relation to Hg tolerance and to identify Hg-tolerant strains. This represents a very important finding because, up to date; no rhizobia strains have been identified with this characteristic. Minimal inhibitory concentrations (MICs) of more resistant strains are in the range of 12.5-30 μM of Cl_2Hg which is significantly high as compared to the standard values. The degree of toxicity of Hg on these strains, as measured by the effective concentration that produces 50% mortality, has permitted to define the concentrations of mercury to be used in trials with these bacteria. Genetic analyses of the 16S ribosomal subunit rDNA (ARDRA and direct sequencing) have determined that there are 3 strains of *Rhizobium leguminosarum* bv. *viciae*, 2 of *Bradyrhizobium canariense*, 7 of *R. leguminosarum* bv. *trifolii*, 6 of *Ensifer (Sinorhizobium) medicae* and 1 of *R. radiobacter* (*Agrobacterium tumefaciens*).

Introducción

Los efectos derivados de la contaminación del suelo por metales pesados son muy diversos, afectando directamente a la calidad de las aguas y el medio natural y constituyendo un grave peligro para salud humana y ambiental. Este es el caso del Distrito minero de Almadén (Ciudad Real), donde la presencia del mayor yacimiento de cinabrio (HgS) del mundo y su explotación durante siglos para la obtención de Hg ha provocado la dispersión de este metal y es reconocido como uno de los lugares del mundo mas contaminados por Hg (Molina *et al.,* 2006). Se han realizado diversos estudios sobre el efecto y la distribución de Hg en suelos y su contenido en plantas de Almadén (Higueras *et al.,* 2003; Molina *et al.,* 2006) pero no se conocen datos de cómo afecta la contaminación por Hg a la diversidad de los microorganismos del suelo, los cuales son esenciales para llevar a cabo los ciclos biogeoquímicos de los elementos mas importantes. Dentro de dichos microorganismos son especialmente relevantes en el ciclo del nitrógeno las bacterias fijadoras de nitrógeno, entre las que destacan las pertenecientes a diferentes géneros de la familia Rhizobiaceae, que generalizamos como rizobios. El objetivo del presente trabajo es determinar cómo afecta la contaminación por Hg a los rizobios del suelo.

Materiales y Métodos

Los aislamientos de rizobios se han realizado a partir de nódulos de leguminosas recogidas en las zonas citadas. La morfología de las colonias fue evaluada en medio Vincent sólido a (28ºC). Además, para la caracterización fenotípica se evaluó la velocidad de crecimiento, el rango de temperatura, el pH y el tipo de flagelación. La caracterización genetica previa se realizó por amplificación de la subunidad 16S del rDNA, análisis de restricción y secuenciación directa (Ruiz-Díez *et al.,* 2009). La tolerancia al Hg se ha analizado por macrodilución añadiendo al medio Vincent líquido un rango de concentraciones de Cl_2Hg de 0-50 μM y por medida espectrofotométrica del crecimiento. Se determinó la concentración mínima inhibitoria (CMI) de metal que se define como la concentración de Hg que produce una inhibición del 90% o más del crecimiento bacteriano (Ruiz-Díez *et al.,* 2009) y la concentracion efectiva (CE50) que es la que produce una mortalidad del 50%.

Resultados y Discusión

Todas las cepas aisladas (Tabla) han sido identificadas como rizobios en base al análisis del 16S rDNA. La variabilidad a nivel de especie comprendió: *Rhizobium leguminosarum* (10 cepas, de las que se muestran 7), 6 pertenecen a *Sinorhizobium medicae*, 2 a *Bradyrhizobium canariense* y una a *R. radiobacter*. Las pruebas de sensibilidad al Hg (Tabla) nos han permitido clasificar las cepas aisladas en tres grupos: sensibles (CMI 3 ó < 3 μM), intermedias (6-12,5 μM) y tolerantes al Hg (12,5-30 μM). Este grupo es el más numeroso (11 cepas) y supera todos los niveles de las cepas empleadas como control. Hasta lo que nosotros sabemos, esta la primera vez que se describen rizobios tolerantes al Hg. El grado de toxicidad celular al Hg, definido como la concentración que produce una mortalidad del 50%, fue diferente en todas las cepas y siempre superior a las cepas control. El que haya una pequeña proporción de celulas supervivientes a estas concentraciones de Hg podría explicar la supervivencia de dichas bacterias en los suelos de origen. Además dichas dosis efectivas nos sirvieron de base para los experimentos con plantas (ver comun. de Quiñones *et al*.).

Tabla. Patrones de resistencia/tolerancia al Hg, expresados como concentración mínima inhibitoria (CMI), y grado de toxicidad, expresado como concentración efectiva (CE50) de 16 cepas de *Rhizobium* aisladas de nódulos de leguminosas en diferentes zonas del área de Almadén (Ciudad Real) y rizobios de referencia.

CEPA	ESPECIE	CMI (μM)[a]	CE50 (μM)[b]
R-7Q	*Rhizobium radiobacter*	30	16,69
L-7AH	*Bradyrhizobium canariense*	12,5	4,39
M-7AH	*Sinorhizobium medicae*	6	2,82
M-7C	*S. medicae*	25	9,96
J-7AH	*S. medicae*	12,5	5,83
J-7C	*S. medicae*	25	9,36
V-7A	*R. leguminosarum*	6	3,77
V-7Q	*R. leguminosarum*	3	1,91
V-7C	*R. leguminosarum*	6	4,54
TT-7A	*R. leguminosarum bv. trifolii*	12,5	5,54
TT-7AH	*R. leguminosarum bv. trifolii*	12,5	6,82
TE-7AH	*R. leguminosarum bv. trifolii*	12,5	6,21
T-7Q	*R. leguminosarum bv. trifolii*	< 3	1,92
TX-7C	*R. leguminosarum bv. trifolii*	12,5	6,85
TT-7C	*R. leguminosarum bv. trifolii*	12,5	7,32
TS-7C	*R. leguminosarum bv. trifolii*	12,5	5,11
CEPAS DE REFERENCIA			
ISLU-16	*B. canariense*	< 3	*
L-3	*B. canariense*	< 3	*
ALF-3	*S. meliloti*	< 3	*
T-3	*R. leguminosarum bv. trifolii*	< 3	*
J-2	*Rhizobium gallicum*	< 3	*

[a]La CMI se define como la menor concentración de Cl_2Hg que permite un crecimiento máximo del 10% con respecto al control sin Hg al final de la fase logarítmica (A 680 nm). [b]CE50 es la concentración de Hg que inhibe el crecimiento exponencial un 50% respecto al control. A: Almadenejos, C: Las Cuevas y Q: San Quintín.

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Mesorhizobium loti is an infrequent symbiont of *Lotus* spp.

Lorite MJ[1], Estrella MJ[2], León-Barrios M[3], Monza J[4], Sanjuán J[1]

[1]Plant-Bacteria Interactions Lab, Dpto. Microbiología, EEZ-CSIC, Granada, Spain; [2]Unidad Biotecnología 3, IIB-INTECH, Chascomús, Buenos Aires, Argentina; [3]Dpto. Microbiología y Biología Celular, Universidad de La Laguna, Tenerife, Spain; [4]Biochemistry Lab, Facultad de Agronomía, UDELAR, Montevideo, Uruguay.

Summary

Mesorhizobium loti has been traditionally considered the type symbiont of *Lotus* spp. like *L. japonicus*, *L. corniculatus* or *L. tenuis*. We have carried out studies on the genetic diversity of rhizobia nodulating various *Lotus* spp. in soils of Spain, Argentina and Uruguay, by characterizing several taxonomic and symbiotic gene markers. In Spain, the rhizobia associated with several *Lotus* species endemic to the Canary Islands were very diverse and taxonomically related to genera *Sinorhizobium* and *Mesorhizobium,* but no *M. loti* were found, whereas rhizobia nodulating *L. corniculatus* and *L. tenuis* in two locations of Granada were related to species like M. *tarimense*/*M. tianshanense*, *M. chacoense*/*M. albiziae* or *M. alhagi*. Likewise, *L. tenuis* symbionts in the Salado River Basin, Argentina, were phylogenetically related to *Rhizobium*, *Agrobacterium*, *Aminobacter* and *Mesorhizobium*, with few isolates clustering with *M. loti* type strains. In Uruguay, bacteria related to *Mesorhizobium* and *Phyllobacterium* were isolated from *L. corniculatus* nodules in several locations throughout the country, but no *M. loti*-like bacteria were isolated. In all countries and sites, however, many of the isolates carried nodulation and nitrogen fixation genes closely related to those of *M. loti*. The results of this work, likely the broader study on diversity of *Lotus* rhizobia ever performed, suggest that *M. loti* is an unusual symbiont of *Lotus* spp.

Introduction

Several *Lotus* species like *L. corniculatus*, *L. uliginosus* and *L. tenuis* are used as pasture-forage worldwide and are phylogenetically related to the model legume *L. japonicus*. Until recently, bacteria nodulating *Lotus* included both intermediate-growing (mesorhizobia) and slow-growing bacteria. The mesorhizobia can form effective symbioses with certain *Lotus* spp. (group I, e.g., *L. corniculatus*, *L. tenuis* or *L. japonicus*) but form tumor-like structures that do not contain bacteria on species like *L. uliginosus*, *L. subbiflorus* and *L. angustissimus* (group II *Lotus*). On the other hand, slow-growing strains are usually efficient with *Lotus* group II species but form no nodules or inefficient nodules in group I species. The narrow host-range rhizobia associated with *L. corniculatus* and other *Lotus* species were initially classified as *Rhizobium loti* and later re-classified as *Mesorhizobium loti*, which is considered the type species (Jarvis *et al.,* 1997). However, diverse evidence indicate that "meso-growing" rhizobia strains classified as *M. loti* do not form a homogeneous group.

Abiotic stresses like salinity or drought are serious and expanding threats to agricultural productivity. Improving crop productivity in stressed soils requires selection of well adapted plant genotypes, and in the case of legumes, highly efficient rhizobial partners adapted to soil conditions. Aiming to isolate and select for stress-tolerant bacteria able to establish efficient symbiosis with *Lotus* spp., we explored the diversity of *Lotus* rhizobia in reference locations of Spain, Argentina and Uruguay.

Materials and Methods

Bacteria were isolated from field plant nodules or from trap plants inoculated with soil suspensions by standard methods. In total, several hundreds isolates were genetically characterised by means of REP-PCR, ERIC-PCR and ARDRA fingerprintings, and *rrs*, *atpD, recA*, *nodC* and *nifH* gene sequencing and corresponding phylogenetic analyses (Versalovic *et al.,* 1991; Tamura *et al.,* 2007).

Results and Discussion

Almost 500 isolates collected from several sites and locations within each country were characterised. The results from genotypic and phylogenetic analyses are summarised below.

Spain: in the Canary Islands, *L. lancerottensis* is mainly nodulated by *S. meliloti* bv. lancerottense, whereas bacteria related to *M. ciceri, M. plurifarium, M. alhagi, M. opportunistum* and *M. caraganae* were associated with other seven endemic *Lotus* species, *L. campylocladus, L. pyranthus/L. callis-viridis, L. sessilifolius, L. berthelotii, L. kunkelii/L. arinagensis*. (León-Barrios *et al.,* 2009; Lorite *et al.,* 2010). Moreover, some isolates were clustered apart from previously described species and therefore could represent novel species. Most of the Canarian isolates were unable to establish effective symbiosis with *L. corniculatus*. In Granada, most isolates could form effective symbioses with both *L. corniculatus* and *L. tenuis*, and were phylogenetically related to M. *tarimense*/*M. tianshanense*, *M. chacoense*/*M. albiziae* or *M. alhagi* (Lorite *et al.,* 2010).

Argentina: bacteria nodulating *L. tenuis* in typical soils of the Salado River Basin were genetically diverse and clustered with species like *R. gallicum, R. etli, R. tropici, A. tumefaciens, A. aminovorans, M. amorphae, M. mediterraneum, M. tianshanense*; few isolates were related to *M. loti* type strain NZP2213 (Estrella *et al.,* 2009).

Uruguay: bacteria nodulating *L. corniculatus* in soils from northern and southern areas of the country were related to *M. huakuii* bv. loti, *M. tianshanense*, *M. gobiense, M. tarimense* or *M. caraganae* (M. Sotelo *et al*., submitted). No bradyrhizobia were isolated from any of the locations and *Lotus* spp. explored, and only a few Uruguayan isolates seemed capable of effectively nodulating *L. uliginosus* in addition to their host of isolation.

Therefore, except a few isolates in Argentina, *M. loti* could not be found among hundreds of isolates from dozens of soil locations in the three countries. In contrast, most isolates carried nodulation genes related with *M. loti*. However, some of them carried well differentiated symbiotic genes and therefore could represent novel symbiotypes or biovars. This is the case of *S. meliloti* bv. lancerottense strains, which form effective symbioses almost exclusively with *L. lancerottensis* (León-Barrios *et al.,* 2009).

Our results agree with other authors who found species other than *M. loti* as symbionts of *Lotus* in China (Hang *et al.,* 2008), and suggest that *M. loti* is an infrequent symbiont of *Lotus* in many countries and environments.

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Identification of common bean-nodulating *Burkholderia phymatum* strains isolated from Moroccan soils

Talbi C[1], Delgado MJ[1], Girard L[2], Ramírez-Trujillo A[2], Caballero-Mellado J[2], Bedmar EJ[1*]

[1]*Departamento de Microbiología del Suelo y Sistemas Simbióticos, Estación Experimental del Zaidin, CSIC, Apartado 419, 18080 Granada, Spain;* [2]*Centro de Ciencias Genómicas, UNAM, Cuernavaca Morelos, México.* **ejbedmar@eez.csic.es*

Summary

The genetic diversity of 52 rhizobial isolates from root nodules of common bean (*Phaseolus vulgaris*) was analysed by repetititve extragenic palindromic (REP) fingerprinting and 16S rRNA gene sequencing. Four strains (GR01 to GR04) clustered in REP I group whose nearly complete 16S rRNA gene sequence classified them into the family *Burkholderiaceae* within the Betaproteobacteria, with *Burkholderia phymatum* strain LMG 21445^T being the closest relative species (99% identity). Phylogenetic analysis showed that sequences of the *nodC* and *nifH* symbiotic genes were also closely related to those of *B. phymatum* LMG 21445^T. The representative strain GR01 was able to fix nitrogen *ex planta* and formed N_2-fixing nodules on roots of *Phaseolus, Mimosa*, *Acacia* and *Prosopis* species, but did not infect *Pisum*, *Cicer*, *Medicago*, *Trifolium*, *Vicia* and *Glycine*.

Introduction

Until 2001 all known bacteria involved in root nodule symbioses with leguminous plants were classified as members of the order Rhizobiales of the Alphaproteobacteria, including *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium* and *Sinorhizobium* (Willems, 2006; Zakhia & de Lajudie, 2006). However, Moulin *et al*. (2001) reported two *Burkholderia* nodule-forming strains, STM678 isolated from *Machaerium lunatum* in French Guiana, and STM815 isolated from the South African legume *Aspalathus carnosa*, this being the first report on the presence of a Betaproteobacteria within root nodules of legumes. Strain STM815^T was formally classified as *B. phymatum*, and strain STM678^T as *B. tuberum* (Vandamme *et al.,* 2002). Most *Burkholderia* species have been found associated with host legumes in the genus *Mimosa*, primarily in the Neotropics (Barret & Parker, 2005, 2006; Chen *et al.,* 2003, 2005; Rasolomampianina *et al.,* 2005). In this study we report on the isolation and characterization of *B. phymatum* from root nodules of bean grown in alkaline soils from Morocco. Our results show that strain GR01 formed effective nodules on species of *Mimosa, Acacia and Prosopis*, and fixed atmospheric N_2 under free-living conditions.

Materials and Methods

Using soil from Oulade Mansour (Oujda province, Morocco) and common beans (*Phaseolus vulgaris* cv. Flamingo) as a trap host, nodules were collected, surface-sterilized and crushed in a drop of sterile water with a sterile glass rod. Then, the resulting suspension was streaked onto Petri dishes containing either yeast extract-mannitol (YEM; Vincent, 1970) medium or peptone-mineral salts-yeast extract (PSY, Regensburger & Hennecke, 1983) medium. After incubation at 30 ºC for 7 d, colony-forming units were selected, which represented all of the colony types that could be distinguished by microscopic observation of living cells. Genomic DNA was isolated from bacterial cells using RealPure genomic DNA extraction kit (Durviz, Spain) following the manufacturer's instructions. Repetive extragenic palindromic (REP)-PCR was performed using primers and conditions described by de Bruijn (1992). PCR amplifications of the 16S rRNA, *nodC* and *nifH* genes were done as indicated by Talbi *et al*. (2010). The sequences obtained were compared with those from the GenBank using the BLASTN program. Phylogenetic analyses were performed as indicated by Talbi *et al.* (2010). Preparation of whole-cell proteins and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) assays were performed as described previously (Estrada de los Santos *et al*. 2001). Acetylene reduction activity (ARA) by nodulated plants was assayed on detached root systems excised at the cotyledonary node as described by Delgado *et al*. (1989). Under free-living conditions, ARA was tested in semisolid JMV medium (Elliot *et al.,* 2007).

Results and Discussion

Out of 52 strains isolated from common bean root nodules, four strains (GR01 to GR04) clustered in REP group I. The nearly complete 16S rRNA gene sequence showed they were members of the family *Burkholderiaceae* within the Betaproteobacteria, with *B. phymatum* STM815^T being the closest relative species (99% identity). The *nodC* and *nifH* genes from each GR strain also showed 99% identity to those of *B. phymatum* strains STM815^T. Strains isolated from common bean nodules showed almost identical SDS-PAGE protein profiles (evaluated by visual comparison) to those from type strain of *B. phymatum* STM815^T, but clearly different to those of other legume-nodulating *Burkholderia* species. No differences were found when API 20 NE and API 50 CH strips were used to check for differences in nitrogen and carbon sources between *B. phymatum* STM815^T and the GR strains. These strains are true symbionts of common bean as, after nodule isolation, they were able to establish new effective symbiosis with common beans, with values of ARA ranging from 492 to 525 µmol ethylene/plant/h. *B. phymatum* STM815^{T}also infected common beans, but the efficiency of the symbiosis, determined as plant dry weight (1.06 ± 0.18 g/plant/h), was half of that found in plants nodulated by the GR strains. These strains also nodulated *M. pigra*, *A. cochliacantha*, *A. bilimeki*, *L. glauca* and *P. laevigata*, but were unable to form nodules on *P. sativum*, *L. culinaris*, *L. corniculatus*, *M. sativa*, *G. max* and *C. arietinum*. Like strain STM815^T, strains isolated from common bean also had nitrogenase activity when grown *ex planta*. Values of activity, however, were about half of that detected in strain STM815^T (83 ± 15 nmol C_2H_4/h) (Elliot *et al.,* 2007).

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Phylogenetic diversity of rhizobial strains nodulating *Cytisus villosus*

Chahboune R[1*], Barrijal S[1], Sánchez-Raya AJ[2], Bedmar EJ[2]

[1]*Departament of Biology, Faculty of Sciences and Techniques, University Abdelmalek Essaadi, Tanger, Morocco;* [2]*Department of Soil Microbiology and Symbiotic Systems, Estación Experimental del Zaidín, CSIC, Apartado 419, 18080 Granada, Spain. *chahboune82rajaa@yahoo.fr*

Summary

The genetic diversity of 82 endophytic bacterial strains isolated from root nodules of *Cytisus villosus* grown in the Moroquian Rif region was analysed by repetititve extragenic palindromic (REP) fingerprinting and 16S rRNA gene sequencing. The isolates were distributed into 19 REP groups, and a representative strain from each group was subjected to nearly complete 16S rRNA gene sequencing. Analysis of these sequences showed that the isolates were related to the genera *Bradyrhizobium* of the order Rhizobiales from Alphaproteobacteria. Further characterization of the isolates by sequencing of the symbiotic genes *nodC* and *nifH* and the house-keeping genes *recA* and *glnII* indicated that eleven isolates were classified as *B. canariense* ecotype *genistearum* and three of them as *B. japonicum* ecotype *genistearum*. The remaining five isolates did not show significant similarity with any *Bradyrhizobium* species.

Introduction

Soil in the north of Morocco, particularly in the central-western region of the Rif mountains, has been heavily degraded as a result of human activities. This situation threatens seriously the existence and survival of Moroquian natural resources and habitats. Native to this region, *Cytisus villosus* has substantial agronomic importance due to its rapid growth and outstanding forage value. This, together with its N_2-fixation capability, renders this plant a very promising candidate not only for cattle fodder but also for soil conservation programs and low-input agroforestry in regions with a Mediterranean climate. Given the potential of those plants in revegetation projects owing to their ecological benefits, the primary objective of this work was to identify for the first time the endosybionts associated with the shrubby legume *C. villosus*.

Materials and Methods

Nodules were collected from wild grown *C. villosus* in different sites near the villages of Aoudal and Fifi in the central-western of the Moroquian Rif (Chefchaouen province, Morocco). They were surface-sterilized and crushed in a drop of sterile water with a sterile glass rod. Then, the resulting suspension was streaked onto Petri dishes containing either yeast extract-mannitol (YEM; Vincent, 1970) medium. After incubation at 30 ºC for 7 d, colony-forming units were selected, which represented all of the colony types that could be distinguished by microscopic observation of living cells. Genomic DNA was isolated from bacterial cells using RealPure genomic DNA extraction kit (Durviz, Spain) following the manufacturer's instructions. REP-PCR was performed using primers and conditions described by de Bruijn (1992). PCR amplification of the 16S rRNA genes was done as indicated by Herrera-Cervera *et al*. (1999), and those of the *recA and glnII* genes as described by Vinuesa *et al.* (2005). The presence of the *nifH* and *nodC* genes was detected according to Laguerre *et al*. (2001). Phylogenetic analyses were performed with the PHYLIP computer program package, version 3.67 (Felsenstein, 2005). The combined *glnII* + *recA* analysis was performed with BioEdit (Hall, 1999), and visualized with MEGA 4.0.2 (Tamura *et al.,* 2007).

Results and Discussion

REP-PCR fingerprinting of 82 strains forming morphologically different colonies showed 28 REP patterns. The nearly complete sequence of 16S rRNA gene from a representative isolate of each REP pattern was obtained and compared with those held in Genbank. BLASTN searches indicated that 19 isolates belong to the genus *Bradyrhizobium*, with the same

similarity value for more than one *Bradyrhizobium* type strain validly published including *B. betae* L7HG1^T, *B. canariense* BTA-1^T, *B. elkanii* USDA 76^T, *B. japonicum* USDA 6^T, *B. liaoningense* LMG 18230^T, *B. yuanmingense* CCBAU 10071^T, *B. pachyrizi* PAC48^T, and *B. jicamae* PAC68^T.

A 16S rRNA-based phylogenetic tree showed that most of the isolates grouped with *B. canariense* BTA-1^T, but affiliation at the species levels could not be inferred. These results agree with those that indicate that limited taxonomic information in the genus *Bradyrhizobium* can be deduced from 16S rDNA sequences because of their highly conserved *rrs* sequences and poor correlation between the groupings formed on the basis of genotypic and phenotypic traits (Willems *et al.,* 2001; Vinuesa *et al.,* 2005).

Both individual and combined *glnII* and *recA* gene phylogenies produced similar trees, which showed that 11 out of the 19 representative isolates could be considered as *B. canariense* and that the other 3 strains could be considered as *B. japonicum*. The similarity values of $\leq$ 95% found in BLAST searches and pairwise alignments suggest that the remaining five isolates may form one or two separate groups within the *Bradyrhizobium* lineage. Further identification of those isolates has not been pursued in this study.

After amplification of the symbiotic *nodC* and *nifH* genes, BLAST searches indicated that all isolates showed identity (91%-99%) with the *B. canariense* BTA-1^T *nodC* and *nifH* genes, respectively. The phylogenetic analyses of *nifH* and *nodC* genes indicate they form a single evolutionary lineage within a monophyletic cluster and support the suggestion that the symbiotic genes arose after bacterial divergence in rhizobia (Turner & Young, 2000). Whether the bradyrhizobial strains in this study acquired their symbiotic genes either from a common ancestor or by lateral transfer from *B. canariense* to *B. japonicum* is unknown.

Acknowledgments

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Genetic diversity of rhizobial symbionts of coastal *Lotus* in the Canary Islands

León-Barrios M[1], Pérez-Debén S[1], Pérez-Galdona R[1], Lorite MJ[2], Sanjuán J[2], del Arco-Aguilar M[3]

[1]Dpto de Microbiología y Biología Celular, Universidad de La Laguna, 38071 La Laguna, Tenerife; [2]Dpto de Microbiología del Suelo y Sistemas Simbióticos, Estación Experimental del Zaidín, CSIC, Apartado 419, 18080 Granada; [3]Dpto de Biología Vegetal (Botánica), Universidad de La Laguna, 38071 La Laguna, Tenerife, Spain.

Summary

Several endemic *Lotus* species grow in arid coastal environments of the Canary Islands and a collection of 42 root nodule bacteria from five *Lotus* species growing in these habitats in Tenerife, Gran Canaria and Lanzarote was characterized. Two species were found preferentially to nodulate the *Lotus* in these habitats: *Sinorhizobium meliloti* (43% of isolates) and *Mesorhizobium alhagi* (31% of isolates). The symbiotic genes harboured by the Canarian isolates from these two rhizobial species constituted two unique biovars, distantly related to bv. *loti.* One of these biovars was recently described as bv. *lancerottense* and its host range included nodulation on *L. lancerottensis, L. corniculatus* and *L. japonicus*, but most isolates showed a non-fixing phenotypes on the non-endemic *Lotus*. The other new biovar showed a more restrictive host range for endemic *Lotus* spp. Thus, in the drier environments of the Canary Islands there appears to be evolution of unique symbiotic genes in association with the specific *Lotus* species growing there.

Introduction

The genus *Lotus* presents in the Canary Islands 17 endemic species, which usually show an insular distribution pattern, exclusive to a single island, and with a limited distribution area. Characterization of the *Lotus* rhizobia on the islands is limited (León-Barrios *et al.,* 2009; Lorite *et al.,* 2010). Here we genetically characterize 42 isolates from five *Lotus* species, *L. arinagensis, L. kunkelii*, *L. lancerottensis*, *L. maculatus*, and *L. sessilifolius*, which grow in arid coastal environments of the Canaries. Natural populations of *L. arinagensis*, *L. kunkelii* and *L. maculatus*, are present exclusively within a very restricted area of the coast (20 m a.s.l.): *L. maculatus* is found on the N of Tenerife *L. arinagensis* and *L. kunkelii* and grow in sandy soils on the SE coast of Gran Canaria; *L. kunkelii* and *L. maculatus* being currently in danger of extinction. *L. lancerottensis* and *L. sessilifolius* are found more widely distributed in Lanzarote and Tenerife, respectively. The isolation of efficient rhizobia is of special interest for the endemic *Lotus*, because they grow in restricted areas and could be associated with specific rhizobial strains only found in a particular environment. The use of appropriate bacterial inoculants might become essential for recovering particular *Lotus* populations.

Materials and Methods

Isolation of rhizobia and growth conditions. The isolates were recovered from the root nodule bacteria, grown and maintained in the laboratory following standard protocols (León-Barrios *et al.,* 2009).

16S rRNA and* nodC *analysis. *rrs* or *nodC* genes were amplified, restricted and analyzed as described (León-Barrios *et al.,* 2009).

Results and Discussion

The isolates were dispersed in eleven branches (Figure). Most branches contained minority genotypes usually represented by one isolate, whereas two branches contained 74% of the isolates. Clade A grouped 43% of the isolates with the species *Sinorhizobium meliloti* and clade B contained other 31% of the isolates with *Mesorhizobium alhagi* species (Figure). *S. meliloti* and *M. alhagi* isolates were recovered from four and five different *Lotus* species, respectively, and in different islands. Therefore, the isolates belonging to *S. meliloti* and *M.*

alhagi dominate the arid coastal environments of the Canary Islands and must be well adapted to dry and salty conditions. The finding of *M. alhagi* isolates in arid environments of the Canaries is not surprising since *M. alhagi* species was described for root nodules isolates of *Alhagi sparsifolia* growing in arid zone of China. On the other hand, *S. meliloti* has been widely recognized as the typical symbiont of *Medicago* and *Melilotus*, but it is interesting that strains of *S. meliloti* were found to nodulate common bean in arid areas of Tunisia, and now our study unequivocally associate this rhizobial species as a symbiont of *Lotus*.

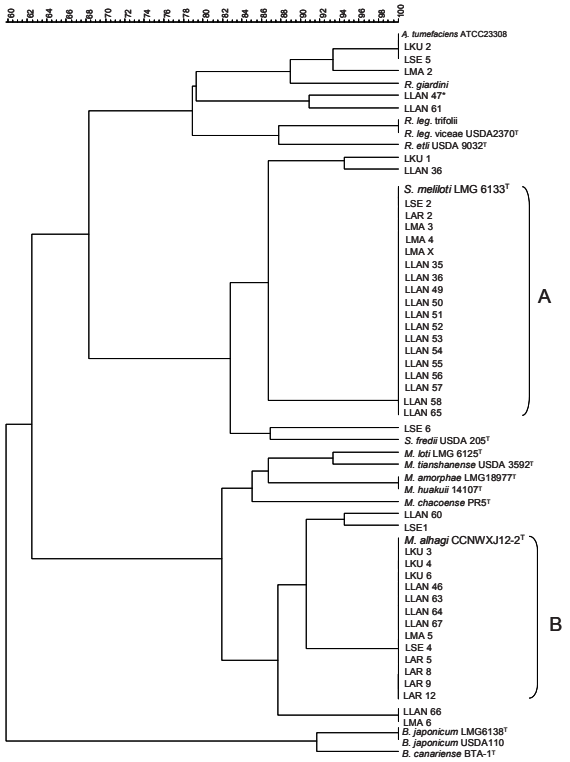

Figure. UPGMA dendrogram showing clusters generated by combined restriction patterns of amplified 16S ARDRA (*HinfI*+*MspI*+*RsaI*) of isolates from endemic Canarian *Lotus* and reference strains.

Two different and unique biovars were detected in isolates of *S. meliloti* and *M. alhagi*. One of them, bv. *lancerottense* (León-Barrios *et al.,* 2009), was found between the *S. meliloti* isolates and its host range included nodulation on *L. lancerottensis*, *L. corniculatus* and *L. japonicus*; however, effective N_2-fixation only occurred on *L. lancerottensis* whereas on the non-endemic *Lotus* the isolates showed moderate N_2-fixing or non-fixing phenotypes. The *M. alhagi* isolates presented a narrower host range almost restricted to endemic *Lotus* and symbiotic genes compatible to represent a new biovar for the *Lotus* rhizobia. Thus, in the drier environments of the Canary Islands there appears to be evolution of unique symbiotic genes in association with the specific *Lotus* species growing there.

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Sinorhizobium meliloti is the main symbiont of *Lotus lancerottensis* in Lanzarote

Pérez-Debén S[1], Pérez-Galdona R[1], Jiménez C[2], del Arco-Aguilar M[3], León-Barrios M[1]

[1]Dpto de Microbiología y Biología Celular, [2]Dpto de Edafología y Geología, and [3]Dpto de Biología Vegetal (Botánica), Universidad de La Laguna, 38071 La Laguna, Tenerife, Spain.

Summary

Lotus lancerottensis is an endemic legume of Lanzarote Island. In a previous study seven strains isolated from the root nodules of *L. lancerottensis* were classified as *S. meliloti*. In this report we characterized a larger sample of 50 rhizobial strains isolated from *L. lancerottensis* grown in soil samples from eleven locations to the aim of investigate more precisely the diversity of the rhizobia nodulating *L. lancerottensis* in Lanzarote. We found that *S. meliloti* was the main symbiont (80% of isolates): it was recovered from all locations and in all but one it was the exclusive or major symbiont of the *Lotus lancerottensis*. Furthermore, the isolated strains of *S. meliloti* appear to be very well adapted to the alkaline soils of Lanzarote since the majority of isolates did not grow at pH ≤ 5.5, whereas most of them showed good growth at pH 9.0.

Introduction

Lotus lancerottensis is an endemic *Lotus* which grows in the most north-eastern islands of the Canarian Archipelago, Lanzarote and Fuerteventura. It is one of the only two *Lotus* species that grows on Lanzarote and is well-distributed all over the Island. Lanzarote is an arid island, and, thus, it is of interest to know the rhizobia able to nodulate this legume in this dry environment. In a previous study seven strains isolated from the root nodules of *L. lancerottensis* were classified as *S. meliloti* (León-Barrios *et al.,* 2009). Due to the small number of isolates it was not possible to draw conclusions about the diversity of the root nodule bacteria associated with this *Lotus* species. The aim of this work was to study the genetic diversity of the rhizobia nodulating *L. lancerottensis* in Lanzarote and determine the importance of *S. meliloti* as *Lotus* symbiont. For this, we collected soil samples at eleven points along the longitudinal axis of the island and by using *L. lancerottensis* as a trap-legume a large collection of root nodule bacteria were isolated.

Materials and Methods

Isolation of rhizobia and growth conditions. Sterilized *Lotus* seeds were grown on soil samples collected where natural populations were growing. The root nodule bacteria were recovered 8 weeks later. The isolates were grown and maintained in the laboratory following standard protocols (Lorite *et al.,* 2010).

Salt and pH tolerance. Tolerance to salinity (NaCl) and pH were assessed according to León-Barrios *et al.* (2009).

16S ARDRA. Near full-length *16S rRNA* gene was amplified, digested with restriction enzymes (*Hinf*I, *Msp*I and *Rsa*I) and analyzed as described (León-Barrios *et al.,* 2009).

Results and Discussion

Eight genotypes belonging to the genera *Rhizobium*, *Sinorhizobium* and *Mesorhizobium* could be detected among the isolates from *Lotus lancerottensis* (Figure). Most genotypes were represented by only one isolate; four isolates from a salty location grouped with species *M. alhaghi*; whereas 80% of isolates were grouped with *Sinorhizobium meliloti*. Moreover, when *L. corniculatus* was used as a trap-legume with soils of Lanzarote *S. meliloti* was recovered again from the root nodules (isolates LCOL). These findings confirm that *S. meliloti* nodulates preferentially *Lotus lancerottensis* in Lanzarote soils.

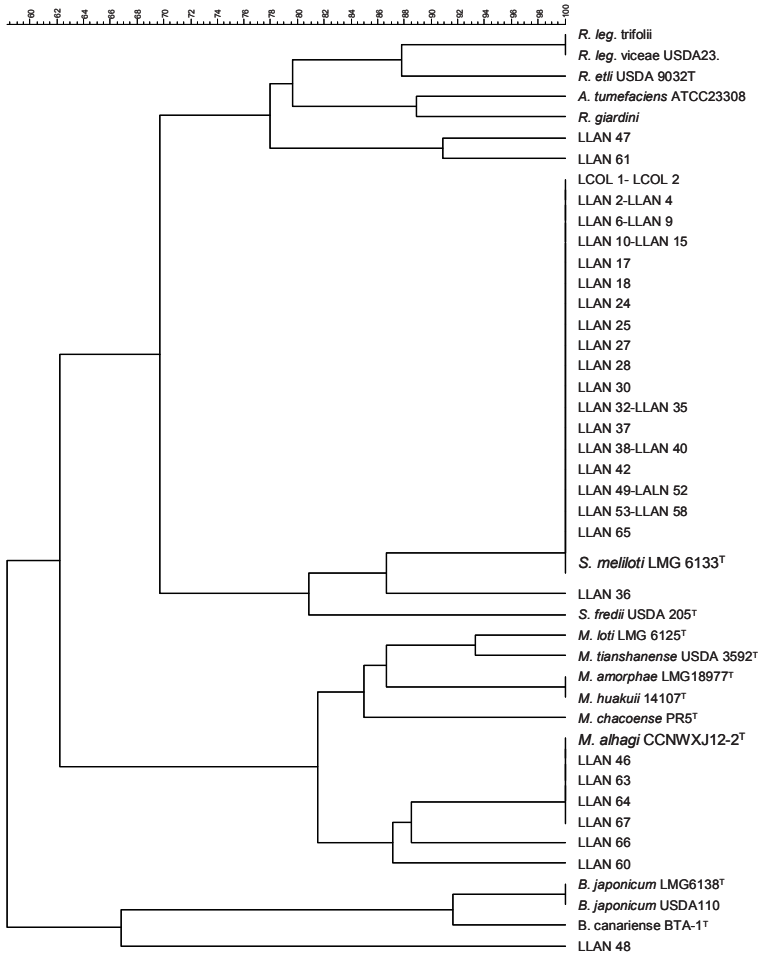

Figure. UPGMA dendrogram showing clusters generated by combined restriction patterns of amplified 16S ARDRA (*HinfI+MspI+RsaI*) of isolates from *Lotus lancerottensis* and reference strains.

Lanzarote soils are characterised by being poor in N, alkaline and carbonated, and salty in some locations. The *S. meliloti* isolates appear to be very well adapted to the alkaline soils of Lanzarote since the majority of isolates did not grow at pH ≤ 5.5, whereas most of them showed good growth at pH 9.0; likewise, the reference strain *S. meliloti* 1021 showed a similar behaviour. On the contrary, reference strains of *M. loti*, NZP2213^T, NZP2037 and MAFF303099, grew at pH 5.5 but were not able to grow at the alkaline pH, which could explain that absence of *M. loti* among the isolates. Most of isolates were moderately tolerant to salt and grew at least up to 2% NaCl.

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Competitive ability and diversity of native rhizobia strains nodulating red clover

Batista L[1], Sánchez M[1], Irisarri P[1], Cuitiño MJ[2], Rebuffo M[2], Sanjuán J[3], Monza J[1]

[1]Laboratorio de Bioquímica, Facultad de Agronomía, Universidad de la República, Montevideo, Uruguay; [2]Instituto Nacional de Investigación Agropecuaria, INIA La Estanzuela, Colonia, Uruguay; [3]Plant-Bacteria Interactions Laboratory, Dpto. Microbiología, EEZ-CSIC, Granada, Spain.

Summary

With the aim of evaluating diversity of red clover (*Trifolium pratense*) rhizobia in Uruguayan soil, the ERIC profiles of isolates from pastures inoculated with *Rhizobium leguminosarum* strain U204 (commercial inoculant) and non-inoculated pastures were compared. Surprisingly, none of the nodule isolates presented ERIC profiles similar to U204. A field experiment was established to evaluate forage production and the competitiveness of the commercial strain. 64% of the primary root nodules and 12% of the secondary ones were occupied by U204 after 6 months, although there were not primary root nodules occupied by U204 after 12 months. The two native strains found more frequently in nodules of inoculated and non-inoculated red clover plants presented charasteristic ERIC profiles previouslly identified in the diversity analysis. There was no difference in red clover forage production due to inoculation after 6 months, indicating that native strains were efficient and may be used for production of more competitive inoculants.

Introducción

En Uruguay, el trébol rojo es usado en el mejoramiento de praderas, y se inocula desde 1967 con la cepa U204 de *Rhizobium leguminosarum* bv *trifolii*, introducida de Estados Unidos. Esta cepa ha sido evaluada por su eficiencia simbiótica en diferentes suelos y sistemas de siembra (García *et al*., 1994). Uruguay es un referente en la producción y uso de inoculantes rizobianos (Date, 2000), debido a las políticas llevadas adelante que involucran la implementación de mecanismos legales, activa participación de la industria y esfuerzos para que los agricultores adopten prácticas de inoculación (García *et al*., 1994). A pesar de esta posición ventajosa, datos recientes sobre la inoculación de leguminosas relevados por Cooperativas Agrarias Federadas pusieron en evidencia que el 22% de los productores no inoculan trébol rojo (Acosta *et al*., 2008). De todas formas, el trébol rojo es nodulado espontáneamente por cepas nativas, no siempre eficientes, presentes en los suelos. El objetivo de este trabajo fue evaluar la diversidad genética de cepas de rizobios que nodulan trébol rojo y comparar la competitividad del inoculante comercial con las cepas presentes en el suelo.

Materiales y Metodos

Los rizobios se aislaron de nódulos de *Trifolium pratense* L. colectados de un sitio sin historia de inoculación y dos con historia de inoculación. El DNA se amplificó con los cebadores ERIC1 y ERIC2 según Agius et al. (1997) y el 16SrRNA según Herrera Cervera *et al.* (1999). El experimento parcelario de campo consistió en dos tratamientos, uno inoculado con la cepa U204 y otro sin inocular, con 4 repeticiones. 6 y 12 meses después de la siembra se determinó el peso seco de la parte aérea, y se muetrearon al azar 25 nódulos de cada tratamiento de las raíces principal y secundaria. De los nódulos se aislaron rizobios y el DNA se amplificó según se describió.

Resultados y Discusión

La evaluación de la diversidad de 60 cepas que nodulan trébol rojo mediante el análisis de sus perfiles genómicos generados por ERIC-PCR, permitió establecer una diversidad del 27% (Figura). Si bien la cepa U204 se usa como inoculante comercial desde hace 43 años, ningún aislado presentó un perfil igual al de ella. De todas formas, la secuencia del gen 16SRNA de 6 aislados analizados y de la cepa U204 fue 100% homóloga a *R. leguminosarum* bv. *trifolii* cepa WSM1325. La competitividad entre la cepa U204 y las cepas nativas se determinó en 50 nódulos de cada tratamiento, a los 6 y 12 meses después de la siembra, tiempos en los que

también se determinó la producción de forraje (Tabla). A los 6 meses, en el tratamiento inoculado el 64% de nódulos de las raíces principales estaban ocupados por la cepa U204, mientras que en las raíces secundarias estaba presente en el 12% de los nódulos. En el tratamiento sin inocular, el 100% de los nódulos estaban ocupados por cepas nativas. Entre las cepas nativas, en los dos tratamientos hubo dos perfiles (I y L) que aparecieron con una frecuencia mayor al 20%. A los 12 meses no se encontraron nódulos ocupados por el inoculante comercial en la raíz principal, pero sí en un 20% de nódulos de las raíces secundarias, y los perfiles I y L mantuvieron la misma frecuencia en ambos tratamientos. De estos resultados se puede inferir que, en las condiciones del ensayo, los nódulos inducidos por la cepa U204 aportan nitrógeno a la planta durante unos 6 meses, pero a partir de ese tiempo el nitrógeno aportado a la planta provendría mayormente de nódulos ocupados por las cepas nativas. De esta manera, si se seleccionan cepas eficientes con mayor competitividad que el inoculante actual, éstas ocuparán en el primer semestre de vida de la planta, además de los nódulos tempranos (raíz principal) los tardíos (raíces secundarias), lo que prolongaría la productividad de la pradera y mejoraría el aporte de nitrógeno a los cultivos posteriores en la rotación. Además es probablemente mejore la persistencia del trébol, dado que la abundante nodulación secundaria inducida por cepas nativas poco eficientes o parásitas puede incidir negativamente sobre este aspecto.

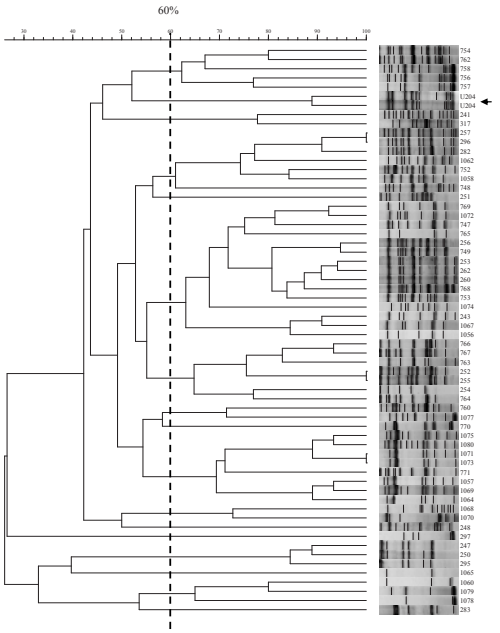

Figura. Dendrograma con 60 perfiles ERIC de aislados de nódulos de trébol rojo. La cepa U204, inoculante comercial, se señala con una flecha. Con el coeficiente de similitud DICE y el método de agrupamiento UPGMA se obtuvieron 16 perfiles genómicos diferentes con una similitud igual o mayor a 60%.

Tabla. Frecuencia de nódulos ocupados por el inoculante comercial (U204) en los tratamientos inoculado y no inoculado a 6 y 12 meses después de la siembra. El rendimiento de forraje se determinó como materia seca.

fuestreo	Tratamiento	R. P.	R. S.	Forraje (kg MS/ha)
meses	Inoculado con U204	64	12	2.486
	No inoculado	0	0	3.013
2 meses	Inoculado con U204	0	20	2.166
	No inoculado	0	0	1.972

Agradecimientos

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Growth and motility of Plant Growth Promoting Rhizobacteria isolated from maca rhizosphere

Ogata K[1], Lorite MJ[2], Sanjuán J[2], Zúñiga D[1*]

*[1]Laboratorio de Ecología Microbiana y Biotecnología Marino Tabusso, Dpto. de Biología, Universidad Nacional Agraria La Molina, Lima 12, Peru; [2]Interacciones Planta-Bacteria, Dpto. Microbiología, Estación Experimental del Zaidin, CSIC, Apartado 419, 18080 Granada, Spain. *dzuniga@lamolina.edu.pe.*

Summary

Maca (*Lepidium meyenii* Walpers) is an ancestral crop of Peruvian Highlands that grows between 3800 and 4400 meters a.s.l. and represents an important income to the farmers in these regions. In 2009, maca showed a rising tendency in exportations, over USD4.449 millions, thus showing the interest of international markets to consume Peruvian native products. Most studies on maca have focused on the chemical composition of the bulbs but not in the agronomic management. Maca has high nutrient requirements, and consequently, its intensive cultivation decreases soil fertility. In this context, the application of microbial inoculants is a biological alternative to improve the nutrition and growth of this crop, enhance its tolerance to certain stresses such as low temperature, and soil acidity, and to prevent soil impoverishment. Here we report some features of plant growth promoting bacteria isolated from the rhizosphere of maca.

Introduction

La maca (*Lepidium meyenii* Walpers) es una Brassicaceae que crece en los andes peruanos, cultivada principalmente en la meseta del Bombón (departamento de Junín), zona que se caracteriza por tener temperaturas promedio entre 4 y 7°C, alta irradiación solar, frecuentes heladas, vientos fuertes y suelos ácidos (pH<5). Esta planta es conocida y empleada desde tiempos precolombinos, principalmente como una planta medicinal y alimenticia (León, 1964). Las rizobacterias promotoras de crecimiento vegetal (PGPR) son microorganismos benéficos que se encuentran en la rizosfera de las plantas. La rizosfera se define como la zona del suelo alrededor de la raíz que se ve influenciada por la actividad metabólica de la planta y donde existen interacciones entre los microorganismos del suelo y las especies vegetales (Kim *et al*., 2006). Las PGPR, como su nombre lo indica, son bacterias de vida libre o simbiótica que tienen la capacidad de incrementar la germinación, estimular el crecimiento de las plantas, realizar actividades de control biológico, inducción de resistencia a patógenos, producción de fitohormonas y mejoramiento en la asimilación de agua y nutrientes (Essaid *et al*., 2000).

Materiales y Métodos

Previamente se aislaron cepas de bacterias diazótrofas, *Pseudomonas, Bacillus* y actinomicetos de diferentes campos de cultivo de maca ubicados en San Pedro de Cajas, Departamento de Junín, y se estudiaron sus características PGPR como solubilización de fosfatos, producción de ácido indolacético (AIA) y efecto en la germinación (Zúñiga *et al*., 2010). En esta investigación se seleccionaron dos cepas con características potenciales para ser usadas como biofertilizantes y se determinaron sus curvas de crecimiento en un sistema Bioscreen a pH 5 y 7 y temperaturas de 20 y 28°C. Además, se estudió la motilidad de las cepas bacterianas, propiedad que podrían estar relacionadas con la capacidad de colonización de la raíz. Para la determinación de las curvas de crecimiento se utilizó el medio TY y para la motilidad los medios Bromsfield (0.3%) y LB (0.6%).

Resultados y Discusión

De 421 cepas aisladas, 132 produjeron altos niveles de AIA y 68 presentaron halos de solubilización de fosfatos (Zúñiga, 2010). De acuerdo a los resultados, algunas cepas fueron utilizadas para ensayos de germinación y emergencia *in vitro*. Se seleccionaron 2 cepas (Ps42 y 5A) que presentaron buenas características PGPR. Con el fin de optimizar algunos parámetros de crecimiento para la producción posterior de biomasa de estas cepas

potencialmente inoculantes de maca, se realizaron curvas de crecimiento de estas bacterias simulando condiciones de temperatura y pH óptimos (28°C, pH 7), en comparación con los pH bajos (4 y 5) y temperatura registrados en los suelos de la zona muestreada. Se observó que la cepa 5A, determinada como un diazótrofo de vida libre, presentó un crecimiento mediano a pH 4 cuando la temperatura de incubación fue de 28°C (Figura 1), mostró una fase lag de aproximadamente 10 h, luego de las cuáles comenzó un crecimiento exponencial que continuó hasta después de 40 h de incubación. Esto se explica por las condiciones de pH del medio de cultivo que genera un estrés en el microorganismo, haciendo que su crecimiento sea más lento que a pH 5 y pH 7. Sin embargo, a la misma condición de pH pero a temperatura más baja (20°C) no se pudo observar crecimiento durante las 25 h de incubación. Cabe resaltar que un pH de 5 no afectó su crecimiento en comparación al pH 7. La cepa Ps42 de *Pseudomonas* fue sensible al pH muy ácido, no pudiendo crecer a pH 4 a temperaura de 28°C, en cambio si lo pudo hacer a pH 5 siendo su comportamiento muy similar al de pH 7 (Figura 2). Ambos microorganismos mostraron un crecimiento superior a 28°C en comparación al de 20°C; también se puede apreciar en ambas figuras que, tanto a pH 5 como pH 7, la cepa 5A casi no presentó fase lag, mientras que la cepa Ps42 tuvo una fase lag de aprox. 10 h. Por otro lado, se determinó la motilidad de las cepas mediante la técnica de swimming y swarming. La cepa 5A presentó swimming con un promedio de 4,54 ± 0,69 cm, pero no se observó swarming, mientras que la cepa Ps42 tuvo bajos niveles de swimming (0,59 ± 0,13 cm) pero swarming positivo. Mientras que el swimming es un comportamiento individual, el swarming es el movimiento de un grupo de bacterias, por lo que es importante para determinar la existencia de flagelos en las bacterias y el rol que estos pudieran cumplir en la colonización de ambientes naturales.

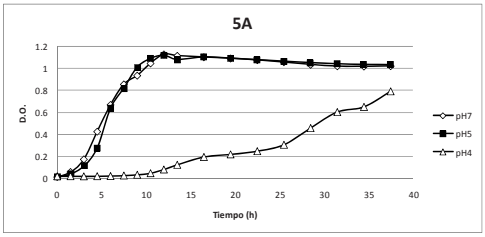

Figura 1. Curvas de crecimiento de la cepa bacteriana diazótrofica (5A) a diferentes pHs a 28°C.

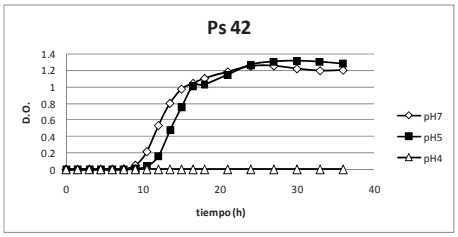

Figura 2. Curvas de crecimiento de la cepa Ps42 de *Pseudomonas* a diferentes pHs a 28°C.

Agradecimientos

Este trabajo ha sido financiado por el proyecto GTZ-Concytec 2009, FDA Biol-111/UNALM y Ecoandino SAC, y por un proyecto de cooperación bilateral CSIC-CONCYTEC.

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Materiales y Métodos

Los muestreos se realizaron en 4 áreas de la cara Sur del Parque Nacional de Sierra Nevada, tomando suelo de cada una de ellas en 3 sitios diferentes con 3 árboles distintos. En el caso de los robles (*Quercus pyrenaica*) las 2 áreas estaban separadas por una diferencia de altitud de 500 m. (CNA y CNB); mientras que en las encinas (*Q. ilex ballota*) se muestreó un área incendiada en el año 2005 (LJQ) y otra zona control próxima, no afectada por el fuego (LJN).

El DNA del suelo se obtuvo utilizando el Power Soil DNA kit de MoBio y se amplificaron los genes *nif*H de la comunidad microbiana empleando los primers nifH(forA), nifH(forB) y nifH(rev) en 2 reacciones de PCR de 29 y 30 ciclos. Para cada área de muestreo se obtuvieron al menos 50 clones diferentes, y después de secuenciar su homología se comprobó mediante BLAST. Con el programa MOTUR (Schloss *et al.,* 2009) se analizó la estructura de las comunidades microbianas de cada rizosfera para conocer su diversidad y riqueza, y se empleó Libshuff para comprobar si existen diferencias en las estructuras de las comunidades de las diferentes muestras.

Resultados y Discusión

La diversidad (índice de Shannon) con un nivel de homología de los OTUs del 90% fue igual en CNA y CNB, con un valor de 2,7; mientras que en las encinas los valores fueron de 2,3 para LJN y de 1,1 para LJQ. Por lo tanto, se puede afirmar que, según la metodología empleada, la diversidad de especies diazotróficas es aproximadamente igual en robles y encinas, pero se ve disminuida por el efecto de los incendios. Sin embargo, se encontraron diferencias significativas en la estructura de las comunidades diazotróficas entre CNA y CNB, entre LJN y LJQ y entre Robles y Encinas. En encinas y CNA las α-Proteobacteria conforman el grupo mayoritario, mientras que en el área de CNB el predominio está compartido por éstas junto a las cianobacterias. Dentro de las α-Proteobacteria, el orden Rhodospirillales predomina en encinas mientras que en robles predominan los géneros pertenecientes al orden Rhizobiales. La presencia de cianobacterias únicamente en robles, puede ser un indicador del grado de humedad o disponibilidad hídrica de estos bosques, que es mayor y menos fluctuante que en encinares.

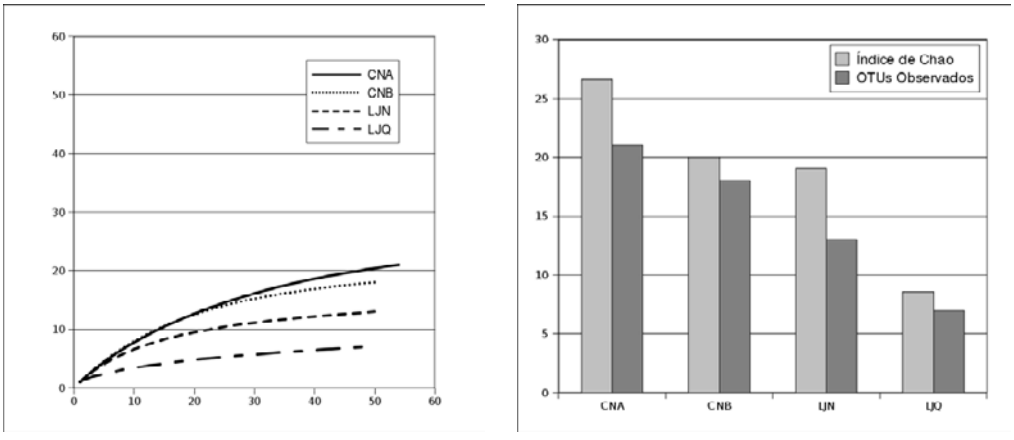

Figura. a) Curva de rarefacción para cada zona muestreada, OTUs>90% b) Comparación de los resultados observados frente a los predichos por el índice de Chao.

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The *celC* gene: a new taxonomic marker for the genus *Rhizobium*

Robledo M, Velázquez E, Ramírez-Bahena MH, García-Fraile P, Rivas R, Martínez-Molina E, Mateos PF*

*Departamento Microbiología y Genética, Universidad de Salamanca, 37007 Salamanca, Spain. *pfmg@usal.es*

Summary

The cellulase genes and the cellulolytic enzymes they encode are widespread and commonly expressed among a large diversity of microorganisms that establish relationships with plants. In particular, we have found that, in *Rhizobium leguminosarum* bv. *trifolii*, the *celC* gene codifies a cellulase that fulfils a very significant role in the infection process of clover. The *celC* gene is located in the *celABC* operon present in the chromosome of the species of the genera *Rhizobium* and *Agrobacterium*, whose genomes have been completely sequenced. In this work we amplified and sequenced the *celC* gene in several species of the genus *Rhizobium* isolated from nodules of different hosts, and the phylogeny of this gene was compared with those obtained after the analysis of other chromosomal and symbiotic genes. Our results showed that *celC* gene phylogeny is congruent to those obtained on the basis of housekeeping genes like *recA* and *atpD*, indicating that this gene is widely conserved among species of the genus *Rhizobium*, in agreement with its involvement in fundamental processes for survival in the environment. As occurs with housekeeping genes, *celC* gene sequencing allows the differentiation of species with close *rrs* gene and thus it is very useful as a taxonomic marker in the genus *Rhizobium.*

Introduction

The *celC* gene codifies a cellulase essential for symbiotic infection of legume host roots in the genus *Rhizobium.* This gene was initially sequenced in different clover-nodulation *Rhizobium* strains. Later, the *celC* gene was localised in several strains from genus *Rhizobium* whose complete sequences have been recently obtained such as *R. leguminosarum*, *R. etli*, *R. radiobacter* and *Rhizobium* sp. NGR234. In these strains the *celC* has been located in the chromosome, except in the strain *Rhizobium* sp. NGR234 in which the gene is located in a plasmid. This gene has not been located in none of the three strains of genus *Bradyrhizobium* sequenced up to date, in *Azorhizobium caulinodans*, in *Mesorhizobium loti* or in *Rhizobium* (formerly *Agrobacterium*) *vitis*. In the case of genus *Ensifer* (formerly *Sinorhizobium*) the *celC* gene was not found in the strain *E. meliloti* 1021, but in the strain *E. medicae* WSM419 was located in a plasmid. We analysed the sequences of this gene in several strains from different species and biovars of the genus *Rhizobium* and the results were compared to those obtained on the basis of chromosomal core genes and plasmidic symbiotic genes. The results of this analysis showed that the phylogeny of *celC* gene coincides with those based on the core genes *recA* and *atpD*, but no correlation exists with phylogeny based on the symbiotic gene *nodC*.

Materials and Methods

A 800 bp fragment of the *celC* gene was amplified and sequenced by using the following primers that were designed in this work from conserved sequences in the *celC* gene available in databanks: CelC1F (5'-atcagccacagcgaagggca-3') and CelC2R (5'-cacagacactccggatgc-3'). The sequence reaction was performed on an ABI377 sequencer (Applied Biosystems Inc., USA) using a BigDye terminator v3.0 cycle sequencing kit as supplied by the manufacturer. The sequences obtained were compared with those from the GenBank using the BLASTN program, and aligned using the Clustal W software. The distances were calculated according to Kimura´s two-parameter model. Phylogenetic trees were inferred using the neighbour-joining method and maximum likelihood (ML) methods. The MEGA 4.0 and PAUP packages were used for all the analyses.

Results and Discussion

Analysis of the* celC *gene. The analysis of this gene was carried out in type strains of genus *Rhizobium* isolated in different geographical locations and from different legumes that belong to different phylogenetic subgroups according to the 16S rRNA gene. Also we included in the study representative strains of different biovars. The type strains of the species of genus *Rhizobium* analysed were clearly differentiated on the basis of the *celC* gene forming clearly different branches in the phylogenetic three. In this analysis were included all strains in which the *celC* gene has been sequenced and whose genomes are completely sequenced. Some of these strains were isolated from *Trifolium* or *Vicia* nodules and their assignment to the species *R. leguminosarum* probably should be reviewed. Nevertheless the more curious cases were the strains *R. etli* CIAT652 and *R. etli* bv. *mimosae* Mim-2 that have a *celC* gene nearly identical to that of *R. phaseoli* ATCC 14482^T. Another example is that of strain *A. radiobacter* (currently *R. radiobacter*) K84 whose *celC* gene is identical to that of *R. rhizogenes* 163C and almost identical to that of the type strain of *R. rhizogenes* ATCC 11325^T. The third interesting case is that of the strain *Rhizobium* sp. NGR234 that clustered with *E. medicae* WSM419.

Analysis of the* rrs *gene. The results of the *celC* gene analysis basically agree with those obtained with the *rrs* gene, nevertheless some considerations should be made. For example, *rrs* gene is not enough to differentiate some species as occurs in the case of *R. phaseoli, R. etli* and *R. pisi* whose *rrs* gene has near 100% identity among them. Since the *celC* gene was able to differentiate these three species, this gene is very useful for species differentiation when they have *rrs* gene closely related. Concerning to the strains whose complete genome has been sequenced, the results of the *rrs* analysis also confirmed those obtained in the case of the *celC* gene. *R. etli* CIAT652 and *A. radiobacter* (currently *R. radiobacter*) K84 have identical *rrs* that *R. phaseoli* ATCC 14482^T and *R. rhizogenes* ATCC 11325^T, respectively. Concerning to the strain *Rhizobium* sp. NGR234 the *rrs* gene analysis confirmed that they belong to genus *Ensifer* but probably represents a new species close to *E. fredii* and *E. saheli*.

Analysis of the* atpD *and* recA *genes. The resulting trees after the phylogenetic analyses of *recA* and *atpD* genes confirmed the results from the *celC* gene analysis showing that strains with high identity (even 100%) in their *rrs* genes could have highly variable protein coding genes. The results of both genes *recA* and *atpD* confirmed that *R. etli* CIAT652 and Mim-2 belong to *R. phaseoli* and not to *R. etli* and that *A. radiobacter* K84 belongs to *R. rhizogenes*. In the case of strain *Rhizobium* sp. NGR234 it is clear that this strain belong to the genus *Ensifer* being its close relatives *E. saheli* and *E. fredii* in agreement with the results of *rrs* gene analysis.

Analysis of the* nodC *gene. From the symbiotic genes pool, the *nodC* has been widely analysed in many rhizobial strains being related with the host range of rhizobia and the promiscuity degree of the hosts. *R. leguminosarum* and *R. pisi*, both isolated from *Pisum* nodules, have *nodC* genes nearly identical; however, these strains have divergent *recA, atpD* and *celC* genes. Even strains of different genera such as *R. mongolense* and *E. medicae* clustered together (90% identity) because they are able to nodulate *Medicago.* All these results showed no relationship between *celC* and *nodC* gene phylogenies in genus *Rhizobium*.

The results of this work show that the taxonomic status of several strains of these genera whose genomes have been completely sequenced should be revised and their names changed accordingly.

Acknowledgments

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Assessment of the bacterial diversity inhabiting *Alnus glutinosa* nodules in the Tormes River basin

Igual JM[1*], Valverde A[2], González-Tirante M[1], Medina-Sierra M[1,2], Santa Regina I[1]

[1]*Instituto de Recursos Naturales y Agrobiología de Salamanca, Consejo Superior de Investigaciones Científicas (IRNASA-CSIC), Salamanca, Spain;* [2]*Grupo Grica, Facultad de Ciencias Agrarias, Universidad de Antioquia, AA 1226 Medellín, Colombia. *mariano.igual@irnasa.csic.es*

Summary

We studied the genetic diversity of *Frankia* strains nodulating *Alnus glutinosa* along the basin of the Tormes River using DGGE as a method to detect 16S-23S rDNA IGS sequence polymorphisms. Our results confirmed those previously obtained by other molecular approaches regarding the low diversity of *Frankia* strains nodulating alders in this region. However, actinobacteria belonging to the genera *Rubrobacter* and *Nocardia* were also detected by DGGE in nodule samples. Therefore, this methodological approach may be useful to gain deeper insights on the actual microbial diversity existing within actinorhizal nodules.

Introduction

Actinomycetes included in genus *Frankia* are the microsymbiont of the N_2-fixing actinorhizal symbioses. Classical physiological testing is of little use in characterizing *Frankia* strains because they are difficult to isolate, or even refractory to isolation, and grow slowly. Polymerase chain reaction (PCR)-based procedures have proved to be very useful to characterize cultured as well nonculturable *Frankia* strains (Hahn *et al*., 1999). Up to date, only very limited research on genetic diversity of *Frankia* strains nodulating actinorhizal plants in the Iberian Peninsula has been conducted (Huguet *et al*., 2004; Igual *et al*., 2006). In a previous work (Igual *et al*., 2006), we assessed the genetic diversity of *Frankia* strains nodulating *Alnus glutinosa* along the Tormes River by PCR–RFLP analysis of the 16S–23S ribosomal spacer region (IGS) and sequencing of 16S rRNA and *glnII* genes. By these approaches there was found a very low genetic diversity among *Frankia* strains symbiotic with *A. glutinosa* along the Tormes River, which is in contrast with results from other World locations. The objective of the study was to further assess the genetic diversity of *Frankia* strains at the same locations using DGGE as method for detecting 16S-23S rDNA IGS sequence polymorphisms.

Materials and Methods

Nodule samples were collected from individual trees of *A. glutinosa* growing along the river Tormes' banks at 12 different locations. Nodule DNA extraction was performed using the methodology proposed by Khan *et al.* (2007). The oligonucleotide PCR primers used to amplify 16S-23S spacer regions of *Frankia* were: FGPS1493 (5'-GGCTGGATCACCTCCTTTCT-3') and FGPL 2054' (5'-CCGGGTTTCCCCATTCGG-3') from Simonet et al. (1991) but with FGPS containing a GC clamp. The PCR programme was: 95ºC for 5 min, 35x (94ºC for 1 min, 55ºC for 25 sec, 72ºC for 1 min), and 72ºC for 7 min. DGGE analysis was performed using the INGENYphorU-2 system. Samples were loaded in 6% (wt/vol) polyacrylamide gels in 1x TAE. The denaturant gradient was 30 to 55%, and electrophoresis was performed for 16 h at 85V and 60ºC. After electrophoresis, gels were removed and silver stained. Bands of interest were excised, eluted, reamplified and sequenced.

Results and Discussion

DGGE separation of 16S–23S IGS sequences amplified from nodule DNA by using the primer set FGPS1493 and FGPL 2054' allowed to distinguish several different patterns according to the number and position of bands (Figure). There were nodule samples from which one single band was detected (Figure, lane 1) while others showed a complex band pattern (Figure, lane 3). By reamplification and sequencing of the DNA eluted from discrete

excised bands, it was possible to determine the taxonomic identities of these nodular endophytes. The genetic diversity of the *Frankia* strains nodulating *A. glutinosa* was found very low since only two different strains were detected. This result confirms those previously obtained by other different approaches (Igual *et al.,* 2006). However, in addition to *Frankia*, other Actinobacteria seem to inhabit nodules of *A. glutinosa* in the region studied (Table), and up to three different strains of non-*Frankia* actinobacteria were detected in a single nodule (Figure, lane 3). This results agrees with reports from other authors (Ghodhbane-Gtari *et al.,* 2010; Valdes *et al.,* 2005) indicating that bacteria phylogenetically different to *Frankia* can be also present in actinorhizal nodules.

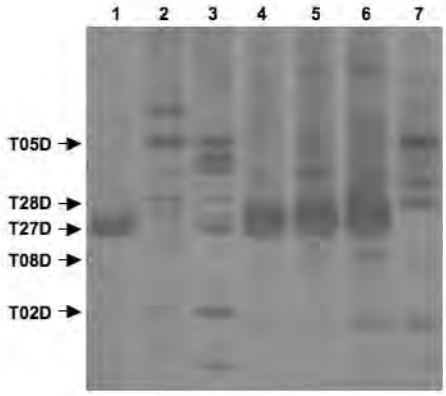

Table 1

Code	Homology (%)	Accession nº
T02D	*Rubrobacter xylanophilus* (87)	CP000386
T05D	*Nocardia vinacea* (81)	GQ853491
T08D	*Frankia* ALRH837 (96)	AY627713
T27D	*Frankia* TFAg5 (99)	DQ141198
T28D	*Nocardia blacklockiae* (82)	GQ853495

Acknowledgments

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Genetic diversity of rhizobia nodulating *Centrosema mole* in Venezuelan soils

Toro M[1], Velázquez E[2], Peix A[3*]

[1]*Laboratorio de Estudios Ambientales, Instituto de Zoología Tropical, Facultad de Ciencias, Universidad Central de Venezuela, Caracas 1041-A, Apartado 47058, Venezuela;* [2]*Departamento de Microbiología y Genética, Universidad de Salamanca, Campus Miguel de Unamuno, 37007 Salamanca, Spain;* [3]*Instituto de Recursos Naturales y Agrobiología (IRNASA), CSIC, Cordel de Merinas 40-52, 37008 Salamanca, Spain. *alvaro.peix@irnasa.csic.es*

Summary

In this study a collection of rhizobial strains from tropical ecosystems in Venezuela were isolated from nodules of *Centrosema mole* and characterized by molecular techniques to analyze their genetic diversity. Results show that they are slow-growing rhizobia within genus *Bradyrhizobium*, and according to 16S rRNA and 16S-23S rRNA intergenic spacer (ITS) analysis, an interesting genetic diversity is found among strains able to nodulate this legume, with strains closely related to different species of *Bradyrhizobium* such as *B. liaoningense*, *B. pachyrhizi*, *B. jicamae* or *B. elkanii*.

Introduction

Centrosema is a shrub leguminous genus within the tribe Phaseolae that includes 33 species, all of them native to America. Most species are widely distributed in the sabanna and forests of tropical regions (Penteado *et al.*, 1996). *Centrosema* is a very nutritive pasture legume occurring naturally in arid tropical ecosystems, thus being tolerant to acid soils (Schultze-Kraft *et al.*, 1990). For this reason, some species as *C. mole* or *C. macrocarpum* are commonly cultivated in agricultural systems in Venezuela as pastures and for their properties to increase nitrogen content in soils in combination with other crops in tropical agroecosystems, where acid soils with poor fertility occur. Even when *Centrosema* has been widely studied as forage legume for its properties, little attention has been paid up to date to their rhizobial endosymbionts. The few studies on this topic report the occurrence of slow growing rhizobia *Bradyrhizobium*-like, but no molecular characterization has been performed in most cases. Therefore, the aim of this work is the isolation and study of the genetic diversity of rhizobial strains nodulating *C. mole* in tropical ecosystems of El Guarico region, Venezuela.

Materials and Methods

Young nodulated plants of *C. mole* naturally growing in the field in El Guarico region were collected. Besides, tropical soils of the same region were sampled and placed in pots in greenhouse, and sterile seeds of *C. mole* were sown in the pots to use the legume as trap plant. The plants were harvested after 40 days and nodules were separated from roots and surface sterilized with 2% $HgCl_2$. A collection of rhizobial isolates were obtained by plating the crushed surface sterilized nodules in Yeast Mannitol Agar Petri dishes. Biodiversity analysis was performed by PCR-RAPD using the primer M13, and by sequencing of 16S-23S rRNA Intergenic Spacer (ITS). Representative strains of the ITS and RAPD groups were subjected to complete 16S rRNA gene sequencing.

Results and Discussion

The whole collection of isolates has not been completely analysed yet. All the isolates were slow-growing strains in YMA medium. Approximately 17 strains have been already analyzed, and according to the results of PCR-RAPD fingerprinting they can be grouped into three subsets. The analysis of 16S-23S rRNA Intergenic Spacer (ITS) sequences of the 17 strains show that they form three genotypic groups, in agreement with the PCR-RAPD fingerprinting. Representative strains of the three groups (strains CMVU02, CM04 and CM31) were subjected to phylogenetic analysis of ITS (Figure). CMVU02 and CMVU31

clustered within *Bradyrhizobium elkanii* group, with high sequence similarity (*c.* 98%) with the type strain of this species in the case of CMVU31, and also closely related to the type strain of *B. pachyrhizi*, isolated from *Pachyrhizus erosus* in Costa Rica (Ramírez-Bahena *et al.*, 2009). The strain CMVU04 cluster in the group of *B. liaoningense*, with a similarity of 99% in ITS sequence with respect to other strains also isolated from *Pachyrhizus* in different American countries such as Nicaragua or Mexico (Rodríguez-Navarro *et al.*, 2004). The three representative strains were subjected to complete 16S rRNA gene sequencing, and the results show that CMVU02 and CMVU31 have identical 16S rRNA sequence, with a similarity of 99.7% with respect to *B. pachyrhizi*, 99.6% with respect to *B. jicamae* and 99.5% with respect to *B. elkanii*. By contrast, CMVU04 is closely related to *B. liaoningense*, with 99.7% sequence similarity. These results reveal an interesting genetic diversity within bradyrhizobia nodulating *Centrosema* in tropical ecosystems, which merits to be thoroughly studied in future.

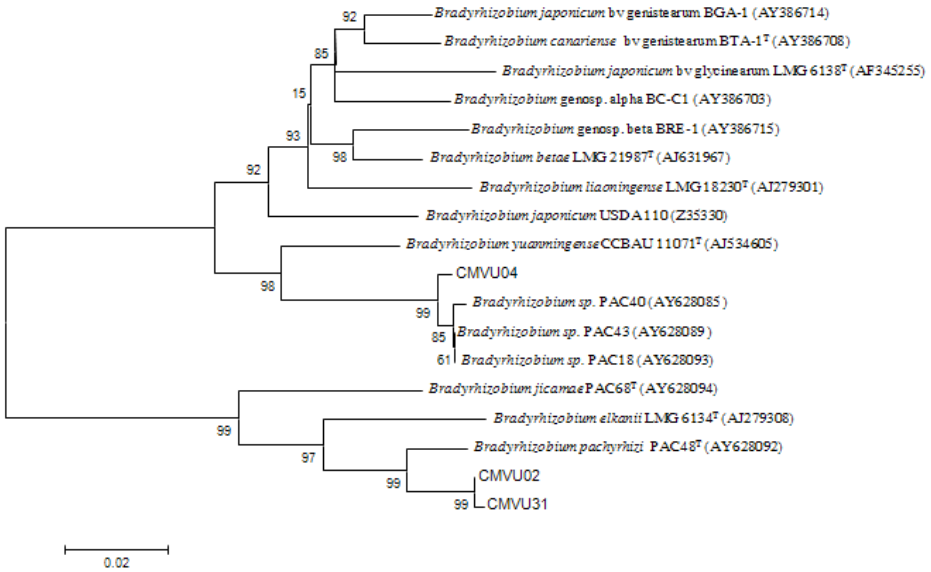

Figure. Neighbour-joining phylogenetic tree based on 16S-23S rRNA gene sequences (ITS) of strains nodulating *Centrosema mole* and closely related species within genus *Bradyrhizobium*.

Acknowledgments

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Rhizobium tropici IIA nodulates common bean in Portugal

Valverde A[1*], Velázquez E[2], Igual JM[1]

[1]*Instituto de Recursos Naturales y Agrobiología de Salamanca, Consejo Superior de Investigaciones Científicas (IRNASA-CSIC), Salamanca;* [2]*Departamento de Microbiología y Genética, Universidad de Salamanca, Spain.* **angel.valverde@irnasa.csic.es*

Summary

After the analysis of 179 strains isolated in six fields from three locations in North Portugal we found a significant prevalence of *R. lusitanum* (*c.* 79% of strains). The remaining 21% of strains were identified as *R. tropici* IIA on the basis of the *rrs* gene and ITS fragment analysis (100% identity with respect to the strain CFN299). The identity in both *rrs* and ITS sequences indicated a common phylogenetic origin of American and Portuguese strains belonging to *R. tropici* IIA. Although strains of *R. tropici* IIA have been previously described in other European soils this is the first time isolated in Portugal.

Introduction

Common bean (*Phaseolus vulgaris*) is a promiscuous legume indigenous from America nodulated by several species of rhizobia. *Rhizobium lusitanum*, a close relative species of *R. tropici*, was isolated from bean nodules formed in soils from a Portuguese region where a local variety of this legume has been cultivated for centuries (Valverde *et al.*, 2006). Surprisingly, strains belonging to other species from genus *Rhizobium* commonly found in the Iberian Peninsula, such as *R. etli, R. leguminosarum* or *R. gallicum* (Rodríguez-Navarro *et al.*, 2000; Velázquez *et al.*, 2001) were not found. In order to assess if rhizobial species other than *R. lusitanum* were present in these soils, we performed the isolation of a wide collection of strains nodulating bean and analyzed their diversity and phylogenetic relationships.

Materials and Methods

The strains were isolated on yeast manitol agar from nodules of a local variety of common bean (var. 'Pinta') grown at three geographical locations in the Northwest of Portugal (Arcos de Valdevez region). A total of 179 rhizobial strains were isolated from effective nodules. Biodiversity analysis by M13-RAPD fingerprinting was performed as previously described (Valverde *et al.,* 2006). *rrs* genes were sequenced according to Rivas *et al*. (2007) and ITS regions according to Kwon *et al*. (2005). Phylogenetic trees were inferred using the NJ method with the MEGA 4 package (Tamura *et al.*, 2007).

Results and Discussion

A total of six different patterns were found among the isolates by the M13-RAPD fingerprinting approach, which indicates the existence of a high genetic diversity in the strains that nodulate beans in the Arcos de Valdevez region (Portugal). The identification of strains was based on the sequencing of both the *rrs* gene and the ITS fragment. The *rrs* gene allows the identification of rhizobia at genus level and to know the closest relative species (Kuykendall *et al.*, 2005) and the ITS fragment permits to analyse the closeness among strains from the same species (Kwon *et al.*, 2005). Strains from the same species with very close ITS sequences probably have the same phylogenetic origin and, therefore, ITS sequences are a good tool to perform biogeography analysis of rhizobial species (Álvarez-Martínez *et al.*, 2009).

According to the results of *rrs* analysis, the strains showing the RAPD patterns A to E were identified as *R. lusistanum* since they had 100% of identity with the type strain of this species, P1-7^T, which also presented the RAPD pattern A. The identification was confirmed after the ITS sequence analysis since all these strains presented near to 100% identity with respect to that of *R. lusitanum* P1-7^T (Figure).

The strains displaying the RAPD pattern F presented a *rrs* gene 100% identical to that of *R. tropici* IIA CFN299 and about 99.3% with respect to *R. tropici* IIB CIAT 899^T (gaps not considered). It has been previously reported the existence of a 70 bp insert in the *rrs* gene of *R. tropici* IIA CFN299 (located between nucleotides 49 and 120) that is absent in *R. tropici* IIB CIAT 899^T (Willems & Collins, 1993). Interestingly, an insert completely identical in length and sequence to that of *R. tropici* IIA CFN299 is present in the Portuguese strains and, therefore, constitutes the first evidence of the common phylogenetic origin of Portuguese and American strains of *R. tropici* IIA. A second evidence was found after the analysis of the ITS fragment in the Portuguese strains (Figure). The strains identified as *R. tropici* IIA have ITS sequences identical to that of *R. tropici* IIA CFN299. Therefore, from a chromosomal point of view, the American origin of strains of *R. tropici* IIA nodulating beans in Portugal seems to be clear. This is a surprising and interesting result not only because it is the first report about the presence of *R. tropici* in Portuguese soils but mainly by the absence in them of *R. etli*.

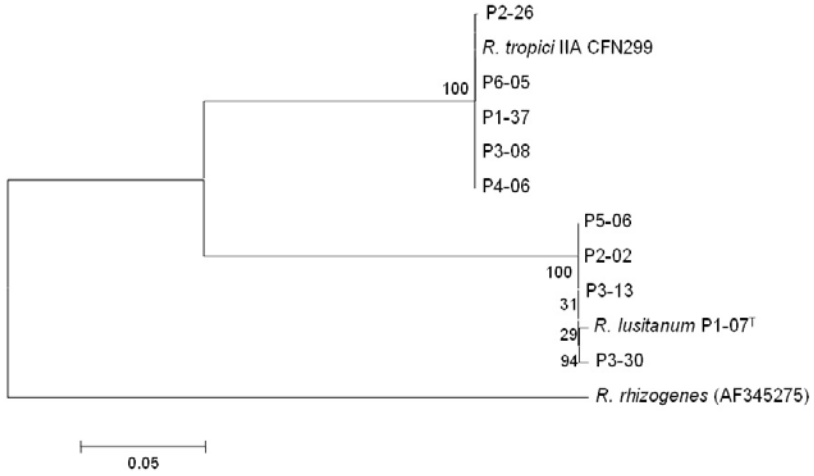

Figure. Neighbour-joining tree based on ITS gene sequences of isolated strains and closely related species. The significance of each branch is indicated by a bootstrap value calculated for 1000 subsets. Bar, 5 substitutions per 100 nt.

Acknowledgments

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Identification of a 70 bp insert in *rrs* gene of *Rhizobium leguminosarum* nodulating several legumes in Salamanca phylogenetically close to that of *R. tropici* IIA

Flores JD, Velázquez E*, García-Fraile P, Rivas R

*Departamento de Microbiología y Genética. Universidad de Salamanca. Spain. *evp@usal.es*

Summary

In this study we isolated 32 fast growing strains from effective nodules of common bean (*Phaseolus vulgaris*) from a Salamanca soil from which we have previously isolated rhizobia nodulating clover, lentils and chickpea. The isolates were distributed into 12 groups after TP-RAPD analysis and representative strains from each group were subjected to *rrs* gene analysis. The results of this analysis showed that the strains isolated were close to *Rhizobium leguminosarum* according to their partial 16S rRNA gene sequences. This species has been already found in bean nodules in León and Ávila, two adjacent regions to Salamanca. However, some strains isolated in this study have an insert at the beginning of the 16S rRNA gene identical to that found in strains isolated in the same region from lentil and phylogenetically related with that of *R. tropici* IIA CFN 299. This finding could have great importance for biogeography studies since it has also found in European and American species nodulating different hosts.

Introduction

Common bean (*Phaseolus vulgaris*) is a legume indigenous from America which is currently cultivated in many countries in different continents. In Spain, the main producer of this legume is the Castilla y León region. However, in this region there are many soils where beans have not been cultivated yet and where, instead, lentil (*Lens culinaris*) and chickpea (*Cicer arietinum*) are common legume crops, as occurs in Salamanca province. Recently we have found that beans are nodulated by *R. leguminosarum* in a zone traditionally cultivated with this host in North Spain (García-Fraile *et al.,* 2010). These results are in contrast with those found in Southern Spain, in which *R. etli*, an American strain, was the most abundant bean endosymbiont in soils without bean cultivation history (Rodríguez-Navarro *et al.*, 2000). Because in Northern Spain there also are soils uncultivated with beans, the aim of this study was to identify the bean endosymbionts in these soils in order to compare them with those isolated in other Spanish regions.

Materials and Methods

The strains were isolated on yeast manitol agar from nodules of a local variety of common bean (var. 'Pinta'). TP-RAPD pattern analysis was performed as was previously described (Rivas *et al.,* 2002). The *rrs* genes were amplified and sequenced according to Rivas *et al*. (2007). The sequences obtained were compared with those from GenBank using the BLASTN program (Altschul *et al.,* 1990), and aligned using the Clustal W software (Thompson *et al.,* 1997). The distances were calculated according to Kimura´s two-parameter model (Kimura, 1980). Phylogenetic trees were inferred using the neighbour-joining method (Saitou & Nei, 1987). Bootstrap analysis was based on 1000 resamplings. The MEGA 4 package (Tamura *et al.,* 2007) was used for all analyses.

Results and Discussion

The strains isolated in this study were distributed into 12 groups according to their TP-RAPD patterns. A representative strains of each group was selected for *rrs* gene sequencing in order to classify them. The sequences obtained were compared with those held in Genbank and all of them were close to the *R. leguminosarum rrs* gene. This species is the most common endosymbiont in other soils from Castilla y León that we analysed in a previous study (García-Fraile *et al.*, 2010). However some of the strain isolated in Salamanca have an insert

that it is absent in the strains nodulating beans up to date. This insert, of about 70 bp, was firstly described by Willems & Collins (1993) in *R. tropici* IIA CFN299 and is located at the beginning of the *rrs* gene. The sequence of this insert is identical in *R. leguminosarum* strains isolated from bean and those isolated from lentils in the same region (Salamanca). However some differences in the sequence of this insert have been found in *R. leguminosarum* strains isolated from beans with respect to those found in *R. tropici* (4 nucleotides in 70). These findings could be crucial for studying the chromosomal origin of strains nodulating legumes from the cross-inoculation group of beans and thus for biogeography studies.

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TTCAAGCAAGCTTGAAGGATTTTTATCCTTGGAAAGGAAGATCAAGAAGAGCTTCTAAGAAGCTTTCTTGATGG MLS05 (lentil, Salamanca)
TTCAAGCAAGCTTGAAGGATTTTTATCCTTGGAAAGGAAGATCAAGAAGAGCTTCTAAGAAGCTTTCTTGATGG PEPV03 (beans, Salamanca)
TTCAAGCAAGCTTGAAGGATTTAC-TCCTTGGAAAAGAAGATCAAGA-GCGCTTCTAAGAAGCTTTCTTGATGG CFN299 (beans, América)
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Figure. Alignment of the insert found in strains of *Rhizobium leguminosarum* isolated in Salamanca and *R. tropici* IIA CFN229.

Acknowledgments

P. García-Fraile was supported by a postdoctoral contract from a MICINN financial project to E. Velázquez.

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Genetic diversity of rhizobia nodulating alfalfa

Rodrigues S[1,2], Ferreira E[1], Figueira E[2], Castro IV[1*]

*[1]UARN, Laboratório de Microbiologia, L-INIA, INRB, IP, 2770-004 Oeiras, Portugal; [2] Departamento de Biologia, Universidade de Aveiro, Aveiro, Portugal. *isabelvcastro@net.sapo.pt*

Summary

The aim of this work was to characterize *Sinorhizobium* (now *Ensifer*) sp. natural population present in an Alentejo soil, from the southern of Portugal, isolated from nodules of different alfalfa (*Medicago sativa*) cultivars. Genetic diversity and symbiotic efficiency were evaluated. Analyzing the different patterns obtained by REP-PCR and ERIC-PCR, the existence of several clusters was verified, indicating a high genetic diversity among rhizobial population studied. The results obtained with PCR amplified 16S rDNA products digested with RsaI enzyme showed a prevalence of *E. medicae* strains (73) in relation to *E. meliloti* strains (18). *E. meliloti* strains had low values of symbiotic effectivness with both *Medicago* species, when compared with *E. medicae* strains, which show to be high effective with *M. polymorpha* and medium to low effective with alfalfa cultivars.

Introduction

The genus *Medicago* is an important forage legume that includes perennial and annual species, such as alfalfa (*M. sativa* L.) and *M. polymorpha* L., respectively. Alfalfa and its symbiont *Sinorhizobium* (now *Ensifer*) *meliloti* have a long history of coexistence and coevolution. In region where alfalfa has been cultivated for centuries, the natural nodulating population of *E. meliloti* plays a major role in satisfying the nitrogen requirements of the plants. Also, natural populations of rhizobia isolated from alfalfa could be related to *Sinorhizobium* (now *Ensifer*) *medicae*. *M. polymorpha* is native to the Mediterranean basin, including Portugal where it grows spontaneously, being natural and effective nodulation restricted to *E. medicae* (Garau *et al.,* 2005). The aim of this work is concerned with the importance to study the genetic structure of natural populations of *Ensifer* sp. and their dynamics in relation to the host plant. Under this point of view the rhizobial natural population present in an Alentejo soil (southern of Portugal) was isolated from nodules of different alfalfa cultivars and characterized genetically and symbiotically.

Materials and Methods

About 100 bacterial isolates from nodules of five alfalfa cultivars (Magali, ABT Coussouls, Melissa and Mamuntanas) were used. The genetic diversity was assessed by REP-PCR and ERIC-PCR (De Bruijn, 1992) and restriction fragment length polymorphism analysis of polymerase chain reaction (PCR/RFLP) of 16S rDNA gene, using the enzyme *Rsa*I. Bacterial DNA was extracted by the "Nucleospin extraction kit" and used in the PCR amplifications. The 16S rDNA gene from all the *Ensifer* strains was amplified with the primers fD1 and rD2 (Weisburg *et al.,* 1991) and the PCR products derived from each strain were digested separately with the enzyme *Rsa*I. *Ensifer* species were assigned to *E. meliloti* and *E. medicae* according to the RsaI pattern of PCR-amplified 16S rDNA as described by Laguerre *et al*. (1997) and Zibri *et al*. (2004). For evaluation nitrogen-fixing activity, *Ensifer* strains were inoculated on their original host alfalfa cultivar and also in *M. polymorpha* (cultivar 66, an annual medic from ENMP). Plants were growing during 8 weeks in controlled environmental conditions and the effectiveness was determined by comparing the mean dry weight of shoots from inoculated plants grown in nitrogen-free medium with those of non-inoculated nitrogen- supplied plants.

Results and Discussion

Analyzing the different patterns obtained by REP-PCR and ERIC-PCR, it was verified the existence of several clusters indicating a high genetic diversity among the population studied. It was also found that the cultivar influenced strongly the cluster distribution of *Ensifer* sp. strains. Gel electrophoresis of the PCR amplified 16S rDNA products digested with the enzyme *Rsa*I show different patterns (Figure, **a**). The results obtained permitted to identify 73 strains as *E. medicae* and 18 as *E. meliloti.*

Symbiotic effectiveness showed differences among *Ensifer* sp. strains tested. Strains from Melissa and Mamuntanas cultivars had the highest values for symbiotic effectiveness and strains from Coussouls cultivar had the lowest values for symbiotic effectiveness (Figure, **b**).

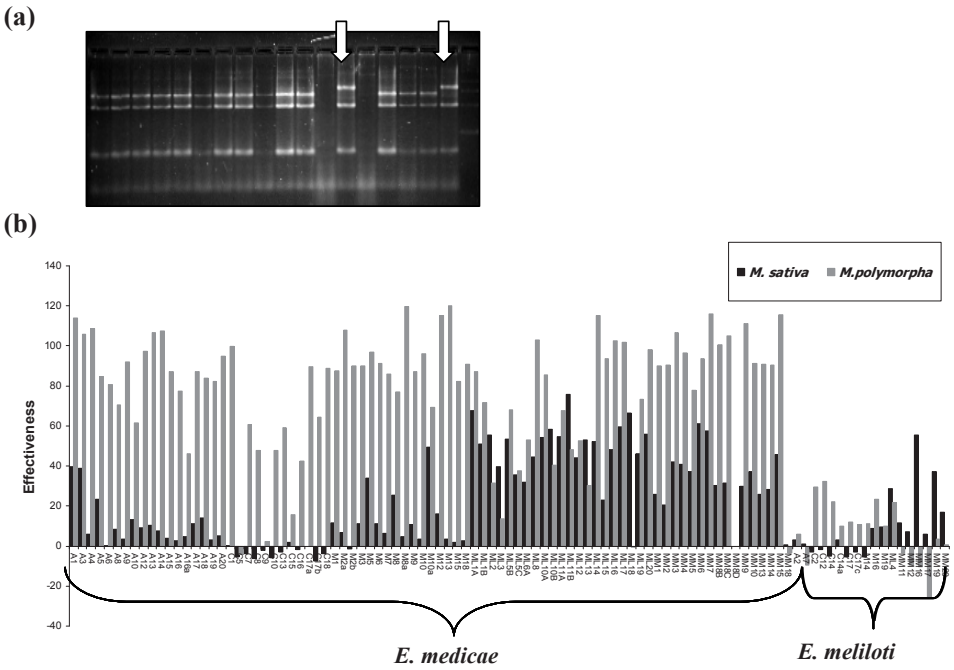

Figure. Identification and effectiveness of *Ensifer* strains. **(a)** PCR/RFLP of 16S rDNA, with RsaI restriction profiles. Lanes with an arrow represents the *E. meliloti* strains and the remaining *E. medicae*. **(b)** Effectiveness of *E. medicae* and *E. meliloti* strains with alfalfa cultivars (ABT - A, Coussouls – C, Magali – M, Melissa – ML, Mamuntanas – MM) and *Medicago polymorpha*.

The results showed that strains of *E. medicae* were more effective with *M. polymorpha* than with alfalfa, although these strains had been isolated from the latter host. Results also showed that *E. meliloti* strains had low values of symbiotic effectivness with both *Medicago* species, when compared with *E. medicae*.

Acknowledgments
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Isolation and characterization of endosymbiotic bacteria from copper contaminated soils in Chile

Durán D[1], Soto C[2], Rubio L[3], Cabrera E[1], Prieto RI[1], Palacios JM[1], Baginsky C[2], Brito B[1*]

*[1]Departamento de Biotecnología, Universidad Politécnica de Madrid, Avenida Complutense s/n, 28040 Madrid and Centro de Biotecnología y Genómica de Plantas, UPM, Campus de Montegancedo - Carretera M40, km 37.7, Pozuelo de Alarcón, 28223 Madrid, Spain; [2]Departamento de Producción Vegetal, Facultad de Ciencias Agronómicas Universidad de Chile, Casilla 1004, Santiago, Chile; [3]Departamento de Química Agrícola, E.T.U. Ingenieros Agrícolas, Ciudad Universitaria s/, 28040 Madrid and Centro de Biotecnología y Genómica de Plantas, Campus de Montegancedo, Pozuelo de Alarcón, 28223 Madrid, Spain. *belen.brito@upm.es*

Summary

Legume endosymbiotic bacteria indigenous of copper (Cu)-contaminated soils from Chile have been isolated using pea (*Pisum sativum*), bean (*Phaseolus vulgaris*) and alfalfa (*Medicago sativa*) as trap host plants. Highly contaminated soils only produced nodules in certain legume hosts, whereas nodulation was observed in the three legume hosts when inoculated with soils containing a low Cu concentration. A collection of 362 strains was isolated, and their levels of Cu resistance were tested in media supplemented with increasing metal concentrations and in disk diffusion assays. By these two approaches, 84 strains displaying levels of Cu resistance higher than those exhibited by the corresponding reference strains were selected. The most resistant strains isolated from alfalfa and bean nodules grew normally at 3 mM and 2.5 mM $CuSO_4$ and were obtained from two different highly contaminated soils. Strains nodulating pea plants showed similar levels of resistance to Cu (2-2.5 mM $CuSO_4$) and were isolated from low-contaminated soils. Our data suggest a reduction of microbial diversity in agricultural Cu-contaminated soils from Chile.

Introduction

The widespread use of Cu in bactericides and fungicides lead to the contamination of agricultural soils mainly due to its accumulation to toxic levels in the surface soil layer. It is known that Cu and other heavy metals have a negative effect on legume endosymbiotic bacteria and on nitrogen fixation process (Laguerre *et al*., 2006), but the level of tolerance to Cu is highly variable among species and strains (Lakzian *et al*., 2002). The Cu extraction processes and wastes affect many agricultural soils in Chile. This situation raised the interest for studies of the biodiversity of legume endosymbiotic bacteria in these soils, and for the isolation of bacteria highly resistance to this heavy metal, in order to identify bacterial mechanisms involved in Cu homeostasis and bacterial survival in contaminated soils.

Materials and Methods

Pea (*Pisum sativum*), bean (*Phaseolus vulgaris)* and alfalfa (*Medicago sativa*) seedlings were inoculated with soils suspensions. Nodules were surfaced-sterilized, crushed and spread in YMB and R minimal media. Cu resistance of strains was analyzed by growing in plates of TY medium with increasing concentrations of $CuSO_4$. For disk diffusion tests (Bauer *et al.,* 1966), exponential cultures were inoculated in TY agar medium and disks soaked in different $CuSO_4$ concentrations. The inhibition zone was measured after 48 hours of incubation. Phylogenetic analysis was carried out with the 16S rDNA sequences of isolated strains. DNA sequences were optimally aligned using the CLUSTALX program. Neighbor-joining matrixes and trees were generated employing the MEGA 4.1 and the phylogenetic web server at www.phylogeny.fr.

Results and Discussion

The aim of this work was to study de diversity of *Rhizobium* species in Cu-contaminated soils of Chile and the identification of rhizobial strains with increased resistance to Cu. To do this, nine soils were prospected from the III, IV and VI regions. Among them three soils, designated as 7-12, 9-11 and Copiapó, contained the highest concentration of Cu and were classified as highly contaminated. Pea, bean and alfalfa plants were used as trap legume hosts to isolate the rhizobia indigenous population. After three weeks of soil inoculation, root

nodulation was checked. In general all hosts inoculated with the less contaminated soils showed a normal nodulation, whereas plants inoculated with suspensions of highly contaminated soils failed to nodulate certain hosts. In fact, no nodulation was observed in any host inoculated with the Copiapo soil sample, the most contaminated soil. A collection of 362 isolates from pea, bean and alfalfa nodules was obtained. Cu resistance was analyzed by growing isolated strains in plates containing concentrations of Cu ranging from 0.5 to 3.5 mM $CuSO_4$ and by disk diffusion tests (Figure). By these approaches 84 strains displaying levels of Cu resistance higher than those exhibited by the corresponding reference strains were selected. The most resistant strains isolated from alfalfa and bean nodules grew normally at 3 mM and 2.5 $CuSO_4$ and were obtained from highly contaminated soils 7-12 and 9-1. All strains obtained from pea nodules showed similar levels of resistance to Cu (2-2.5 mM $CuSO_4$) and were isolated from non-contaminated soils.

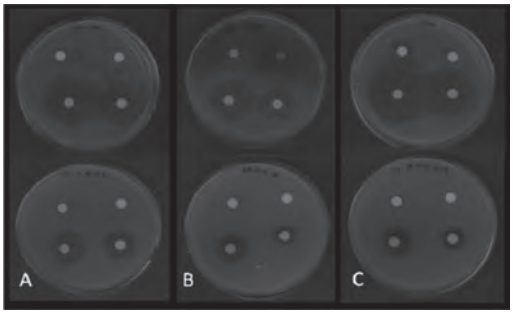

Figure. Analysis of Cu resistance of endosymbiotic bacteria isolated from pea (strain G.M.D.4, Panel **A**, bottom), bean (strain J.9-11.C.14, Panel **B**, bottom), and alfalfa (strain A.7-12.C.19, Panel **C**, bottom) nodules by disk diffusion tests. Plates in the top are inoculated with the respective control strains: *R. leguminosarum* strain UPM791 (Panel **A**), *R. etli* CFN42 (Panel **B**) and *S. meliloti* 1021(Panel **C**). Each plate contains disk soaked in water, 100 mM, 500 mM, 1 M $CuSO_4$.

According to the phylogenetic characterization based on 16S rRNA sequences, the pea isolates are grouped together forming a monophyletic group together with the corresponding strains of *R. leguminosarum* bv. *viciae*, which agrees with the results of the tests of nodulation. The sequences of the bean strains showed a high sequence similarity with the corresponding of *R. etli* and *R. leguminosarum* bv. *phaseoli* strains. Sequences belonging to the alfalfa isolates grouped together with sequences coding for 16S rRNA in strains of *S. meliloti*, and *Sinorhizobium* sp. Our data suggest a reduction of microbial diversity in agricultural Cu-contaminated soils from Chile.

Acknowledgments

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Phenotypic and phylogenetic characterization of endosymbiotic bacteria from *Lupinus mariae-josephi*

Sánchez C[1], Rey L[1], Durán D[1*], Navarro AJ[2], Sánchez P[1], Temprano F[3], Imperial J[1,4], Ruiz-Argüeso T[1]

[1]Departamento de Biotecnología, Universidad Politécnica de Madrid, Avenida Complutense s/n, 28040 Madrid and Centro de Biotecnología y Genómica de Plantas (CBGP), UPM, Campus de Montegancedo-Carretera M40 km 38, 28223 Pozuelo de Alarcón (Madrid), Spain; [2]Centro de Investigación y Experimentación Forestal (CIEF), Avenida Comarques del País Valencià, 114, 46930 Quart de Poblet (Valencia), Spain; [3]IFAPA Las Torres-Tomejil, Carretera de Sevilla-Cazalla Km 12.2, 41200 Alcalá del Rio (Sevilla), Spain; [4]CSIC.
**david.duran.wendt@alumnos.upm.es*

Summary

Lupinus mariae-josephi is a *Lupinus* species that thrives in a Southeastern area of Spain (Valencia) in soils of singularly high pH and active lime content. It is nodulated by extra-slow growing bacteria symbiotically and phylogenetically distant to endosymbiotic strains nodulating other *Lupinus* sp. native of the Iberian Peninsula and adapted to growth in acid soils. Cross-inoculation experiments revealed that the *L. mariae-josephi* endosymbiotic bacteria are unable to nodulate or efficiently fix nitrogen with well-known *Lupinus* spp. Their species affiliation was examined by a multilocus sequence analysis of four housekeeping genes (16S rDNA, *glnII, recA, atpD*) and the symbiotic *nodC* gene. Single and concatenated phylogenetic analyses of these genes consistently revealed that *L. mariae-josephi* endosymbiotic bacteria belong to a clade, within the *Bradyrhizobium* genus, highly differentiated from the *Bradyrhizobium* clade that includes currently named *Bradyrhizobium* species as well as the endosymbiotic bacteria from *Lupinus* species tested in this study. Within this new clade the *L. mariae-josephi* bacteria nested in several subgroup that may correspond to novel sister species. The phylogenetic analysis based on the *nodC* gene showed that *L. mariae-josephi* endosymbiotic bacteria define a novel branch of the *nodC Bradyrhizobium* tree and likely have a common unique ancestor for the symbiotic genes with nodule isolates from *Retama* spp.

Introduction

Based on their legume-host ranges and on phylogenetic analysis, a significant heterogeneity among bradyrhizobia nodulating *Lupinus* spp. has been unraveled in the last few years. Most isolates have been related to the *B. canariense* and the *B. japonicum* lineages (Jarabo-Lorenzo *et al.,* 2003; Vinuesa *et al.,* 2005; Stepkowsky *et al.,* 2007). *Lupinus mariae-josephi,* a recently described species of *Lupinus* (Pascual, 2004), thrives in soils of high pH and active lime content in a southeastern area of Spain (Valencia), and it is endangered due to its reduced habitat.

Materials and Methods

Isolates of *L. mariae-josephi* endosymbiotic bacteria were obtained using trap plants from soils of five spots of native plant population of Llombai area (Valencia). Phylogenetic trees were generated by Neighbor-joining (NJ) and Maximum-likelihood (ML) methods employing the MEGA 4.1 and PAUP software and the phylogenetic web server at www.phylogeny.fr.

Results and Discussion

L. mariae-josephi is nodulated by extra-slow growing bacteria with symbiotic and phylogenetic characteristics singularly different from *Bradyrhizobium* strains nodulating other *Lupinus* sp. native of the Iberian Peninsula and adapted to grow in acid soils. Cross-inoculation experiments performed with five representative isolates (Table) showed that *L. mariae-josephi* strains do not nodulate or efficiently fix N_2 with other *Lupinus* spp. Their species affiliation was examined by a multilocus sequence analysis of four housekeeping genes (16S rDNA, *glnII, recA, atpD*) and the symbiotic *nodC* gene. Single and concatenated

(*glnII+recA* and *glnII+recA+atpD*) analysis consistently revealed that *L. mariae-josephi* endosymbiotic bacteria are members of the *Bradyrhizobium* genus and belong to a single evolutionary clade (Clade I) highly differentiated from the *Bradyrhizobium* Clade II that includes currently named *Bradyrhizobium* species and, singularly, all the endosymbiotic bacteria from *Lupinus* species adapted to acid soils in the Iberian Peninsula.

Table. Legume host-range analysis of representative isolates of *Lupinus mariae-josephi* endosymbiotic bacteria strains.

Strains / Legume hosts	LmjA2 Nodulation + NF	LmjB2b Nodulation + NF	LmjC Nodulation + NF	LmjD2b Nodulation + NF	LmjH2p Nodulation + NF
L. mariae-josephi	Yes + high	Yes + high	Yes + high	Yes + high	Yes + high
L. angustifolius	No	No	No	No	No
L. luteus	No	No	No	No	No
L. micranthus	Yes + poor	No	Yes + poor	No	Yes + poor
L. hispanicus	No	No	No	No	No
L. cosentinii	Yes + poor	Yes + poor	Yes + high	No	Yes + poor
L. gredensis	No	No	No	No	No
L. albus	Yes + poor	Yes + poor	Yes + high	Yes + poor	Yes + poor
M. atropurpureum	No	No	Yes + poor	ND	No
O. compressus	No	No	No	ND	No

The combined results from these analyses showe that the tested *L. mariae-josephi* isolates nest in three sub-groups that might correspond to novel sister *Bradyrhizobium* species.

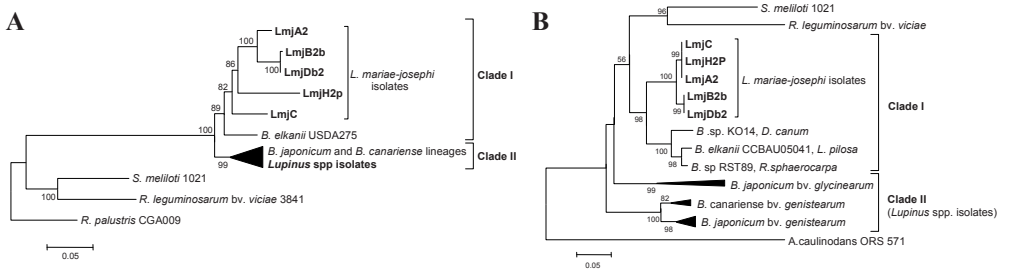

Figure. NJ trees showing the phylogenetic relationship of *L. mariae-josephi* isolates with isolates from *Lupinus* spp. and rhizobial reference strains based on concatenated *glnII+recA+atpD* genes **(A)** and on symbiotic *nodC* gene **(B)**.

The 16S rDNA topology tree showed that the Clade I also includes bradyrhizobia isolates from *Retama* spp. (Boulila *et al.,* 2009) and *Phaseolus lunatus* (Ormeño-Orrillo *et al.,* 2006) as well as *B. elkani, B. pachyrhizi* and *B. jicamae* species. The phylogenetic analysis based on the *nodC* gene showed that all *L. mariae-josephi* endosymbiotic bacteria studied defined a novel branch of the *Bradyrhizobium* tree. In contrast, the symbiotic genes of isolates from other *Lupinus* spp. of the Iberian Peninsula are clearly related with the *B. canariense* lineage. The allopatric speciation of *L. mariae-josephi* bradyrhizobia may have its origin in the colonization by its singular legume host of a singular habitat, such as the basic and high calcium carbonate soils of Valencia area.

Acknowledgments

We thank Drs. Fos and Ferrando (Conselleria de Medi Ambient, Aigua, Urbanisme i Habitatge, Valencia), Dra. de la Rosa (Centro Nacional de Recursos Naturales, INIA, Alcalá de Henares) and Dr. Pascual for providing the *Lupinus mariae-josephi* seeds and soil samples. This research was financed by Fundación del BBVA (FBBVA) 2009-2012.

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A mathematical model for *Rhizobium*-legume mutualism

Marco DE[1 *], Carbajal JP[2], Cannas S[3]

*[1]Laboratorio de Ecología Matemática, Area de Producción Orgánica, Facultad de Ciencias Agropecuarias, Universidad Nacional de Córdoba, Ciudad Universitaria, CC 509, 5000 Córdoba, Argentina; [2]Artificial Intelligence Laboratory, Dept. of Informatics, University of Zurich, Andreasstrasse. 15, CH-8050 Zurich, Switzerland; [3]Instituto de Física de la Facultad de Matemática, Astronomía y Física (IFFMAMAF-CONICET), Universidad Nacional de Córdoba,Ciudad Universitaria, 5000 Córdoba, Argentina. *dmarco@agro.unc.edu.ar*

Summary

We analyze the ecology stability of *Rhizobium*-legume symbiosis, using population dynamic models. We include in the model two rhizobial strains differing in their ability to fix N_2 for the plant, and explored the case of sanctions applied by the plant to ineffective rhizobia. Other factors addressed are horizontal transfer of *sym* genes, competition between fix+ and fix- for nodulation, co-occupation of the same nodule by the two strains, and plant stress. Our model predicts a critical a fraction of fixing rhizobia in soil, represented by the fixing rhizobia needed to provide a minimum N_2 amount for plant population persistence. We show that a simple population model can explain the coexistence between fixing and non-fixing strains observed at field, without the need of incorporating any sanction towards non-fixing rhizobia.

Introduction

Biology of *Rhizobium*-plant symbiosis has been extensively studied. In contrast, fewer attempts have been made to understand its ecology and in particular, mathematical models are scarce in the literature. In this work, we model the ecology of *Rhizobium*-legume symbiosis. As rhizobial strains can vary in their N_2 fixation ability, we considered two strains, fixing (fix+) and no fixing (fix-), and explored the case of sanctions applied by the plant to ineffective rhizobia. Other relevant factors addressed are horizontal transfer of *sym* genes, competition between fix+ and fix- strains for nodulation, co-occupation of the same nodule by the two strains, and the effect of plant stress.

Materials and Methods

We considered a mutated *Rhizobium* lacking fixation activity but showing similar competitiveness respect to the effective wild-type (Marco *et al.,* 2009a,b). We modelled the system using three simple logistic mappings, representing the plant population and the two the populations of free fixing (fix+) and non-fixing (fix-) bacteria in soil. Initial values of plant and bacteria populations set the values of bacteria in nodules, with a potential and a final number of nodules which provide N_2 to the plants. New populations are calculated based on the produced seeds and the released bacteria. Sanction of the plant to fix- strain was incorporated as 0% (complete sanction), 50% (moderate sanction) and 100% (no sanction) survival of released bacteroids from nodules to the soil. Horizontal transfer of *sym* genes occurs from fix+ to fix- strains. Competition between strains was included as $\log N_{fix+}:N_{fix-} = \log C_{fix+\ fix-} + k \log I_{fix+}:I_{fix-}$, where N_{fix+}, N_{fix} = nodules formed by fix+ and fix- strains, and I_{fix+}, I_{fix-} = number of cells of the two strains in soil. Plant seed production decreases with the percentage of co-occupied nodules and stress severity.

Results and Discussion

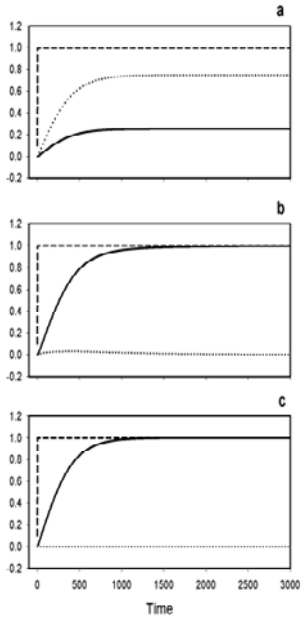

Figure. Dynamics of plant (dashed line), fix+ strain (solid line) and fix- strain populations (dotted line) for no sanction (a), moderate sanction (b), and total sanction (c).

Figure. In **(a)** we show the typical behaviour of plant and strains populations with no sanction. Even starting with low population values of fixing rhizobia and comparatively high population values of non-fixing rhizobia coexistence between strains is met in the long term. Plants persist at the carrying capacity values since early simulation steps. With intermediate sanction, i.e., half of the nodules prevented from releasing bacteria into the soil, the plant population survives equally well, but, as expected, a substantial reduction in fix- numbers can be seen and its population remains very close to zero (**Figure, b**). With extreme sanction, the plants halt all the non-fixing bacteria inside the nodules coming into the soil and thus fix- rhizobia go extinct very fast (**Figure, c**). The fixing bacterial populations grow faster due to the reinsertion to the soil of the bacteria coming from the senescent nodules. It is clear that the number of fixing bacteria, will increase with time and eventually go to 1. This means that, in the long term when the plant population persists by applying sanctions, only fixing rhizobia will be present in the system.

Our results predict a minimum fraction of effective rhizobia needed to maintain plant populations, and show that the coexistence between fixing and non-fixing strains observed at field can be explained without the need of incorporating any sanction towards non-fixing rhizobia. Inclusion of sanction in the model leads to the unrealistic result of non-fixing strains disappearing from the soil.

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Acknowledgments

This research was supported by grants from Secyt , Agencia Córdoba Ciencia and CONICET (Argentina). We thank Dr. Juan Sanjuán, Prof. José Olivares and staff of Estación Experimental del Zaidín (CSIC, Spain) for helpful discussions. D.E.M. and S. A. C. are members of the National Scientific Council (CONICET) of Argentina

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Influence of nitrogenous fertilization in the biodiversity of soils used for rice cultivation in the marshes of the Guadalquivir

Romero Cuadrado L*, Del Castillo I, Megías M

*Departamento de Microbiología y Parasitología, Facultad de Farmacia, Sevilla, Spain. *lrc@us.es*

Summary
Denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rRNA gene segments was used to profile microbial populations inhabiting different areas of the marshes of the Guadalquivir. DGGE allowed a rapid evaluation of the distributions of amplifiable sequence types. The use of ribosomal RNA (rRNA) as molecular marker to identify microbial populations is now routinely used in microbial ecology. One of the strategies herein is cloning of PCR products obtained from environmental DNA to explore the diversity of microbial communities without the need of cultivation. By using this approach, we know how the population change along the growing of the rice. We can know the variability of microbial diversity depending on using a type of fertilization.

Introducción

El cultivo del arroz sigue siendo, hoy en día, un cultivo de subsistencia, y permite alimentar a gran parte de la población mundial. Por otro lado, presenta escasas exigencias edafológicas circunscribiéndolo a suelos poco fértiles, de tierras anegables y marismas, por ello no aptos para otros tipos de cultivos. La producción arrocera en la CEE (Comunidad Económica Europea) se centra en los países del Mediterráneo, destacando Italia y España con un 55,91% y un 26,72% respectivamente del total comunitario. Con altibajos ligados a las disponibilidades hídricas, la producción media del arrozal sevillano se estima en unas 330.000 Tm de arroz-cáscara con unos rendimientos de 8.000-9-000 Kg/ha (Federación de Arroceros de Sevilla) lo que representa más del 40% del total nacional.

Las comunidades microbiológicas existentes en el suelo participan en la movilización de nutrientes poniéndolos a disposición de las plantas, por lo que nos indican la fertilidad del mismo. Por tanto, la alteración de las mismas puede repercutir en perjuicio de los cultivos de las plantas. Este estudio se ha realizado mediante la técnica PCR-DGGE, que nos permite elaborar un perfil de la diversidad microbiana del suelo de los arrozales para poder estudiar los cambios en las poblaciones en relación con el tipo de fertilizante empleado y la manera de aplicarlo, a lo largo de las fases del desarrollo de la planta.

Materiales y Métodos

Las muestras fueron recogidas en distintas parcelas de experimentación y en diferentes etapas del desarrollo de la planta. Se procedió a la extracción del ADN del suelo según el protocolo del kit comercial *Ultraclean Soil DNA MoBio*. A continuación, se procedió a la amplificación del ADN de las muestras y por último a la realización de la DGGE (Denaturing Gradient Gel Electrophoretic), usando un gradiente del 40-70% de agentes desnaturalizantes (urea y formamida). La DGGE se desarrolla en el tampón TAE 0.5X, a 60ºC y a un voltaje constante de 70v durante 16h. Tinción del gel con bromuro de etidio para la detección de bandas.

Resultados y Discusión

En este estudio se realizaron dos tipos de ensayos en parcelas experimentales en las Marismas del Guadalquivir. El primero de ellos fue usar distintos tipos de fertilizantes químicos: amonio, nitrato y urea. El segundo fue aplicarlos de dos maneras distintas: en fondo y en fondo más cobertera. Los ensayos se realizaron en cinco estadíos fenológicos de la planta: antes del laboreo, post-inundación, ahijado, floración y grano lechoso-pastoso. Con

todo esto, pretendemos discutir cómo varia la población microbiana con respecto al tipo de abono y a la manera de aplicarlo en las distintas etapas del cultivo del arroz variedad puntal.

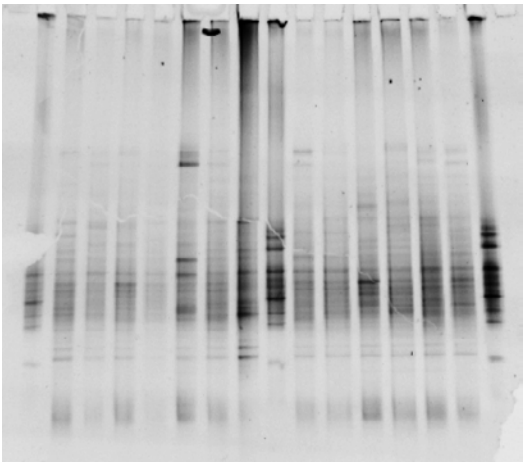

Figura. Gel de parcela experimental en el estado de floración con las muestras de suelo con sus distintos tratamientos.

Observamos que en todas las parcelas experimentales siempre se produce un aumento de la diversidad biológica en el estado fenológico Máximo Ahijado con respecto a los estados fenológicos restantes estudiados. Dicho aumento podría deberse al aporte de nutrientes suministrados por la planta. Sin embargo en los estados fenológico (Antes del Laboreo y Post-Inundación) se observan resultados más variables y menos concluyentes. La influencia de los fertilizantes es variable en las diferentes parcelas experimentales estudiadas y se ve marcada las diferencias por las capacidades de aparición de microorganismos movilizadores de nitrógeno entre la población bacteriana existente, principalmente bacterias ureolíticas en las parcelas fertilizadas por urea

Agradecimientos

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Session 2

Genetics, genomics,
and proteomics of
diazotrophic microorganisms
and associated plants

Genomic analysis of the actinorhizal symbiosis partners: *Frankia alni* and *Alnus glutinosa*

Alloisio N[1], Fournier P[1], Pujic P[1], Kucho KI[2], Hocher V[3], Bogusz D[3], Normand P[1*]

*[1]Ecologie Microbienne, UMR5557 CNRS, Universite Lyon1, 69622 Villeurbanne cedex, France; [2]Department of Chemistry and Bioscience, Graduate School of Science and Engineering, Kagoshima University, Korimoto 1-21-35, Kagoshima 890-0065, Japan; [3]Equipe Rhizogenese, IRD-UMR DIA-PC, 911 Avenue Agropolis, BP 64501, 34394 Montpellier Cedex 5, France. * Philippe.Normand@univ-lyon1.fr*

Summary

The actinorhizal and legume symbioses share a number of similarities, morphological and functional, that have led to investigations of the evolutionary link between the host and symbiont determinants involved. On the plant side, recent work has shown that at least part of the signalling pathway was conserved between legumes and actinorhizal plants, namely the SymRK kinase. Due to the lack of a classic genetic transformation approach for *Frankia*, alternatives have been used to identify the symbiotic determinants. The analysis of the recently determined genomes of three strains did not permit to find canonical *nod* genes. A DNA array transcriptomic analysis showed, as expected, upregulation of *nif* genes, as well as *suf, hup,* and genes involved in the biosynthesis of the hopanoid lipids for synthesis of specialized cells vesicles. Concomitantly, an EST analysis of symbiotic *Alnus* and *Casuarina* has been undertaken to identify other upregulated symbiotic genes.

Introduction

Legumes and actinorhizal plants share a number of similarities, morphological and functional, that have led to investigations of the evolutionary link between the host and symbiont determinants involved. Both types of symbioses have two infection routes, one through deformed root hairs and another one through intercellular spaces; both use an infection thread that delivers the microbial symbiont to the cortex; and both involve a thermoresistant low molecular weight soluble effector that deforms root hairs (Ceremonie *et al.,* 1999).

On the plant side, recent work has shown that at least part of the signalling pathway was conserved between legumes and actinorhizal plants, namely the SymRK kinase (Gherbi *et al.,* 2008), known to be involved in transmission of the *Rhizobium* and mycorhizal signals and the subsequent development of symbiotic organs.

Frankia is an actinobacterium that belongs to the suborder *Frankinae* (Normand & Fernandez, 2009) and that has as phylogenetic neighbours the extremophilic bacteria *Acidothermus* from acid hydrothermal springs and *Geodermatophilaceae* from desert sands.

To determine whether the bacterial symbiosis determinants were phylogenetically related to the rhizobial *nod* genes, we undertook the sequencing of the genomes of three strains in collaboration with the Genoscope and the JGI (Normand *et al.,* 2007a). This approach did not permit to find canonical *nod* genes, all the *nod* homologs present were found to be spread around the genomes, away from the *nif* genes, and to have low similarity levels (Normand *et al.,* 2007b).

A DNA array transcriptomic analysis of *Frankia alni* was undertaken to identify genes that are upregulated in *Alnus glutinosa* nodules. This approach permitted to show, as expected, upregulation of *nif* genes, as well as *suf, hup,* and genes involved in the biosynthesis of the hopanoid lipids for synthesis of specialized cells vesicles (Alloisio *et al.,* 2010). Concomitantly, an EST analysis of symbiotic *Alnus* and *Casuarina* has been undertaken to identify other upregulated symbiotic genes. In this presentation, I will discuss preliminary results.

Materials and Methods

Frankia alni strain ACN14a was grown in defined medium with and without nitrogen (Alloisio *et al.,* 2007). Genomes of the three *Frankia* strains were determined and assembled as previously described (Normand *et al.,* 2007a). Plants of *Alnus glutinosa* were inoculated with *F. alni* ACN14a and grown in hydroponic pots in the greenhouse for 21d according to Alloisio *et al*. (2010). RNAs from pure culture of *F. alni* and from *F. alni* in symbiosis with *A. glutinosa* were extracted and hybridized with a DNA array designed by Nimblegen as described elsewhere (Alloisio *et al.,* 2010).

Results and Discussion

The first aim of this work was to analyse *Frankia* genomes for the presence of *nod* genes. There are in the three genomes a few *nodB* homologs at a similarity level of *c.* 45% and *nodC* homologs at a similarity levels of *c.* 30%, but no *nodA* homolog. These genes are all spread out on the genome, far away from the other genes related to the symbiosis such as the *nif* cluster (Figure). It can thus be said that there are no canonical *nod* genes and no readily identifiable symbiotic island.

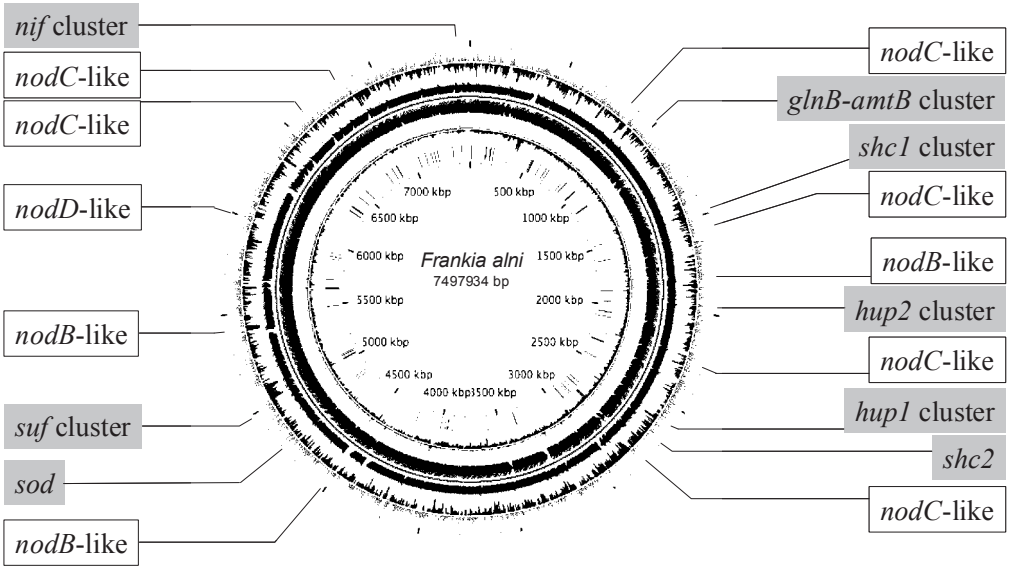

Figure. Circular representation of the *Frankia alni* ACN14a chromosome. Circles display (from the outside): (1) GC percent deviation (GC window - mean GC) in a 1000-bp window; (2) Predicted CDSs transcribed in the clockwise direction; (3) Predicted CDSs transcribed in the counterclockwise direction. Genes displayed in (2) and (3) represent clockwise and counterclockwise MaGe validated annotations; (4) GC skew (G+C/G-C) in a 1000-bp window; (5) RNAs, transposable elements and pseudogenes. Made on the Mage application (www.genoscope.cns.fr/agc/mage). The genes or clusters indicated in white boxes are the *nod* homologs scattered around the genome while the grey boxes represent other genes related to symbiosis.

A transcriptomic approach was then followed to identify genes that are upregulated in symbiosis and thus putatively involved in it. This permitted to see that, by far, the most upregulated genes were those belonging to the 15 genes *nif* cluster (nitrogenase) with a fold change of up to 396. Another related upregulated cluster was *suf*, which permits the synthesis of the sulfur-iron cofactors at the core of nitrogenase with a fold change of up to 11. There are two hydrogenase uptake clusters in the *Frankia* genome: one of them, which plays a role in recycling hydrogen produced by nitrogenase, was upregulated up to 21-fold, whereas the

other cluster was not. Finally, the scattered genes involved in the biosynthesis of bacteriohopane-tetrol necessary for build-up of the nitrogen-fixing vesicles were upregulated by up to 12-fold. The *nod* genes homologs were not upregulated. These results were all confirmed by quantitative PCR. Preliminary EST results will be presented.

Even though with these genomic and transcriptomic approaches we have gained a global vision of *Frankia* biology, in particular from an evolutionary point of view, the question of the *Frankia* symbiotic effectors remains unsolved so far. Nevertheless, the modern genomic tools should permit chemical mutagenesis or gain-of-function approaches that constitute the logical development to decipher the *Frankia* symbiotic determinants.

Acknowledgments

We thank JSPS-CNRS for bilateral funding. We thank the Genoscope for the EST project, the Genoscope and the JGI for the sequencing the bacterial genomes, and the CNRS EC2CO program for funding of the plant EST program.

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Common bean-*Rhizobium* symbiosis: functional genomics of legume responses to abiotic stresses

Hernández G[1*], Ramírez M[1], Valdés-López O[1], Aparicio-Fabre R[1], Yang SS[2], Naya L[1], Mendoza AB[1], Nova-Franco B[1], Leija A[1], Fuentes SI[1], Arellano J[1], Girard L[1], Sánchez F[3], Reyes JL[3], Vance CP[2, 4]

*[1]Centro de Ciencias Genómicas, Universidad Nacional Autónoma de México (UNAM), Cuernavaca, Mor. 62209, México; [2]USDA-Agricultural Research Service, Plant Science Research Unit, St. Paul, MN 55108, USA; [3]Instituto de Biotecnología-UNAM, Cuernavaca, Mor. 62209, México; [4]Department of Agronomy and Plant Genetics, University of Minnesota, St. Paul, MN 55108, USA. *gina@ccg.unam.mx*

Summary

Common bean (*Phaseolus vulgaris*) is the world's most important grain legume for direct human consumption and a main source of proteins in Latin America and Africa. Environmental factors like nutrients deficiency, soil acidity and metal toxicity are important constraints for bean symbiotic nitrogen fixation (SNF) and for crop production. Our research is aimed to the identification of genes, gene networks and signaling pathways that are relevant for the response/adaptation to abiotic stresses of SNF bean plants. We have developed a platform for bean functional genomics that includes: expressed sequence tag (EST) sequencing, transcriptomics, transcription factor genes and microRNA (miRNA) expression profiles, and reverse genetics in bean composite plants. Phosphorus (P) deficiency is widespread in regions where bean is produced and is perhaps the factor that most limits SNF. We have analyzed the global gene expression and metabolic responses of nodules from bean plants inoculated with *Rhizobium tropici* grown under P-deficient and P-sufficient conditions. Recently, we have used a miRNA-macroarray hybridization approach to identify miRNAs expressed in nodules of bean grown under full-nutrient or abiotic stress conditions. Our data provide the foundation to evaluate the individual roles of miRNAs in bean.

Introduction

Common bean is the world's most important grain legume for direct human consumption, comprising 50% of the legumes consumed worldwide. In several countries of Central/South America and Africa, beans are staple crops serving as the primary source of protein in the diet (Broughton *et al.,* 2003; Graham *et al.,* 2003). Beans, as well as other legumes, are vital in agriculture as they form associations with N_2-fixing bacteria. The original microsymbiont of beans is *Rhizobium etli*; both symbionts originated in America and have co-evolved for thousands of years (Segovia *et al.,* 1993; Broughton *et al.,* 2003). Beans can also establish symbiosis with other rhizobia such as *R. tropici, R. leguminosarum* bv. *phaseoli*, etc. In bean, SNF rates, seed protein levels and tolerance to nutrients deficiencies are low in comparison to other legumes. The reduced yield of bean production is due, in part, to disease and insect pressures but also to edaphic constraints that include soil nutrients deficiencies, soil acidity and metal toxicity (Graham *et al.,* 2003).

Our research is aimed to the identification of genes, gene networks and signaling pathways that are relevant for the response/adaptation of bean SNF to abiotic stresses. The understanding of genes involved in the response of bean SNF to abiotic stresses will not only increase our knowledge of processes integral to crop productivity, but also will identify new targets for crop improvement.

Platform for functional genomics of bean

Despite the agronomical importance of beans, genomic resources were few even ten years ago. Work from our group at UNAM-México, in collaboration with others, has been oriented to develop a suitable platform for bean genomics. We have contributed to ESTs sequencing (Ramírez *et al.,* 2005; Graham *et al.,* 2006). The DFCI Bean Gene Index (v. 3.1,

http://compbio.dfci.harvard.edu/tgi/) now includes a unigene set of 21,576. Bean transcriptomic analysis has been performed using macro-arrays printed with *c*. 2000-3000 ESTs from different organs (Ramírez *et al.,* 2005; Hernández *et al.,* 2007; 2009). More recently we have shown that the GeneChip® Soybean Genome Array can be successfully used for bean transcript profiling (Yang *et al.,* 2010). To assess gene expression of trancription factor (TF) genes we established a platform based in qRT-PCR (Hernández *et al.,* 2007; 2009). This includes a set of 372 TF genes, identified from the Bean Gene Index, coding for proteins with Inter-Pro domains characteristic or diagnostic for TF. We have used miRNA-macroarray hybridization and Northern blot approaches to analyze the expression profile of miRNAs in bean organs (Valdés-López *et al.,* 2010). Because beans are recalcitrant for stable genetic transformation, we are using the alternative method of generation of "composite plants", with transformed roots elicited by *Agrobaterium rhizogenes*, to assess gene function through reverse genetics that includes RNAi gene silencing (Estrada-Navarrete *et al.,* 2007; Valdés-López *et al.,* 2008).

Functional genomics of bean SNF in response to P deficiency

P deficiency (-P) is widespread in regions where beans are produced and is perhaps the most limiting factor for SNF. Global gene expression and metabolome approaches were used to investigate the responses of nodules from bean plants inoculated with *Rhizobium tropici* CIAT899 grown under P-deficient and P-sufficient conditions (Hernández *et al.,* 2009). P-deficient inoculated plants showed drastic reduction in nodulation and nitrogenase activity. Nodule transcript profiling, performed through hybridization of macro-arrays, showed a total of 459 genes with significant differential expression in response to P. The P-responsive genes represent different biological processes according to updated annotation using the UniProt Knowledgebase database. Thirty-seven TF genes were differentially expressed in -P nodules; only one gene was repressed. Data from performed non-targeted metabolic profile indicated that amino acids and other N metabolites were decreased while organic and polyhydroxy acids accumulated in P-deficient nodules. Bioinformatic analyses using MapMan and PathExpress software tools, customized to beans, were utilized for the analysis of global changes in gene expression that affected overall metabolism. Glycolysis and glycerolipid metabolism, and starch and sucrose metabolism, were identified among the pathways significantly induced or repressed in P-deficient nodules, respectively.

The comparative analysis of transcript and metabolic profiles between -P nodules (Hernández *et al.,* 2009) and -P roots from non-inoculated bean plants (Hernández *et al.,* 2007) revealed a very small proportion of common responsive genes (24/585). In agreement, the metabolic profile of –P roots was very different from that of -P nodules. Our data suggest a rather different response of each organ to the same nutrient stress. We conclude that the main response of –P roots is addressed to maintain P homeostasis, whereas responses in nodules are mainly oriented to maintain an adequate C/N flux between the symbionts and to avoid oxidative stress.

Expression of miRNAs in bean nodules

Plant miRNAs are essential post-transcriptional regulators of gene expression and are involved in the response and adaptation to adverse environmental conditions. The information about the role of miRNAs in bean is yet scarce (Arenas-Huertero *et al.,* 2009). Our current research is oriented to identify miRNAs expressed in nodules of bean plants grown under control or full-nutrient condition as compared to those of plants grown under P or Fe deficiency, or metal toxicities (Al, Cu or Mn).

A hybridization approach of miRNA-macroarrays was used to detect miRNAs in leaves, roots or nodules from control or stressed bean plants. miRNA-macroarrays were printed with 88 synthetic DNA oligonucleotides corresponding to reverse complementary sequences of known mature miRNAs: 24 conserved miRNAs, 35 from soybean, 20 from *Medicago truncatula* and 9 from bean. We detected 62 miRNAs (24 conserved, 8 from bean, 19 soybean, and 11 from *M. truncatula*) expressed in bean nodules. Forty miRNAs were differentially regulated in stressed nodules, including 5 miRNAs (gma-miR1511, gma-miR1516, gma-miR1536, mtr-miR1509 and mtr-miR2590) that were only detected under stress conditions. The expression of selected nodule miRNAs evidenced by miRNA-macroarray hybridiztion was validated by Northern-blot analysis. The miRNA expression ratios (stress/control) were analyzed by principal component and hierarchical cluster analyses. Novel miRNA target genes were proposed for bean and the expression of selected targets was evaluated by qRT-PCR (Valdés-López *et al.,* 2010). In addition to the detection of previously reported stress-responsive miRNAs, we discovered novel bean stress-responsive miRNAs, *i.e.* for Mn toxicity. Our data provide the foundation to evaluate the individual roles of miRNAs in bean.

Acknowledgments

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How to bring a sensitive metal cluster to work: the role of the metallochaperone NafY from *Azotobacter vinelandii* in the biosynthesis of nitrogenase

Hernandez JA[1*], Erbil WK[2], Phillips AH[2], Zhao D[3], Demuez M[4], Zeymer C[2], Pelton JG[2], Wemmer DE[2], Rubio LM[4]

[1]Department of Biochemistry. Midwestern University, Glendale, AZ 85308, USA; [2]Department of Chemistry, University of California, Berkeley, CA 94709, USA; [3]Department of Pharmaceutical Chemistry, University of California, San Francisco, CA, 94158, USA; [4]Instituto Madrileno de Estudios Avanzados - Energia, Madrid, 28023, Spain. [*]jherna@midwestern.edu

Summary

The molybdenum nitrogenase is the major contributor to the biological nitrogen fixation, an essential process of the biogeochemical nitrogen cycle. The iron-molybdenum cofactor (FeMo-co), located at the active site of the molybdenum nitrogenase, is one of the most complex metalloclusters known in biology. More than twenty genes are involved in the synthesis of the nitrogenase cofactor, including enzymes, molecular scaffolds and carriers. NafY is an escort protein that has been shown to participate in the final steps of the process. In this work, we present a complete biochemical and structural characterization of the NafY protein to show the solution structure of the apo-dinitrogenase-interacting domain and to characterize NafY domains interaction by NMR. We demonstrate that the N-terminal domain of NafY displays an apo-dinitrogenase binding activity by itself. Moreover, we show that the binding of the N-terminal domain of NafY to apo-dinitrogenase prevents the insertion of FeMo-co. Furthermore, NMR analyses of the N-terminal domain of NafY reveal it belongs to the sterile alpha motif (SAM) family of domains, involved in protein-protein interactions. Altogether, these results support a role for NafY in the delivery of FeMo-co to the apo-dinitrogenase and give new insights to the mechanism of cofactor insertion into proteins.

Introduction

Most of the bacterial nitrogen fixation (BNF) is carried out by the activity of the molybdenum nitrogenase, found in a very diverse group of bacteria. The molybdenum nitrogenase enzyme complex is composed of two oxygen-labile metalloproteins: dinitrogenase (also referred to as MoFe protein or NifDK) and dinitrogenase reductase (also referred to as Fe protein or NifH). The molybdenum nitrogenase contains the iron-molybdenum cofactor (FeMo-co) at its active site, the location where BNF physically takes place. FeMo-co is arguably the most complex bioinorganic cofactor in nature. FeMo-co biosynthesis is a very complex process that involves the activities of at least 11 nitrogen fixation (*nif*) gene products (Rubio & Ludden, 2008). The nitrogenase accessory factor (NafY), which is able to independently bind apo-NifDK and FeMo-co, has been proposed to have a role in the last steps of nitrogenase biosynthesis. Ultimately, the world-wide BNF relies on nitrogenase biosynthesis, which has made it an active field of research for years.

Materials and Methods

Overproduction of NafY and NafY domains in *E. coli* was accomplished by fusing a glutathione-*S*-transferase (GST) domain to the respective protein sequences, in derivatives of vector pRHB153 (Hernandez *et al.,* 2007). The *in vitro* FeMo-co insertion reactions were performed with purified components in serum vials sealed with rubber stoppers. The resulting activation of apo-dinitrogenase present in the reaction mixture was analyzed by the acetylene reduction assay after addition of 0.4 mg of NifH and 0.8 ml of ATP-regenerating mixture (Shah & Brill, 1973). NMR data sets were collected at 298K on either a Bruker Advance 800, DMX 600 or II 900 equipped with a cryoprobe. NMR data were processed using NMRpipe or rNMRtk, and analyzed with NMRDraw and CARA.

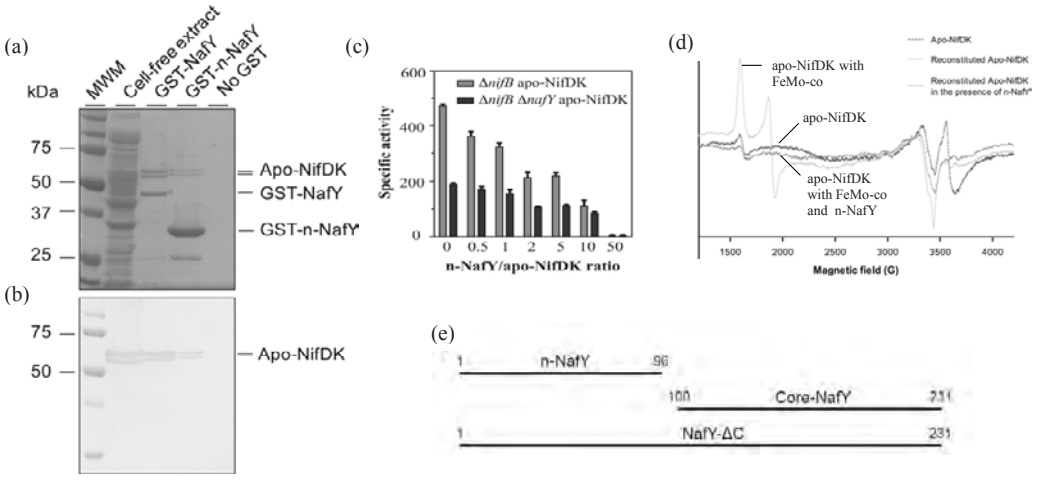

Figure 1. Binding and effect of n-NafY on apo-NifDK reconstitution. **(a)** SDS-PAGE analysis of the interaction between n-NafY and apo-NifDK. **(b)** Immunoblot analysis of SDS-gel from panel (a) developed with antibodies to NifDK. The position of apo-NifDK is indicated by arrows to the right. **(c)** Effect of n-NafY on the activation of Δ*nifB* apo-NifDK and Δ*nifB* Δ*nafY* apo-nifDK by sub-optimal amounts of FeMo-co. Specific activity is given in nmol C_2H_4 produced per min per mg of apo-NifDK added to the reaction mixture. Data are the average of two to four independent determinations. **(d)** CW-EPR spectra from apo-NifDK (dashed line) and apo-NifDK samples incubated with FeMo-co (dotted line) or n-NafY plus FeMo-co (solid line). **(e)** NafY domain architecture.

Results and Discussion

In this work, we describe the biochemical and structural characterization of the N-terminal domain of the NafY protein (n-NafY). Whereas the C-terminal domain (core-NafY) was shown to bind the iron-molybdenum cofactor of nitrogenase, we demonstrate that the N-terminal domain of NafY is able to interact with apo-dinitrogenase by itself. Moreover, by tracking the EPR signature of FeMo-co, we show that the binding of the N-terminal domain of NafY to apo-dinitrogenase prevents the insertion of FeMo-co. The solution structure of the N-terminal domain of NafY is solved and showed to belong to the Sterile Alpha Motif (SAM) family of domains, reported to be frequently involved in protein-protein interactions. NafY domain structure and dynamics, described in this work, and its interaction with apo-NifDK are consistent with the function of NafY in FeMo-co delivery to apo-NifDK.

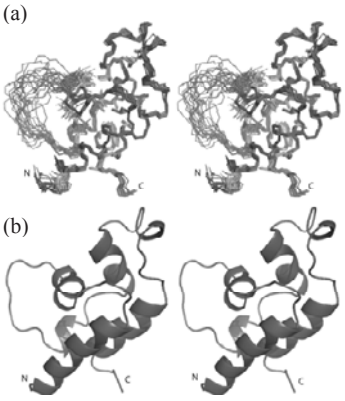

Figure 2. Structure of n-NafY in solution. (a) Stereo representation of the superposition of the 20 lowest energy structures. (b) Cartoon model of the lowest energy structure.

Acknowledgments

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Impact of the *Sinorhizobium meliloti* RNA chaperone Hfq in transport, metabolism and symbiosis

Jiménez-Zurdo JI[1*], Torres-Quesada O[1], Oruezabal RI[2], Peregrina A[1], Jofré E[3], Lloret J[2], Rivilla R[2], Toro N[1]

*[1]Grupo de Ecología Genética de la Rizosfera, Estación Experimental del Zaidín, CSIC, 18008 Granada, Spain; [2]Departamento de Biología, Universidad Autónoma de Madrid, 28049 Madrid, Spain; [3]Departamento de Ciencias Naturales, Universidad Nacional de Río Cuarto, X5804BYA Río Cuarto, Córdoba, Argentina. *jijz@eez.csic.es*

Summary

The bacterial RNA-binding protein Hfq has emerged as a global post-transcriptional regulator of gene expression controlling diverse cellular processes, including virulence. As a first approach to understand the role of Hfq in *Sinorhizobium meliloti*, both as free-living and nitrogen-fixing endosymbiotic bacterium, Hfq-dependent phenotypes were analyzed. Transcriptome and proteome profiling of *S. meliloti hfq* knock-out mutants revealed a general down-regulation of genes of sugar transporters and some enzymes of the carbon metabolism, whereas genes specifying the uptake and metabolism of nitrogen sources were up-regulated as compared to the wild-type strains. Symbiotic tests showed that Hfq influences rhizospheric competence, survival of bacteria within the nodule cells and efficiency of symbiosis. Furthermore, full expression of the *nifA* and *fixK1/K2* regulators requires Hfq as revealed by RT-PCR experiments. Finally, we show that some of the recently identified *S. meliloti* small non-coding RNAs co-inmunoprecipitate with a FLAG-epitope tagged Hfq protein, thus anticipating the involvement of these molecules in the rhizobial Hfq regulatory network.

Introduction

Hfq is a ubiquitous bacterial protein originally identified in *Escherichia coli* as a host factor essential for Qβ RNA bacteriophage replication (Franze de Fernández *et al.,* 1972). In uninfected bacteria Hfq retains the ability to bind many mRNAs and *trans*-acting antisense small non-coding regulatory RNAs (sRNAs), thereby influencing on the stability and/or translation of functionally diverse RNA molecules (Valentin-Hansen *et al.,* 2004). This variety of interactions place Hfq at a crucial node in bacterial post-transcriptional regulatory networks underlying a wide range of cellular processes and pathways. In several enterobacteria and other facultative intracellular mammal pathogens, including α-proteobacterial representatives, these deficiencies ultimately compromise virulence traits such as motility, host invasion or survival in the intracellular niche (Chao & Vogel, 2010). Despite the increasing evidence for a major role of Hfq in the establishment and maintenance of chronic intracellular infections, the function of this RNA chaperone in rhizobia has remained largely unexplored. We have determined global Hfq-dependent changes in gene expression and protein accumulation coupled with the characterization of the symbiotic behavior of *hfq* knock-out mutants to pinpoint the function of this RNA chaperone in the alfalfa symbiont *S. meliloti* (Torres-Quesada *et al.,* 2010). We also provide experimental evidence of Hfq binding to some of the sRNAs identified in this bacterium (del Val *et al.,* 2007).

Materials and Methods

S. meliloti mutants 2011-3.4 and 1021Δ*hfq* were obtained by pK18*mobsacB*-mediated disruption and marker-free deletion of the *hfq* gene in the wild-type strains 2011 and 1021, respectively, following standard molecular genetics protocols. Similarly, a 1021 derivative strain expressing a chromosomal C-terminal FLAG-epitope tagged Hfq protein was also constructed. Transcriptomics (1021Δ*hfq*), proteomics (2011-3.4), symbiotic tests, nodule microscopy, RT-PCR experiments and co-inmunoprecipitation assays were performed as detailed in Torres-Quesada *et al*. (2010).

Results and Discussion

Loss of Hfq affected free-living growth of *S. meliloti*, thus confirming the pleiotropy of this mutation in bacteria. Independent transcriptome profiling of mutant 1021Δ*hfq* and proteome analysis of 2011-3.4 converged in the identification of genes coding for periplasmic solute binding proteins of ABC transporters and metabolic enzymes as the dominant functional categories influenced by an Hfq mutation. Knock-out of *hfq* resulted in the general down-regulation of genes of the main central energy production pathways based on sugar uptake and catabolism which imposes a metabolic rearrangement favouring TCA and gluconeogenesis supported by the up-regulation of amino acid supply and nitrogen metabolism (Figure).

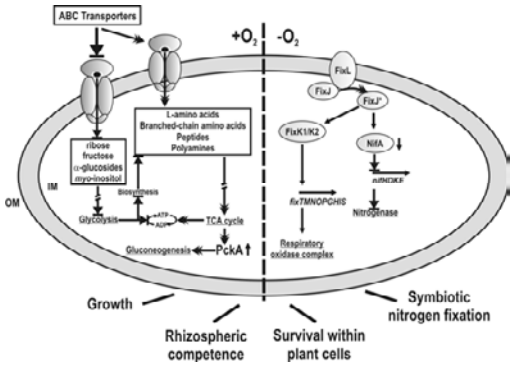

Figure. Hfq-dependent pathways and phenotypes in *S. meliloti*. Double arrowheads denote favoured pathways and blocked arrows unfavoured pathways in the absence of Hfq. $+O_2$, aerobic conditions; $-O_2$, microaerobic conditions.

Symbiotic tests did not reveal significant differences in the onset of nodulation or the average number of nodules induced per plant when 1021 and 1021Δ*hfq* strains were compared. However, competition for nodulation of the 2011-3.4 strain when co-inoculated with its parent 2011 was found to be severely compromised. A large proportion of nodules elicited by the 1021Δ*hfq* mutant were non-fixing, with scarce content in bacteroids and signs of premature senescence of endosymbiotic bacteria, thus suggesting that Hfq contributes to adaptation of *S. meliloti* to the intracellular environment of the nodule. RT-PCR experiments on RNA from bacteria grown under aerobic and microoxic conditions revealed that Hfq also contributes to regulation of *nifA* and *fixK1/K2*, the key regulators of nitrogen fixation, although the Hfq-dependent regulation of *fixK* was only observed in aerobiosis and could not be relevant to symbiosis. We have also found that four of the recently identified *S. meliloti* sRNAs co-inmunoprecipitate with a FLAG-epitope tagged Hfq protein, which renders these molecules as new players in the complex *S. meliloti* Hfq-dependent post-transcriptional regulatory network.

Acknowledgments

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Transcriptome profiling of a *Sinorhizobium meliloti fadD* mutant reveals the role of rhizobactin 1021 biosynthesis and regulation genes in the control of swarming

Nogales J*, Domínguez-Ferreras A, Amaya-Gómez CV, van Dillewijn P, Cuéllar V, Sanjuán J, Olivares J, Soto MJ

*Departamento de Microbiología del Suelo y Sistemas Simbióticos, Estación Experimental del Zaidín, CSIC, 18008 Granada, Spain. *quina@eez.csic.es*

Summary

Swarming is a multicellular phenomenom characterized by the coordinated and rapid movement of bacteria across semisolid surfaces. In *Sinorhizobium meliloti* this type of motility has been described in a *fadD* mutant. To gain insights into the mechanisms underlying the process of swarming in *Rhizobium*, we compared the transcriptome of a *S. meliloti fadD* mutant grown under swarming conditions (semisolid medium) to those of cells grown under non-swarming conditions (broth and solid medium). More than a thousand genes were identified as differentially expressed in response to growth on agar surfaces including genes for several metabolic activities, iron uptake, chemotaxis, motility and stress-related genes. These data suggest the existence of striking differences in the physiology of *S. meliloti* growing in broth compared with agar surfaces. Under swarming-specific conditions, the most remarkable response was the up-regulation of iron-related genes. We demonstrate that the pSymA plasmid and specifically genes required for the biosynthesis of the siderophore rhizobactin 1021 are essential for swarming of a *S. meliloti* wild-type strain but not in a *fadD* mutant. Moreover, high iron conditions inhibit swarming of the wild-type strain but not in mutants lacking either the iron limitation response regulator RirA or FadD.

Introducción

El swarming es un tipo de translocación bacteriana caracterizado por el movimiento rápido y coordinado de toda una población sobre una superficie. Las señales y genes de las rutas que participan en la diferenciación a célula swarmer son en su gran mayoría desconocidos. Estudios recientes revelan que el swarming es mucho más que un tipo de motilidad, ya que se han detectado grandes alteraciones en rutas metabólicas y expresión génica. En bacterias patógenas, el swarming se ha asociado a virulencia en parte debido a que la diferenciación a célula swarmer conlleva una expresión acoplada de factores de virulencia. Nuestro grupo fue pionero en evidenciar la existencia de swarming en un *Rhizobium*, concretamente en un mutante *fadD* de *S. meliloti*. Este mutante además de mostrar swarming en condiciones en las que la cepa silvestre normalmente no muestra esta motilidad, se encuentra afectado negativamente en su capacidad infectiva y competitiva en plantas de alfalfa (Soto *et al.,* 2002). Posteriormente, también se ha demostrado la existencia de swarming en una cepa silvestre de *R. etli* y se han identificado nuevos determinantes genéticos implicados en el swarming de esta bacteria, entre los que se encuentran genes implicados en la síntesis y transporte de polisacáridos, motilidad y metabolismo de aminoácidos y poliaminas (Braeken *et al.,* 2008). Con el objetivo de conocer el proceso de adaptación que ocurre durante la migración multicelular de *Rhizobium*, en este trabajo se ha analizado el patrón de expresión génica global de un mutante *fadD* de *S. meliloti* en condiciones inductoras de swarming.

Materiales y Métodos

Cepas de *S. meliloti* utilizadas en este estudio: Rm1021, Rm2011, GR4 (cepas silvestres), 1021FDC5 (mutante fadD derivado de Rm1021), A818 (Rm2011 curada del pSymA), 2011rhtA1 (derivada de Rm2011 mutada en el gen *rhtA* que codifica el receptor de rhizobactina 1021), 2011rhrA26 (derivada de Rm2011 mutada en RhrA, activador transcripcional de los genes de biosíntesis de rhizobactina 1021), 2011rhbA62 y 2011rhbE11 (derivadas de Rm2011 mutadas en los genes *rhbA* y *rhbE* implicados en biosíntesis de rhizobactina 1021),

G212rirA (derivada de Rm1021 mutada en el regulador global de hierro RirA). Las muestras para realizar el análisis transcriptómico se obtuvieron creciendo las células de 1021FDC5 en medio mínimo (MM) líquido, semisólido (0.6% agar purificado) y sólido (1.3% agar purificado). La extracción de ARN, síntesis de cDNA, hibridación del microarray y comprobación de genes por RT-qPCR se ha hecho según Domínguez-Ferreras *et al.* (2006). La mutación *fadD*$^-$ se transfirió mediante transducción con el fago ΦM12 (Finan *et al.,* 1984). Los ensayos de motilidad swarming se hicieron siguiendo la metodología descrita en Soto *et al.* (2002).

Resultados y Discusión

El primer análisis transcriptómico de un *Rhizobium* tras crecimiento sobre una superficie ha revelado la expresión diferencial de más de mil genes implicados en distintas actividades metabólicas, captación de hierro, quimiotaxis, motilidad y respuesta a estrés (Nogales *et al.,* 2010). Esto sugiere que las células de *S. meliloti* sufren notables cambios en su fisiología cuando crecen en una superficie con respecto a las células que crecen en líquido. El análisis de genes que responden específicamente a condiciones inductoras de swarming ha permitido demostrar que el plásmido simbiótico pSymA y concretamente genes requeridos para la biosíntesis del sideróforo rhizobactina 1021 desempeñan un papel clave en el control del swarming en una cepa silvestre de *S. meliloti*, pero no en un mutante *fadD* (Figura, **A**, **B** y **C**). Además, hemos demostrado que los niveles de hierro presentes en el medio son importantes en el control del swarming de *S. meliloti*. La presencia de condiciones de suficiencia de hierro inhibe el swarming en una cepa silvestre de *S. meliloti* pero no en un mutante *fadD* ni en un mutante *rirA* (afectado en regulador general de respuesta a hierro y en el que la producción de rhizobactina es constitutiva) (Figura, **D**). Estos resultados nos han llevado a proponer que la presencia de rhizobactina 1021, cuya síntesis es reprimida en condiciones de suficiencia de hierro, es importante para que *S. meliloti* pueda mostrar migración en superficie, ejerciendo su función fuera de la célula como surfactante o molécula señal. En un mutante *fadD*, la función ejercida por el sideróforo parece ser reemplazada por un compuesto de naturaleza aún desconocida, cuya síntesis no se encuentra regulada por hierro, y que no se encuentra presente o es inactivo en la cepa silvestre.

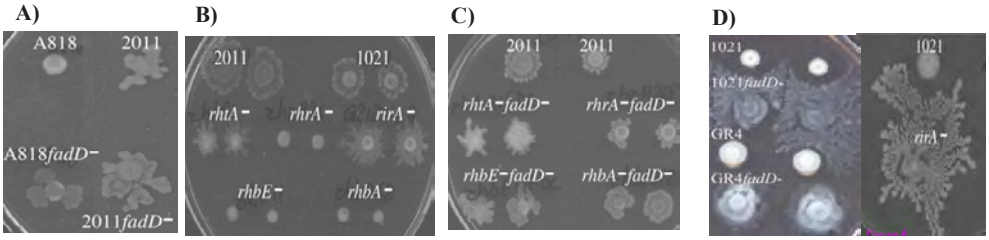

Figura. Motilidad swarming de distintas cepas de *S. meliloti*. Efecto en swarming de **A**) pSymA y mutación *fadD*; **B**) genes relacionados con el sideróforo rhizobactina 1021 en un fondo *fadD*$^+$; **C**) genes relacionados con rhizobactina 1021 en un fondo *fadD*$^-$; **D**) alta concentración de hierro (220 μM $FeCl_3$) en el medio. La concentración de $FeCl_3$ presente en el medio utilizado en los paneles **A**), **B**) y **C**) fue de 22 μM. La descripción de las distintas cepas se encuentra en Materiales y Métodos.

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Genomic sequence of *Pseudomonas fluorescens* F113. Differences with other *P. fluorescens*

Redondo-Nieto M[1*], Morrissey J[2], Barret M[2], Dowling D[3], O'Gara F[2], Barahona E[1], Navazo A[1], Martínez-Granero F[1], Martín M[1], Rivilla R[1]

*[1]Departamento de Biología, Universidad Autónoma de Madrid, Madrid, Spain; [2]Biomerit Research Centre, University College Cork, Cork, Ireland; [3]Institute of Technology, Carlow, Ireland. *miguel.redondo@uam.es*

Summary

P. fluorescens F113 was isolated from sugar beet rhizosphere in Ireland. Its importance lies in its biocontrol abilities based on the production of several antibiotic and antifungic compounds and in its capacity to colonize the rhizosphere. In order to determine the whole metabolic pathway map and find out new abilities, *P. fluorescens* F113 genome has been sequenced using NGS systems: 454 (Roche) and Solexa (Illumina). Currently bioinformatic tools targeted to genome assembly and orf prediction have allowed the achievement of a first draft. According to this F113 genome consists in a single replicon, a circular chromosome of around 6.9 Mb and contains more than 5587 ORFs, being at least 839 of them not present in any of the already sequenced and annotated *P. fluorescens* strains.

Introduction

P. fluorescens F113 isolated from sugar beet rhizosphere (Shananan *et al.*, 1992) has already proven its biocontrol importance by secretion of antibiotic and antifungic compunds. Besides, its genome plasticity thanks to phase variation events, provides it with a natural advantage against other microorganisms regarding rhizosphere colonitation and competition. Currently, Next-Generation Sequentiation (NGS) systems such as 454 (Roche) and Solexa (Illumina), together with the modern computation tools, make possible a microorganism genome sequenciation with a relatively low cost of money and time. Here we present the first draft of *P. fluorescens* F113 and a comparison of its genome with other *P. fluorescens* already sequenced.

Materials and Methods

P. fluorescens F113 genome material was obtained from O/N cultures grown in SA medium, using REALPURE Extracción DNA Genómico (REAL^{Tm}, Durviz). The DNA suspension was sent to the NGS sequencing service following specific conditions for each technology. Genome was sequenced both by 454 FLX Titanium (Roche) and Solexa (Illumina) technologies. Once 454 and Solexa reads were received, were assembled using MIRA3 software (Chevreaux *et al.*, 2004) and the obtained contigs were annotated using GRC software (Warren & Setubal, 2009). *P. fluorescens* PF0-1 (NC_007492), PF-5 (NC_004129) and SBW25 (NC_012660) genome and protein sequences were downloaded from NCBI to be used as blast databases for strains comparisons.

Results and Discussion

As described previously (DiGuistini *et al.*, 2009), we used a combination of NGS technologies to sequence *P. fluorescens* F113 in order to reduce sequencing costs without losing sequence reliability. Though 454 Titanium can provide long reads up to 2000 pb, these are not totally accurate. On the other hand Solexa, produces better quality reads though smaller, 36 pb (Figure, A-B). Thus, we have used 454 reads as an scaffold over which Solexa reads can be assembled to increase sequence quality. By using MIRA software together with a combination of iterative blastn searches and perl scripts, a first draft comprising 11 contigs with a size of around 6.9 Mb and a average depth coverage of 70 has been obtained. GRC software was used to predict orfs and their corresponding annotation within those contigs. As a first approximation 5587 ORFs have been found. Those ORFs were compared by Blastp against a local database of *P. fluorescens* PF0-1, PF-5, and SBW25 annotated proteins in

order to identify those ORFs in *P. fluorescens* F113 not appearing in other *P. fluorescens* strains (Figure, C). *P. fluorescens* F113 ORFs with blast results having at least a 50% of homology and covering more than 85% of ORF length were discarded. On doing so we have been able to determine 839 ORFs that either are not conserved with other *P. fluorescens* or are somehow distant orthologs that could been acquired by gene horizontal transfer events. Among the "new" sequences in *P. fluorescens* F113, we have found new clusters that might provide interestings functionalities to this strain, most of them concerning competitiviness and biocontrol subjects. We can highlight several clusters of genes encoding denitrification activiy, plant and insect pathogenesis, aromatic compounds degradation, toxin synthesis related proteins, ectoin synthesis, and a cluster directed to the formation of a new flagellum with an homology closer to *Azotobacter vinelandii* than to the ones described in any Pseudomonads.

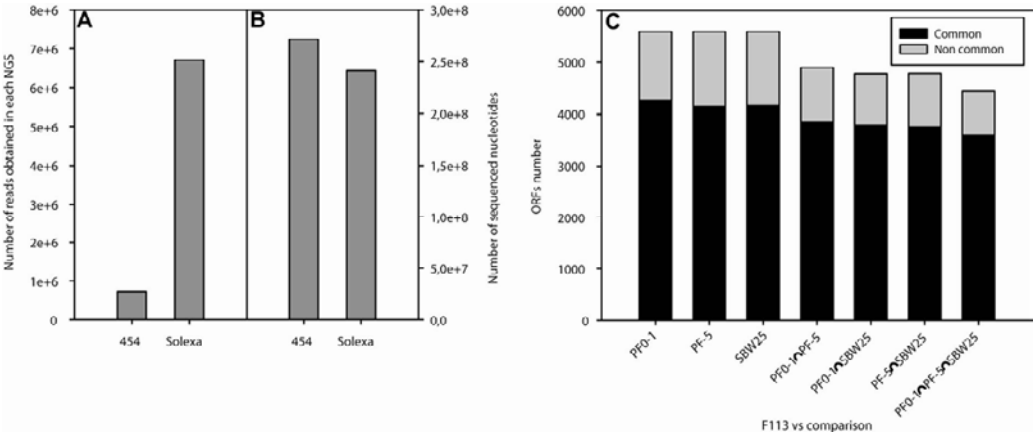

Figure. Total number of reads **(A)** and nucleotides **(B)** sequenced with both technologies 454 and Solexa. **(C)** Blastp comparison of F113 ORFs against the already annotated *P. fluorescens* and the different combinations of common genes (∩) shared by the *P. fluorescens*. In black number of F113 ORFs common to the compared groups, in grey number of F113 not present in those groups.

Acknowledgments

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Genetic determinants of swarming in *Sinorhizobium meliloti*

Amaya-Gómez CV*, Nogales J, Cuéllar V, Sanjuán J, Olivares J, Soto MJ

*Departamento de Microbiología del Suelo y Sistemas Simbióticos, Estación Experimental del Zaidín, CSIC, 18008 Granada, Spain. *carol.amaya@eez.csic.es*

Summary

Swarming motility is considered to be a social phenomenon that allows the rapid and coordinated movement of bacterial populations across semisolid surfaces. Different studies suggest that either components essential for this multicellular motility and/or factors which are co-regulated during swarmer cell differentiation may play a role in the ability of pathogenic and symbiotic bacteria to associate with their eukaryotic hosts. In an attempt to identify new bacterial genes playing a role in the *Rhizobium*-legume symbiosis, we have initiated the genetic characterization of swarming in *S. meliloti*. In this *Rhizobium*, swarming motility has been described in a *fadD* mutant. We carried out transposon mutagenesis of *fadD* mutant derivatives of *S. meliloti* GR4 and Rm1021 strains, and selected transposants showing an altered swarming motility. Nineteen mutants defective in swarming or showing a hyperswarmer phenotype were identified. Genetic characterization of these mutants revealed that the transposon had targeted 14 different genes involved in chemotaxis, bacterial motility, environmental stresses, regulation of polysaccharide production and genes with unknown function. Although all the mutants are able to establish nitrogen-fixing symbiosis with alfalfa plants, some of them show altered nodule formation efficiency or competitiveness compared to the parental strain.

Introducción

El swarming es un tipo de translocación bacteriana caracterizado por el movimiento rápido y coordinado de toda una población sobre una superficie llegando a formar biopelículas. Requiere un proceso de diferenciación celular disparado por una serie de señales que conlleva grandes alteraciones metabólicas y de expresión génica. Diversos datos sugieren que componentes esenciales en swarming y/o factores co-regulados durante el proceso de diferenciación, pueden ser importantes para que bacterias patogénicas o mutualistas puedan interaccionar con sus correspondientes hospedadores eucariotas. En *Rhizobium* es muy poco lo que se conoce sobre las bases moleculares del swarming y el papel que este proceso desempeña en el establecimiento de simbiosis con leguminosas. Hasta la fecha, esta motilidad multicelular sólo se ha descrito en *Sinorhizobium meliloti, R. etli*, y en *R. leguminosarum* biovar viciae. En *S. meliloti*, el swarming se ha asociado a un mutante en el gen *fadD* que codifica una acil-CoA ligasa específica de ácidos grasos de cadena larga (Soto *et al.,* 2002). Este mutante, además de mostrar swarming condicional, es menos infectivo y competitivo en la nodulación de alfalfa. Existen evidencias de que la regulación del swarming es distinta en las cepas GR4 y Rm1021 de *S. meliloti*. (Nogales *et al.,* 2010). Con el objetivo de identificar nuevos componentes bacterianos implicados en swarming de este *Rhizobium*, hemos aislado y caracterizado mutantes derivados de las cepas *fadD*$^-$ de GR4 y Rm1021 con alteraciones en este tipo de motilidad. El fenotipo simbiótico de estos mutantes también ha sido analizado.

Materiales y Métodos

1021FDCSS y GR4FDCSS son mutantes *fadD* de *S. meliloti* derivados de las cepas Rm1021 y GR4, respectivamente, obtenidos por deleción e inserción de un cassette de resistencia a estreptomicina/ espectinomicina. La mutagénesis generalizada con Tn5 se realizó usando el plásmido pSUP2021. La motilidad swarming se ensayó en medio mínimo semisólido (0.6% de agar) y el swimming en medio Bromfield (0.3% agar). El gen afectado por la inserción del Tn5 se identificó mediante secuenciación del producto generado en una reacción de PCR arbitraria. El fenotipo simbiótico se ensayó en plantas de alfalfa crecidas en condiciones axénicas en tubos con solución mineral sin nitrógeno. El grado de infectividad se obtuvo determinando diariamente el número de nódulos desarrollados por planta tras inocular con una suspensión de 10^6 UFC/planta. En los ensayos de competitividad las plantas se inocularon con una suspensión bacteriana que contenía una

mezcla 1:1 de la cepa a ensayar y la correspondiente cepa parental portando el plásmido pGUS3, lo que permite distinguir nódulos blancos y azules, tras la correspondiente tinción GUS.

Resultados y Discusión

Con el objetivo de obtener mutantes de *S. meliloti* alterados en swarming hemos llevado a cabo mutagénesis generalizada con Tn*5* en las cepas 1021FDCSS y GR4FDCSS. Tras analizar 4000 transposantes en cada uno de los fondos genéticos, se han identificado 19 mutantes defectivos en swarming o hiperswarmers (Figura).

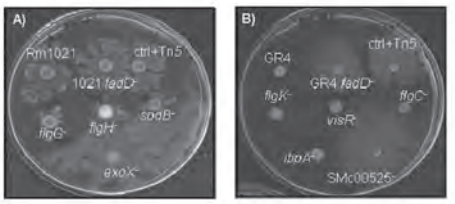

Figura. Swarming en distintas cepas de *Sinorhizobium meliloti*. **A)** Transposantes derivados de 1021FDCSS (1021*fadD*$^-$), **B)** Transposantes derivados de GR4FDCSS (GR4*fadD*$^-$). En los mutantes afectados en *flgG*, *flgH*, *sodB* hay una disminución del swarming comparado con la cepa parental 1021FDCSS; en los mutantes en *flgK*, *visR*, *flgC* el swarming se vio abolido por completo, y los mutantes *exoX*$^-$ y SMc00525$^-$ presentan un aumento en motilidad tipo swarming. Ctrl+ Tn*5*: Transposantes no afectados en swarming.

La caracterización genética de los mutantes ha revelado que el Tn*5* ha afectado a un total de 14 genes diferentes implicados en estructura y movimiento del flagelo, quimiotaxis, respuesta a estreses ambientales, reguladores de la producción de exopolisacárido o genes de función desconocida (Tabla). Por otra parte, el análisis del fenotipo simbiótico de los transposantes, ha puesto de manifiesto que aunque mutaciones que afectan el swarming mostrado por mutantes *fadD* de *S. meliloti*, no impiden el desarrollo de nódulos fijadores de nitrógeno en plantas de alfalfa, sí pueden alterar la eficiencia de formación de nódulos y la capacidad competitiva de la bacteria.

Tabla. Descripción del gen afectado en los transposantes alterados en swarming, sus fenotipos en motilidad y simbiosis. (++) Fenotipo de la cepa silvestre, (+++/++++) aumento en el fenotipo, (+) disminución del fenotipo, (–) no swarming/swimming. (Nod) Eficiencia de nodulación. (Fix). Fijación de Nitrógeno. (Comp) Capacidad Competitiva por la nodulación de alfalfa del transposante frente a su parental mutante *fadD* .

Gen interrumpido	Descripción	swarming	swimming	Fenotipo simbiosis
Transposantes 1021FDCSS::Tn*5*				
flgG	Proteína cuerpo basal flagelar	+	-	Nod^{+}, Fix^{++},Comp^{++}
motC	Proteína de motilidad	+	-	Nod^{++}, Fix^{++}, Comp^{++}
flgH	Proteína anillo L del flagelo	+	-	Nod^{+}, Fix^{++}, Comp^{++}
sodB	Superóxido dismutasa	+	+	Nod^{++}, Fix^{++}, Comp^{+}
cheA	Proteína de quimiotaxis	+++	+	Nod^{+}, Fix^{++}, Comp^{++}
exoX	Regulador Post-transcripcional	++++	++	Nod^{++}, Fix^{++}, Comp^{+++}
Transposantes GR4FDCSS::Tn*5*				
flgK	Proteína gancho flagelar.	-	-	Nod^{++}, Fix^{++}, Comp^{++}
flgC	Proteína cuerpo basal flagelar.	-	-	Nod^{+++}, Fix^{++}, Comp^{+}
visN	Regulador máster del regulón flagelar	-	-	Nod^{++}, Fix^{++}, Comp^{+}
visR	Regulador máster del regulón flagelar	-	-	Nod^{+++}, Fix^{++}, Comp^{++}
fliQ-flhA	Proteínas biosíntesis de flagelos.	-	-	Nod^{+++}, Fix^{++}, Comp^{++}
emmA	Proteína de regulación de EPS y motilidad	+	+	Nod^{+++}, Fix^{++}, Comp^{++}
ibpA	Transportador ABC de myo-inositol	+	++	Nod^{+++}, Fix^{++}, Comp^{++}
Smc00525	Hipotética proteína trasmembrana.	+++	++	Nod^{+}, Fix^{++}, Comp^{+++}

Agradecimientos

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Involvement of RegR in redox control of denitrification in *Bradyrhizobium japonicum*

Torres MJ, Bueno E, Mesa S, Bedmar EJ, Delgado MJ*

*Estación Experimental del Zaidín, CSIC, Apartado 419, 18080 Granada, Spain. *mdelgado@eez.csic.es*

Summary

Bradyrhizobium japonicum is a gram-negative soil bacterium, associated symbiotically with soybean plants, which is also able to grow under denitrifying conditions. In *B. japonicum,* the *napEDABC*, *nirK*, *norCBQD* and *nosRZDYFLX* genes, which encode reductases for nitrate, nitrite, nitric oxide and nitrous oxide, respectively, are required for denitrification. Recent results showed that the NifA regulatory protein, which induces gene expression at a very low-O_2 concentration, is required for maximal expression of *napEDABC, nirK* and *norCBQD* genes, suggesting a role for RegSR-NifA regulatory cascade in the control of the denitrification process in *B. japonicum*. RegSR belongs to the family of two-component regulatory systems, present in a large number of proteobacteria in which they globally control gene expression mostly in a redox-responsive manner. In this work, we have demonstrated that expression of NapC and NorC haem *c* stained proteins was higher in membranes from cells grown with succinate as carbon source than in those from cells grown with butyrate. Furthermore, a *B. japonicum regR* mutant displayed lower expression of NapC and NorC proteins under denitrifying conditions, either with butyrate or succinate as carbon sources. By real-time reverse transcription-PCR, we have observed a decreased expression of *norC* and *nos* genes in the *regR* mutant grown under denitrifying conditions with succinate compared to wild-type cells. We propose that expression of *B. japonicum* denitrification proteins is under redox control and that the RegR regulatory protein is involved in this control.

Introducción

En *Bradyrhizobium japonicum*, se han descrito dos cascadas regulatorias de respuesta a oxígeno (Sciotti *et al.,* 2003), el sistema FixLJ-FixK_2, que responde a bajas concentraciones de oxígeno y está implicado en la inducción de los genes de la desnitrificación, entre otros, y el sistema RegSR-NifA, que responde a concentraciones de oxígeno aún más limitantes y se relaciona con la activación de genes requeridos principalmente en la fijación de nitrógeno. En nuestro grupo de investigación, se ha desmostrado, recientemente, que la proteína NifA se requiere para que ocurra la máxima expresión de los genes de la desnitrificación en *B. japonicum* (Bueno *et al*., 2010). Sin embargo, se desconoce el papel de las proteínas RegSR en dicho proceso. RegSR de *B. japonicum* pertenece a la familia de sistemas reguladores de dos componentes de respuesta a potencial redox descrita en bacterias. Recientemente, se ha descubierto que el sistema RegBA de *Rhodobacter capsulatus*, que es homólogo a RegSR de *B. japonicum*, es capaz de sensar el estado de óxido-reducción del pool de ubiquinonas. En este trabajo se ha investigado la implicación del potencial redox en la regulación de la desnitrificación en *B. japonicum* y el papel que desempeña la proteína RegR en este control.

Materiales y Métodos

Las cepas bacterianas usadas en este trabajo son la cepa parental de *B. japonicum* USDA110*spc*4 y la cepa mutante *regR* 2426 (Bauer *et al.,* 1998). Las células se crecieron en precultivos de medio completo PSY, y posteriormente se incubaron en medio mínimo Bergersen en condiciones anaeróbicas con nitrato como aceptor de electrones y con una fuente de carbono oxidada (succinato) o reducida (butirato).

Análisis de proteínas con grupos hemo c. Tras el aislamiento de las proteínas de las membranas, se llevó a cabo la tinción de la actividad peroxidada dependiente de hemo *c*, según se describe en Bueno *et al*. (2010).

Análisis de expresión génica. Tras el aislamiento de ARN y síntesis de cDNA (Bueno *et al.,* 2010), se realizó la PCR cuantitativa a tiempo real (qRT-PCR) utilizando el kit PCR QuantiTect Sybr Green y un termociclador Rotor-Gene 3000.

Resultados y Discusión

Mediante tinción de los citocromos *c* presentes en la membrana, se observó que la expresión de NapC (25 kDa) y NorC (16 kDa) fue significativamente mayor en la cepa parental crecida con succinato que cuando se cultivó con butirato como fuente de carbono (Figura, carriles 1 y 3). La expresión de NapC y NorC fue considerablemente menor en la mutante *regR* respecto a la detectada en la cepa parental en presencia de butirato o succinato (Figura). Estas observaciones coinciden con los resultados obtenidos en los análisis de actividad nitrato reductasa periplásmica y de actividad β-galactosidasa de una fusión transcripcional *napE-lacZ* (resultados no mostrados), e indican que la expresión de las proteínas nitrato reductasa periplásmica y óxido nítrico reductasa de *B. japonicum* es dependiente del estado redox de la célula y que la proteína RegR es esencial para la máxima expresión de dichas proteínas, la cuál ocurre en presencia de sustratos carbonados oxidados.

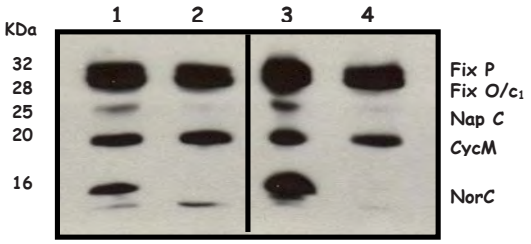

Figura. Detección de los citocromos NapC y NorC en *B. japonicum* 110*spc4* (carriles **1** y **3**) y en la mutante *regR* (carriles **2** y **4**) crecidas en medio mínimo Bergersen con butirato (carriles **1** y **2**) o succinato (carriles **3** y **4**) como fuente de carbono y en condiciones desnitrificantes con 10 mM de KNO_3 durante 48 h.

También se realizaron análisis de expresión génica mediante qRT-PCR, observándose que la expresión de los genes *norC, nosZ, y nosY* disminuyó 28, 10 y 8 veces respectivamente, en la mutante *regR* respecto a la cepa parental (Tabla). Estos resultados confirman los mostrados en la Figura y demuestran la implicación de la proteína RegR en la expresión de la desnitrificación en *B. japonicum.*

Tabla. Expresión génica mediante qRT-PCR de los genes *nap*, *nos* y *norC* en la mutante *regR* respecto de la cepa parental de *B. japonicum*. Las células se incubaron durante 24 horas en condiciones desnitrificantes con succinato como fuente de carbono.

		Cantidad relativa de transcrito	
Gen	**Descripción**	**Cepa parental**	**Mutante *regR***
napE	Nitrato reductasa periplásmica	1.000 ± 0.000	0.733 ± 0.150
napA	Nitrato reductasa periplásmica	1.000 ± 0.000	0.550 ± 0.097
norC	Óxido nítrico reductasa	1.000 ± 0.000	0.037 ± 0.005
nosZ	Óxido nitroso reductasa	1.000 ± 0.000	0.103 ± 0.018
nosY	Óxido nitroso reductasa	1.000 ± 0.000	0.127 ± 0.024

Agradecimientos

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Microscopic and transcriptomic analysis of the arsenic effect on the *Sinorhizobium* - *Medicago* symbiotic interaction

Lafuente A, Pajuelo E, Rodríguez-Llorente ID, Caviedes MA

Departamento de Microbiología y Parasitología, Facultad de Farmacia, Universidad de Sevilla, 41012 Sevilla, Spain

Summary

The phytotoxic effects of arsenic (As) on nodulation of *Medicago sativa* have been examined *in vitro* using the highly As resistant and simbiotically effective *Sinorhizobium medicae* MA11 strain. Concentrations of arsenite from 25 to 35 μM produced a 75% decrease of the total number of nodules, due to a 90% reduction in the number of rhizobial infections. This effect was associated to root hair damage and a shorter infective root zone. We have also examined the molecular mechanism underlying this toxic effect. In the presence and absence of As, the expression patterns of seven nodulin genes, markers for the different events leading to nodule formation, were analyzed by RT-PCR and real-time RT-PCR. A significant decrease was observed, especially from days 1-5 after the inoculation, in the expression of four early nodulins (*nork*, *NIN*, *N6* and *Enod2*). A reduction in the expression level of *Legbrc* gene (leghemoglobin) was also observed. On the contrary, the expression of *Enod40* and the cyclin gen ccs52 was not significantly altered. Finally, a transcriptomic analysis of the As effect on the *Sinorhizobium-Medicago* symbiotic interaction using *Medicago truncatula* microarrays has been developed.

Introduction

There is increased interest in the use of legume plants (usually the first colonizers of poor or degraded soils) associated with microorganisms (the major factor affecting metals solubility, bioavailability and mobility) for bioremediation (Khan *et al.,* 2009). In addition, the rhizobia-legume symbiotic interaction is an efficient soil nitrogen improvement system. In order to use legumes for this purpose, it is important to determine the effect of soil contaminants on the interaction process.

Material and Methods

Sinorhizobium medicae MA11 isolation and As tolerance characterization, *Medicago sativa* growth conditions and microscopy of nodulation in the presence of As are described in Pajuelo *et al*. (2008). RT-PCR and real time RT-PCR analyses are described in Lafuente *et al*. (2010).

Results and Discussion

The nodulation and nitrogen fixation properties of the *S. medicae* MA11 strain resistant to As have been evaluated determining parameters such as number of primordia and nodules and nitrogenase activity, both in the presence and absence of As (Figure, **A**). Arsenic decreased to 25% the total number of nodules, due to a reduction in the number of rhizobial infection close to 90%. The first steps of nodulation of alfalfa were more sensitive to As than nitrogen fixation and, once nodulation was established, nodule development was normal, since nodules were effective (as demonstrated by nitrogenase activity).

A detailed microscopic study of nodulation has been conducted using the *S. medicae* MA11 strain carrying *hemA::lacZ* reporter gene fussion, both in the presence and absence of As. In the presence of As, roots showed deep symptoms of necrosis and root hairs looked damaged, showing deformities specially at the root hair tips (Figure, **B**). Infection threads (3 dpi) and nodule primordia (6dpi) were normal (Figure, **C-D**), being primordia in the presence of As more developed than those found in the absence of As. In mature fixing nodules (28 dpi), a big senescence zone without staining was observed (Figure, **E**). Results suggest that an earlier senescence can be observed in the presence of the toxic.

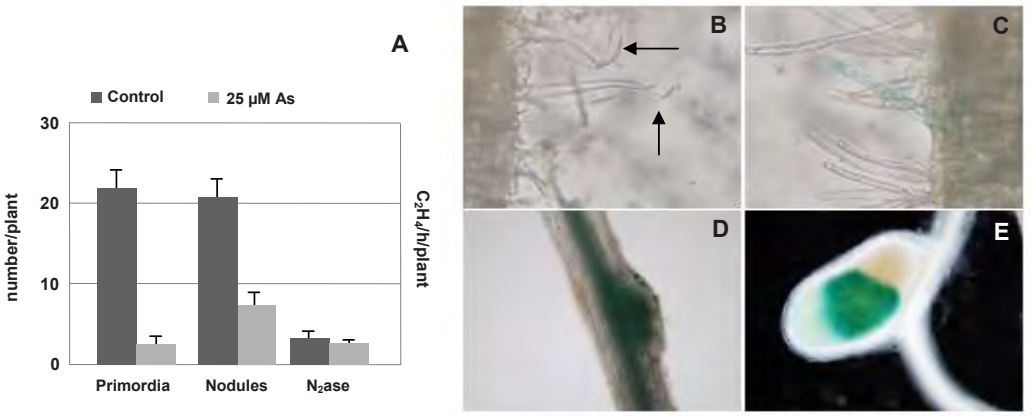

Figure. Effect of the presence of arsenic on *M. sativa* nodulation. **A**) Effect of As on nodule features (primordia were counted on plants grown on square plates, while nodules and nitrogenase activity were estimated on plants grown in Leonard jars). **B-E**) Microscopic observation of nodule development in plant grown in the presence of As (β-galactosidase staining). **B**) Aspect of root hairs (arrows: tips were specially affected). **C**) Infection thread. **D**) 6dpi nodule primordia. **E**) Mature fixing nodule (28 dpi). Note the big senescence zone without β-galactosidase staining.

We have examined the molecular mechanisms underlying As toxic effect. In the presence and absence of As, the expression pattern of seven nodulin genes, markers for the different events leading to nodule formation, were analyzed by RT-PCR and real-time RT-PCR. A significant decreased was observed, especially from days 1-5 after the inoculation, in the expression of four early nodulins: the genes coding the Nod factor receptor (*nork*), the transcription factor *NIN* and the markers for infection progression (*N6*) and nodule organogenesis (*Enod2*). The expression of markers for primordium initiation (*Enod40*) and differentiation (*ccs52*) was not significantly altered (Table). Finally the expression of a marker for nitrogen fixation (*Legbrc*) was also reduced. These results suggest that As affects the expression of genes associated with processes that take place in the epidermis and the outer cortical cells.

Table. RT-PCR analysis of nodulation marker gene expression in the root from 1 to 5 dpi. Gene expression in the absence of As represents 100% of the gene of interest for each day.

Gene	*nork*	*NIN*	*Enod2*	*N6*	*Enod40*	*ccs52*	*Legbrc*
% expression (+As)	20-40	20-40	15-25	70-100	96-102	98-103	30-35

To complete the transcriptomic profile of roots inoculated in the presence of As, we have hybridized Mt16kOLIPlus *M. truncatuula* microarrays. Samples were obtained from 5 dpi roots, inoculated with *S. medicae* MA11 in the presence or absence of As. Preliminary results showed that As induced the overexpression of 168 genes (≥5-fold; 2 of them related with nodulation) and the repression of 167 genes (≥5-fold; 18 of them related with nodulation).

Acknowledgments

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Glutathione-*S*-transferase activities of two *Rhizobium leguminosarum* bv. *viceae* strains differing in cadmium-glutathione complexation

Corticeiro S, Figueira E*

*Center for Cell Biology, University of Aveiro, Campus de Santiago, 3800-193 Aveiro, Portugal. *efigueira@ua.pt*

Summary

Recently we have demonstrated the existence of a novel intercellular cadmium (Cd) detoxification mechanism in *Rhizobium leguminosarum*: the formation of Cd-bisglutathionate complexes. In yeasts the binding between glutathione (GSH) and Cd was shown to be mediated by glutathione-*S*-transferases (GSTs), but in bacteria this issue has never been addressed. In order to elucidate the role of GSTs in the efficiency of Cd-GSH binding, GST activity was measured with three specific substrates, in two *Rhizobium leguminosarum* strains differing in Cd tolerance. Our results show that the two strains differed in their Cd binding efficiency, which was related to their Cd tolerance. GST activities varied between strains when using CDNB, ETHA and DCNB as substrates, and Cd affected differently these activities, suggesting that GST isoforms may be differently related to Cd tolerance. These findings may lead to a better understanding of the metal tolerance mechanisms in free-living *Rhizobium* and may contribute to the maintenance of *Rhizobium* populations in metal contaminated soils.

Introduction

The permanent increase in heavy metal pollution, especially in agricultural soils, due to the current use of fertilizers and industrial and domestic sludges, is becoming one of the most troublesome environmental problems, which negatively affects the soil microbial community. Among the nonessential metals, Cd poses a most concerning threat due to its high mobility, bioavailability and persistence. It affects free-living *Rhizobium* survival and when in symbiosis with a legume, nitrogen fixation is seriously impared. Previous results from our lab showed that GSH played a dual function in the tolerance of *Rhizobium leguminosarum* to Cd: protection from oxidative stress, induced by the metal (Corticeiro *et al.,* 2006), and chelation of metal ions (Lima *et al.,* 2006), thus alleviating the stress imposed by Cd in the cell metabolism. Understanding the factors affecting the complex formation between Cd and GSH in *Rhizobium* is thus crucial to understand the Cd tolerance differences among strains and can be used to develop strategies to improve metal tolerance in *Rhizobium*. A strong candidate for mediating the GSH-Cd binding, and consequently affecting the efficiency of the chelation mechanism, are GSTs, a group of detoxifying enzymes that mediate the complexation of toxicants with GSH. In yeasts the bound between GSH and Cd was shown to be mediated by GSTs (Adamis *et al.,* 2004), but this activity was never reported in bacteria. Thus, the aim of this work is to determine the efficiency of Cd chelation and to study GST activities in strains presenting different Cd tolerance in order to highlight the involvement of these enzymes in the Cd-GSH complex formation, thereby contributing to better understand this tolerance mechanism of *Rhizobium* when exposed to an excess of metals.

Materials and Methods

Two *Rhizobium leguminosarum* strains E20-8 (tolerant) and NII-1 (sensitive) were grown in YEM medium (Lima *et al.,* 2006) supplemented with Cd concentrations displaying 70% of growth reductions (1 mM and 0.25 mM $CdCl_2$ for the E20-8 and NII-1, respectively). The Cd chelated to GSH was obtained by size exclusion chromatography (Lima *et al.,* 2006). Total intracellular and chelated Cd was determnied by ICP-AES, after acid extraction in teflon bombs (Lima *et al.,* 2006). GST activity was measured spectrophotometrically at 25ºC using CDNB, ETHA and DCNB according to the method of Habig & Jacoby (1974). Specific activity was expressed as U mg^{-1} protein. The protein concentration was determined according to Bradford (1976). Significant

differences between means were determined by a one-way ANOVA, using the Tukey test (P <0.05) in the Sigma Stat 3.5 program.

Results and Discussion

Although GSH-mediated Cd chelation was present in both tolerant and sensitive strains, its efficiency was not the same (Table). The tolerant strain was able to complex 75% of the intracellular Cd through GSH coordination, which unequivocally demonstrates the importance of this mechanism on the intracellular Cd detoxification in this *R. leguminosarum* strain. However, in the sensitive strain the cell only chelated 23% of its intracellular Cd to GSH. This contrast in metal complexation can certainly contribute to the Cd tolerance difference displayed by the two strains.

Table1: Amount of Cd chelated to GSH and its relation to the total intracellular Cd in two Rhizobium strains: one tolerant and one sensitive exposed 1mM and 0.25 $CdCl_2$, respectively. Data are the means of three replicate experiments, with standard errors.

	Cd (μmol/mg protein)	
	Tolerant	Sensitive
Total intracellular	151.75 ± 19.04	16.22 ± 3.27
Percentage complexed	75	23

GST activity varied between strains, and was influenced by the substrate and Cd (Figure). GST activity in *Rhizobium* was determined towards different substrates, since the catalytic ability to selected substrates is considered an important parameter to distinguish among different GST isoforms (Dainelli *et al.,* 2002). The activity in the presence of the CDNB substrate was the one displaying the most significant increases in the presence of Cd, but only in the tolerant strain.

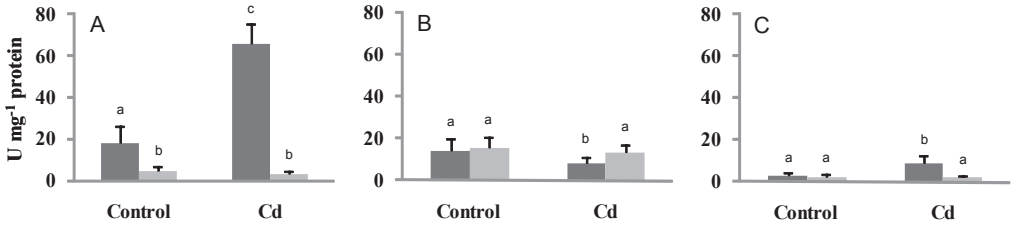

Figure. Total GST activity towards different specific substrates, CDNB (**A**), ETHA (**B**) and DCNB (**C**), of two *Rhizobium* strains, one tolerant (■) and one sensitive to Cd (■) growing under control conditions and under Cd stress. Values are the means ± SE of three or more replicates. Different letters indicate significant differences between means.

Thus, results suggest that the GST isoforms with higher affinity to CDNB can mediate GSH-Cd complexation and thus be responsible for the high Cd tolerance in the tolerant strain. These findings may lead to a better understanding of the metal tolerance mechanisms in free-living *Rhizobium* and may contribute to the persistance of *Rhizobium* populations and to the maintenace of fertility in metal contaminated soils. They also open new perspectives for enhancing metal tolerance abilities in bacteria.

Acknowledgments

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Lima AIG, Corticeiro SC, Figueira EMAP (2006) *Enz Microb Tech* 39: 763-769.

Defense-related genes involved in the *Casuarina glauca-Frankia* actinorhizal symbiosis

Graça I[1,2*], Liang J[1,3], Pinto Ricardo CP[3], Melo A[1], Pereira AS[2], Ribeiro A[1,3]

[1]Eco-Bio/IICT, Oeiras, Portugal; [2]FCT-UNL, Monte da Caparica, Portugal; [3]ITQB/UNL, Oeiras, Portugal.
**inesgraca@hotmail.com*

Summary

Eleven putative defense-related genes have been cloned from actinorhizal nodules of *Casuarina glauca*. Based on their expression patterns, three genes, *CgChi3* (chitinase), *CgHin1* (hairpin-inducible protein) and *Cg-GST* (glutathione *S*-transferase), that were found to be up-regulated in nodules (relative to roots) were selected for further functional analysis through the characterization of the encoded proteins. At a broader scale, a study of the nodule proteome under physiological and salt stress conditions is being carried out.

Introduction

Actinorhizal nodules are induced by nitrogen-fixing bacteria of the genus *Frankia* on roots of several woody plants, called actinorhizal plants. These plants have a great plasticity for environmental adaptation and are widely used in agro-forestry and for ecological purposes, being important elements in plant communities. Worldwide, several groups have been working in the field of actinorhizal symbioses aiming at understanding the mechanisms controlling the symbiotic process, and at evaluating the genetic diversity of macro- and microsymbionts as well as the potentialities of the symbiotic systems for ecological purposes. Under this context, our group has initiated a study on the role of defense-related genes during the symbiosis between *C. glauca* and *Frankia,* under optimal growth conditions and following exposure to abiotic stress.

Materials and Methods

Expression of recombinant proteins. The open reading frames (ORFs) of *CgChi3* and *CgHin1* were cloned into *pET-28a* and *pET-21c* expression vectors (Novagen), respectively, and expressed in *Escherichia coli* BL21(DE3) cells. These contained the pRec-cDNA-ORFs and were grown in 100 ml of LB medium at 37ºC to optimize experimental conditions: growth time, concentration of inducer (isopropyl-D-thiogalactopyranoside, IPTG), and induction time. After optimization, cultures were scaled-up to 1 l for further protein purification and biochemical characterization.

Proteomics. The *C. glauca* nodule proteome is being studied by 2 D-electrophoresis. Total protein was extracted from leaves, roots and nodules, and proteins were resolved by isoelectric focusing (IEF; 13 cm strips and pH 3-11), followed by SDS-PAGE. Gels were stained with Coomassie Brilliant Blue G-250.

Results and Discussion

CgChi3, CgHIN1and CgGST proteins were successfully expressed in small-scale experiments (100 ml cultures) under the following conditions. For CgChi3: OD_{600} = 0.5, 0.1 mM IPTG, 3 h-induction at 15ºC; for CgHin1: OD_{600} = 2, 0.5 mM IPTG, 3 and 21 h-induction at 37ºC; and for CgGST: OD_{600} = 0.5, 0.5 mM IPTG, 3 h-induction at 37ºC. After scaling up the cultures, CgChi3 and CgHIN1 recombinant proteins were found at low levels in the cell membrane fractions and at high levels in inclusion bodies. This was probably due to the motifs of the proteins, which are not properly recognized by the recombinant bacteria. Because the amount of protein in the membrane fractions was not sufficient, the recombinant proteins were further purified from the inclusion bodies by denaturation/renaturation in an urea gradient (8 to 0 M). In order to confirm if CgGST is soluble (as predicted by bioinformatic analysis of the putative

protein sequence), a fractionation assay is being tested before scaling up the recombinant cultures. A protocol for activity studies was then developed with several putative enzymatic substrates such as chitinases (CgChi3), components of the *Frankia* surrounding plant-matrix and *Frankia* factors (Cghi3 and CgHin1), and glutathione (CgGST). Concomitantly, the corresponding antibodies are being produced in rabbits to perform immunoblotting and immunolocalization studies during the nodulation process, under normal conditions or salt stress. To do this, a salt-stress experiment with increasing NaCl concentrations (0 to 600 mM) was set up. In a larger approach we have established a protocol for nodule proteome analysis by 2 D-electrophoresis. Following this strategy, it is expected to complement the nodule trancriptome analysis that is being carried out at IRD-Montpellier, and to identify some of the key elements that are required for a successful symbiosis between *C. glauca* and *Frankia*.

Acknowledgments

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Structural and regulatory analysis of quorum sensing in *Rhizobium leguminosarum*

Sánchez-Cañizares C[1], Morata A[2], Cantero L[3], Ruiz-Argüeso T[1], Imperial J[1,4*], Palacios JM[1]

*[1]Centro de Biotecnologia y Genómica de Plantas and Departamento de Biotecnología Universidad Politécnica de Madrid, Campus de Montegancedo, M-40, km 37.7, 28223 Pozuelo de Alarcón, Madrid, Spain; [2]Departamento de Tecnología de Alimentos, E.T.S. Ingenieros Agrónomos, Universidad Politécnica de Madrid, 28040 Madrid, Spain; [3]Centro de Investigaciones Príncipe Felipe, Valencia, Spain; [4]CSIC, Madrid. *juan.imperial@upm.es*

Summary

In the present work, we have studied the role of quorum-sensing regulatory systems in *Rhizobium*-legume symbiosis. Competition assays suggest that inactivation of quorum sensing systems significantly affects the competitiveness of *Rhizobium leguminosarum* bv. *viciae* UPM791 (*Rl* UPM791) when compared to other strains. Structural analysis through HPLC / mass spectrometry revealed that the signals produced by *Rl* UPM791 correspond to: C_6-HSL, C_7-HSL, C_8-HSL and 3OH-C_{14}-HSL; also, small amounts of C_4-HSL have been detected. We are also analyzing the complex regulation of AHL signal molecules. We have evidence indicating that an *Rl* UPM791 plasmid (pUPM791d) participates in a regulatory network acting on the chromosomal system *cin*RI. We are using concurrent strategies (sequencing of pUPM791d and random mutagenesis) to identify the mechanism responsible for the control of pUPM791d over AHL production in *Rl* UPM791.

Introduction

Bacteria are able to detect changes in their own population density and respond to them by activating transcription of different target genes involved in different bacterial functions. This process relies upon an intercellular communication system known as quorum sensing (Gonzalez & Marketon, 2003). In the case of our laboratory reference strain, *Rl* UPM791, two functional systems of quorum sensing regulation have been described (Cantero *et al.,* 2006). Both systems are similar to the *lux*RI model system described in *Vibrio fischeri* and are mediated by N-acyl-homoserine lactone (AHL) signals: *cin*RI system, located in the chromosome, and *rhi*RI system, encoded in the symbiotic plasmid. In this work we present studies on the structure and regulation of AHL signals involved in quorum sensing in this endosymbiotic bacterium.

Materials and Methods

AHLs were obtained from spent supernatant from stationary phase cultures and extracted with ethyl acetate. Solvent was evaporated and extracted compounds were dissolved in methanol. Samples were then subjected to HPLC analysis (C_{18} reverse pahse, water-methanol gradient) followed by electrospray-mass spectropmetry (Gould *et al.,* 2006). Competition assays were carried out using *Rl* UPM791 derivative strain *Rl* UPM1156, carrying a constitutively expressed *gus*A gene, resulting in blue nodules when incubated with X-gluc substrate (Wilson *et al.,* 1995). DNA has been obtained using DNeasy Blood & Tissue Kit columns (QIAGEN Ltd.). Genome sequencing is being carried out through 454 massive sequencing at the Institute for Genome Sciences (Maryland, USA).

Results and Discussion

To study the role of quorum sensing regulatory systems in *Rhizobium*-legume symbiosis, different competition assays for nodulation of pea roots by *Rl* UPM791, *Rl* 3841 and derivative strains affected in AHL production have been carried out. The strains we used carried the plasmid pME6863 containing the gene *aii*A (Cha *et al.,* 1998), which codes for a AHL-hydrolyzing lactonase enzyme, or vector pMP6000 as control. The introduction of pME6863 plasmid resulted in the elimination of virtually all AHLs produced by the bacteria. The analysis revealed that hydrolysis of quorum sensing signals significantly affects the

competitiveness of strain *Rl* UPM791 vs *Rl* 3841, thus suggesting a relevant role of AHLs in nodule occupancy.

We have also characterized the type of AHLs produced by *Rl* UPM791 by structural analysis through HPLC / mass spectrometry. The analysis of spectra revealed that the signals produced by *Rl* UPM791 correspond to: C_6-HSL, C_7-HSL and C_8-HSL, produced by *rhi*RI system encoded in the symbiotic plasmid; and 3OH-C_{14}-HSL, a bacteriocin synthesized by the chromosomally-located system *cin*RI; small amounts of C_4-HSL have also been detected.

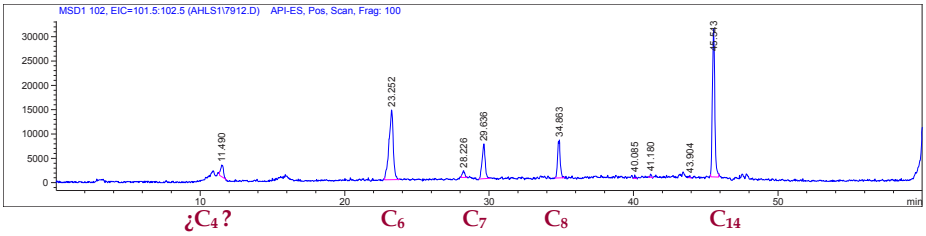

Figure. AHL signal spectra produced by *Rl* UPM791 identified by their retention time and characteristic molecular ions.

The regulation of AHL signal molecules is a complex process. We have evidence indicating that plasmid pUPM791d is involved in the regulation of 3OH-C_{14}-HSL production. To study this regulation we have constructed reporter gene fusions to *cin* and *rhi* systems. These fusions are being analyzed in different genetic backgrounds in order to evaluate the effect of the presence of the different plasmids in *Rl* UPM791. Preliminary data indicate that the presence of pUPM791d affects 3OH-C_{14}-HSL production at the post-transcriptional level.

Finally, we are developing several strategies to identify the mechanism responsible for the control of pUPM791d over 3OH-C_{14}-HSL production in *Rl* UPM791. This includes the determination of pUPM791d sequence and random mutagenesis of this plasmid.

Acknowledgments

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Optimizing Gateway™ technology (Invitrogen) to construct *Rhizobium leguminosarum* deletion mutants

Lanza M*, Alborno M, Rey L, Imperial J

*Centro de Biotecnología y Genómica de Plantas, UPM-INIA and Departamento de Biotecnología, Universidad Politécnica de Madrid, Campus de Montegancedo, Carretera M-40, km 38, 28223 Pozuelo de Alarcón, Madrid, Spain. *monica.lanza@upm.es*

Summary

The study of the role of different genes in *Rhizobium leguminosarum* requires the generation of mutants by homologous recombination. In this communication we describe a novel approach to obtain deletion mutants of genes in *Rhizobium* using Gateway™ Cloning technology (Invitrogen) and a new vector (pK18-attR), both conjugative and *Rhizobium* specific, that carries the recombination tails of Gateway system. This tool is a new alternative to the classic approach based on cloning using restriction enzymes. The first step consists of designing directed oligonucleotides with specific tails for isolating recombination fragments and a resistance marker cassette to an antibiotic by PCR. The three inserts are cloned by homologous recombination in three specific vectors, in a single step. The last step consists of multisite-directed recombination of the three donor vectors to the pK18-attR destination vector. After recombination, this vector loses the *ccdB* gene, whose expression results in synthesis of a DNA gyrase that is lethal to carrier cells and thus guarantees the effectiveness in obtaining clones that carry the homologous construction to the subsequent recombination in *Rhizobium*.

Introduction

Gateway™ cloning technology uses the specific recombination of λ phage to introduce directed inserts in *E. coli* expression vectors. This strategy provides a number of advantages over traditional technology based on the use of restriction enzymes and allows incorporation of an insert for a set of expression vectors in one step. The incorporation of the insert also means the extrusion of lethal gene *cddB* after recombination. The product of the *cddB* gene is a DNA gyrase lethal to *E.coli* cells where it is expressed. This system guarantees the effectiveness of the cloning, because colonies only survive if they lose this lethal marker, and this only happens when homologous recombination has occurred and the *ccdB* gene has been replaced by the corresponding insert.

In recent years Gateway cloning technology has evolved, and new specific expression vectors have been developed for a wide variety of prokaryotic (especially *E. coli*) and eukaryotic organisms, such as yeasts and plants. Recently, the Invitrogen company has started commercializing the "multisite Gateway cloning technology" as an improvement over the previous Gateway technology, because this new system allows cloning two, three, or four inserts in a single step. This strategy is very useful to generate gene deletion mutants in organisms, where they are produced by homologous recombination from the adjacent areas of the gene to be deleted. In this study we used this method to generate deletion mutants in *Rhizobium.*

Materials and Methods

The pK18-attR expression vector was generated in this work. To construct it, the pK18mobsacB (Schafer *et al.,* 1996) conjugative vector was digested with restriction enzyme *Hind*III and the Gateway cassette, carrying the recombination-selection *ccdB* gene and a chloramphenicol resistance gene, introduced in this site. This cassette has been previously amplified by PCR and digested with *Hind*III (Figure). Vector pK18-attR can be used to generate deletion mutants in *Rhizobium leguminosarum* using the Invitrogen multisite Gateway cloning technology.

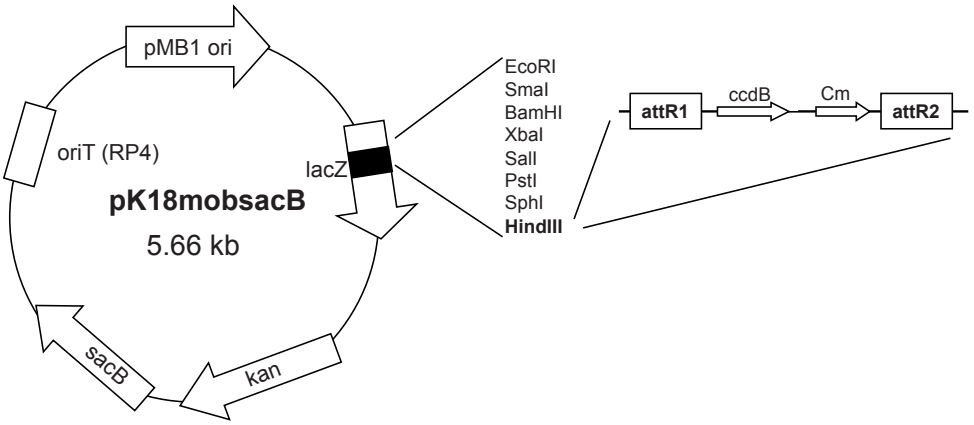

Figure. PK18-attR vector map.

Deletion mutants were generated in *Rhizobium* by conjugatively introducing the plasmid pK18-attR construct that carries the deletion construction for homologous recombination. This construct consisted of two adjacent recombination fragments of the gene to be mutated and a spectinomycin antibiotic resistance gene between those fragments. Specifically, this construction was generated as follows: First, specific primers were designed to amplify, by PCR, the three directed fragments for recombination by adding queues for the Invitrogen multiple system: tails P1 and P4 for insert 1, tails P4r and P3r for the antibiotic resistance gene, and tails P3 and P2 for the third fragment. They were all specifically recombined by BP reaction with vectors pDONR221 P1-P4, pDONR221 P3r-P4R and pDONR221 P3-P2, respectively. In this way, three independent clones, with compatible specific tails, were obtained that recombine with each other through a second recombination reaction, called LR, with the pK18-attR *Rhizobium*-specific destination vector. Both donor and target vectors endow carrier cells with resistance to the antibiotic kanamycin. Because of it, donor vectors were previously digested by the restriction enzyme *Nsi*I that breaks the of kanamycin resistance gene cassette. The subsequent selection of clones, after the LR reaction, was done with spectinomycin. Additionally, for the LR step of some vectors the pDEST22 destination vector (Invitrogen), that confers resistance to the antibiotic ampicillin but it is not conjugative to *Rhizobium*; was used. For that reason, an additional LR reaction was carried out using the pK18-attR vector as target and selecting the resulting clones with the antibiotic kanamycin.

Results and Discussion

In this paper we demonstrate an alternative strategy to generate deletion mutants of genes in *Rhizobium leguminosarum* taking advantage of Gateway technology (Invitrogen). For this purpose, a specific conjugative vector for *Rhizobium* has been generated (as described in Materials and Methods) to serve as a molecular tool for introduction of the deletion into the bacteria. The resulting mutants were confirmed by PCR and Southern blot in order to validate the method.

This system of mutant generation allows the quick mutation of several genes from an operon, because clones generated after the BP reaction are interchangeable so one of the genes can be kept and another one varied depending on whether one, two or more genes from the same operon are to be deleted. On the other hand, resistance cassettes are interchangeable so that if necessary, they could be replaced by another marker in a single LR reaction. In this way, a collection of specific directed clones is generated and these are placed in a concrete position and are interchangeable with clones of the same position. Furthermore, the sequences of the recombination tails which are generated after LR reaction are compatible with all the vectors that carry the same Gateway tails.

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Analysis of a periplasmic thiol oxidoreductase in *Rhizobium leguminosarum*

Rey L*, Vega RM, Alborno M, Ruiz-Argüeso T, Palacios JM

*Departamento de Biotecnología, E.T.S.I. Agrónomos, Centro de Biotecnología y Genómica de Plantas (UPM-INIA), Campus de Montegancedo, Universidad Politécnica de Madrid, Pozuelo de Alarcón, Madrid, Spain. *luis.rey@upm.es*

Summary

In this work the expression and cellular localization of a predicted periplasmic thiol oxidoreductase encoded by *Rhizobium leguminosarum* 3841, ORF RL1083, were analysed. Based on the homology of the encoded protein with DsbA proteins from other bacteria we named it *dsbA*. The genetic organization of *dsb*A region showed that it is a monocystronic gene. A putative σ^{70} promoter was predicted upstream *dsb*A gene. The promoter region fused to *gus*A reporter gene revealed that *dsb*A is expressed in free-living conditions in different media and also, although at a lower level, in pea bacteroids. *R. leguminosarum* DsbA contains a potential Tat-dependent signal peptide. To localize this protein in different cellular fractions the protein was labelled by means of a C-terminal Strep tag. The DsbA-Strep protein was localized in the periplasmic fraction. At present three type of experiments are in progress: first, the study of DsbA Tat-dependence by using a *tat* mutant strain harbouring *dsb*A-Strep; second, the construction of a *dsb*A mutant and third the evaluation of periplasmic disulfide oxidoreductase activity of different strains: wild-type, *tat* mutant and *dsb*A mutant.

Introduction

Periplasmic proteins play a very important role for the *Rhizobium*-legume symbiosis. Some of the proteins located in the periplasmic space require a translocation system designated Tat, able to translocate folded proteins containing signal peptides with a "*twin-arginine*" conserved motif (Sargent *et al.,* 1998). In *Rhizobium leguminosarum* 3841 more than one hundred proteins are predicted by bioinformatic tools to be transported by the Tat system (Jiménez, 2006). One of these is RL1083, a DsbA-like protein, that would catalyze disulfide bond formation in the periplasm. Dsb proteins are involved in plant and animal pathogenesis (Jiang *et al.,* 2008) and could have an important role in the *Rhizobium*-legume symbiosis.

Materials and Methods

Generation of a *gus*A fusion to *R. leguminosarum dsb*A promoter (plasmid pJP2pdsbA) was performed by cloning a 516-bp PCR DNA fragment containing 185 pb of *dsbA* promoter and 331 pb of *dsb*A gene in front of *gus*A gene of plasmid pJP2. A DsbA-Strep fusion protein was produced in *Rhizobium* using a derivative of plasmid pJN105 harbouring a DNA fragment encoding DsbA with a Strep-tag coding sequence fused to its C-*terminus*. DsbA-Strep was identified by immunoblot using Streptavidin conjugates. Disulfide oxidoreductase activity was determined by Ellman´s assay (Jiang *et al.,* 2008). For more details on Material and Methods, see Vega (2009).

Results and Discussion

Analysis of *R. leguminosarum dsb*A upstream region by NNPP (Neural Network Promoter Prediction server) predicted the presence of a putative σ^{70} promoter. Experimental analysis of this promoter using a fusion with *gus*A reporter gene indicated that *dsbA* is expressed in different media and in symbiosis with peas. These data are consistent with a constitutive expression of DsbA, suggesting that periplasmic protein disulfide bond formation will be required under most conditions. Additionally the promoter activities were determined at different OD_{600} in free-living conditions (TY medium). The results indicated a similar activity at different OD_{600} including exponential and stationary phases.

A protein compatible with the expected size of DsbA-Strep without signal peptide (26 kDa) was identified in total protein extracts and in periplasmic fractions by immunoblot. Also, this protein was partially purified from periplasmic cellular fractions by Streptactin chromatography (Figure).

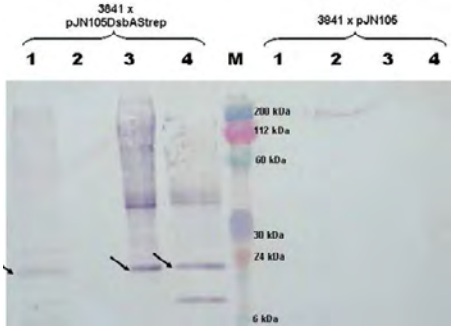

Figure. Partial purification of DsbA-Strep protein by Streptactin chromatography. DsbA-Strep (arrows) was identified by Western blot using AP-conjugated Streptavidin. Lanes: **1,** Periplasmic proteins before chromatography. **2**, Streptactin column passthrough. **3-4,** Streptactin-binding proteins.

At present the Tat dependence of DsbA is being investigated using a *tat* defective strain (Meloni *et al.,* 2003). Generation of a *dsb*A mutant by recombination of a plasmid containing an internal fragment of *dsb*A gene is also in progress.

Acknowledgments

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Overexpression of FurA affects heterocyst differentiation in *Anabaena* sp. PCC 7120

González A, Bes MT, Peleato ML, Fillat MF*

*Departamento de Bioquímica y Biología Molecular y Celular, Universidad de Zaragoza, 50009 Zaragoza, Spain. *fillat@unizar.es*

Summary

Heterocyst differentiation and its spacing pattern formation in filamentous nitrogen-fixing cyanobacteria occur as result of multiple signals and the influence of several regulators. Previous studies have suggested a transcriptional regulatory connection between iron and nitrogen metabolism in *Anabaena* sp., since the ferric uptake regulator FurA bound *in vitro* to iron boxes located in the promoter region of the master nitrogen regulator NtcA, and overlapping genes were identified in FurA and NtcA regulons. In the present study we analyzed the effect of FurA overexpression on the heterocyst development in *Anabaena* sp. PCC 7120, and we investigated the *in vitro* affinity of FurA to promoters of some other key regulators implicated in heterocyst differentiation. By using a shuttle vector with the copper-responsive *petE* (plastocyanin) promoter fused to a *furA* gene copy, we observed that overexpression of FurA decreased the heterocyst development rate and changed the pattern formation in *Anabaena* filaments growing under nitrogen deprivation. Electrophoretic mobility shift assays showed specific *in vitro* binding of FurA to promoters of *hetR* and *asr1734*, but did not to promoters of *devH*, *nrrA*, *patS* or *hetN*. The results provide new evidences of a possible cross-talk between iron and nitrogen regulatory networks orchestrated by Fur in cyanobacteria.

Introduction

Some filamentous cyanobacteria can fix atmospheric nitrogen under combined nitrogen deprivation in specialized differentiated cells called heterocysts. Heterocyst differentiation and its spacing pattern formation occur as result of a concerted series of signals and the influence of multiple regulators, including both inducers and repressors.

Ferric uptake regulator (Fur) proteins are considered global transcriptional regulators in prokaryotes. This family of proteins controls not only the iron uptake and storage machineries, but also modulates the expression of genes involved in a variety of other cellular processes, usually functioning as iron-dependent transcriptional repressors. In *Anabaena* sp. PCC 7120, FurA is a constitutive protein that controls iron uptake and seems to be essential for *in vitro* growth, since attempts to eliminate all wild-type copies of the *furA* gene from the polyploid genome have resulted only in a partial segregation of mutated chromosomes.

Previous studies have suggested a transcriptional regulatory connection between iron and nitrogen metabolism in this cyanobacterium, since FurA bound *in vitro* to iron boxes located in the promoter region of the master nitrogen regulator NtcA, and overlapping genes were identified in FurA and NtcA regulons. In the present study, we have analyzed the effect of FurA overexpression on the heterocyst development in *Anabaena* sp. PCC 7120, and have investigated the *in vitro* affinity of FurA to promoters of some other key regulators implicated in heterocyst differentiation.

Materials and Methods

Vector pAM2770FurA, which contains a copy of the *furA* gene under the control of the *Anabaena* sp. copper-inducible *petE* (plastocyanin) promoter, was constructed from the shuttle vector pAM2770 (Lee *et al.,* 2003), and transferred to wild-type *Anabaena* sp. PCC 7120 by conjugation (Elhai *et al.,* 1997). FurA ovexpression phenotype of exconjugant clones was screened by Western blotting using a rabbit anti-FurA polyclonal antiserum. The clone selected as the *furA*-overexpressing strain was named AG2770FurA. For physiological studies, *Anabaena* sp. strains were photoautotrophically grown in BG-11 medium (supplemented with neomycin 50 μg ml^{-1} in case of strain AG2770FurA) using an incubator shaker under controlled conditions (30ºC, 120 rpm, 20 μE m^{-2} s^{-1} continuos white light). To induce heterocyst differentiation, triplicate cultures of wild-type strain

PCC 7120 and its derivative *furA*-overexpressing strain AG2770 were grown to log phase (OD_{750}= 0.3-0.4) in BG-11 medium, washed two times using BG-11 without combined nitrogen ($BG\text{-}11_0$), resuspended in $BG\text{-}11_0$ and incubated under the same culture conditions during 6 d. Samples were aseptically taken every 24 h and heterocyst formation was followed by bright-field and fluorescence microscopic. Promoter regions of *ntcA*, *hetR*, *devH*, *nrrA*, *patS*, *hetN* and *asr1734* were obtained by PCR. Electrophoretic mobility shift assays (EMSA) with recombinant FurA were performed as described previously (Hernández *et al.,* 2006).

Results and Discussion

A derivative strain of *Anabaena* sp. PCC 7120 showing a high level of overexpression of FurA was generated using the shuttle vector pAM2770; the resulted strain was named AG2770FurA (Fig. 1A). Bright-field and fluorescence microscopy analyses (Fig. 1B) of wild-type and AG2770FurA strains growing under combined nitrogen deprivation revealed a decrease in the heterocyst development rate as well as changes in the pattern formation in the *furA*-overexpressing strain. While the wild-type strain developed differentiated cells in ~72h, AG2770FurA late more than 5 d. In wild-type, heterocysts appeared followed the typical pattern of one differentiated cell separated by 10-12 vegetative cells. This typical pattern did not occur in AG2770FurA, which occasionally developed just one heterocyst per filament.

By using EMSA analyses, we showed *in vitro* specific binding of recombinant FurA to at least three regulators involved in the heterocyst differentiation process: NtcA, HetR and Asr1734 (Fig. 2). No *in vitro* affinity of was observed with other key nitrogen regulators like DevH, NrrA, HetN or PatS.

NtcA is a transcriptional factor with a dual function, it senses nitrogen starvation in the cell and leads to the expression of HetR, the main positive factor involved in heterocyst development and pattern formation (Muro-Pastor *et al.,* 2002). Under a FurA overexpression background, the expression of both trigger nitrogen regulators could be partially repressed, leading to affectation in the heterocyst differentiation process. Thus, FurA coud stablish a connection between iron status, oxidative stress and nitrogen metabolism in *Anabaena* sp. PCC 7120.

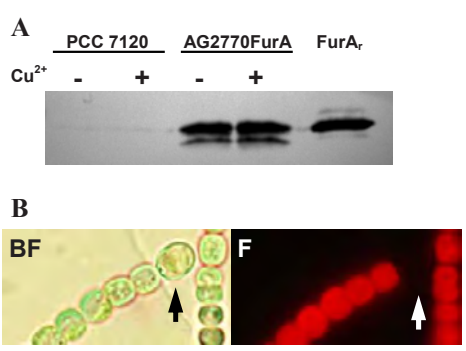

Figure 1. (A) Overexpression of FurA as revealed by Western blotting analysis. Recombinant FurA was included as control. Levels of P*petE-furA* induction were similar in BG-11 (-) and BG-11 supplemented with additional copper (+). **(B)** Heterocyst as observed by bright-field (BF) and fluorescence (F) microscopy.

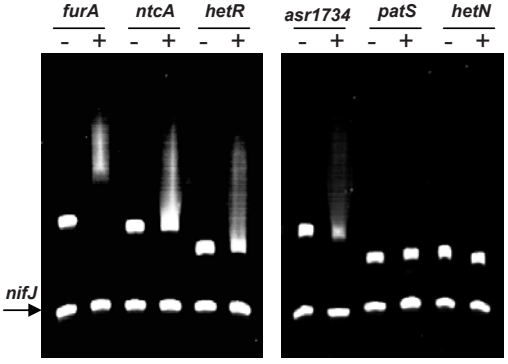

Figure 2. EMSA analyses showed specific *in vitro* binding of FurA to promoters of regulators NtcA, HetR and Asr1734. Free DNA (-), DNA plus FurA (+). Promoter of NifJ was included as non-specific DNA competitor in all assays. Binding of FurA to its own promoter was included as positive control.

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MicroRNAs expression profile during symbiotic nitrogen fixation in nutrient- or metal-stressed common bean plants

Naya L[1*], Valdés-López O[1], Mendoza AB[1], Nova-Franco B[1], Yang SS[2], Aparicio-Fabre R[1], Vance CP[2,3], Reyes JL[4], Hernández G[1]

[1]Centro de Ciencias Genómicas, Universidad Nacional Autónoma de México (UNAM), Ap. Postal 565-A, 62210 Cuernavaca, Mor. México; [2]USDA-Agricultural Research Service, Plant Science Research Unit and [3]Department of Agronomy and Plant Genetics, University of Minnesota, St. Paul, MN 55108, USA; [4]Departamento de Biología Molecular de Plantas, Instituto de Biotecnología, UNAM, Cuernavaca, Morelos, Mexico.
**aloreto@ccg.unam.mx*

Summary
Plant microRNAs (miRNAs) are essential post-transcriptional regulators of gene expression and are involved in the response and adaptation to adverse environmental conditions. So far, relatively little information is available about legume miRNAs. The aim of this work was to identify miRNAs expressed in nodules of common bean (*Phaseolus vulgaris*) plants grown under control conditions, phosphorous and iron deficiencies, and metal toxicities (aluminium, copper, and manganese). In this work we have detected 62 miRNAs (24 conserved, 8 from common bean, 19 from soybean, and 11 from *Medicago truncatula*) expressed in nodules using a miRNA-macroarray approach. Forty miRNAs were differentially regulated in stressed nodules, including 5 miRNAs (gm-miR1511, gm-miR1516, gm-miR1536, mt-miR1509, and mt-miR2590) that were only detected under stressful conditions. Subsequently, 5 miRNAs were validated by Northern-blot analysis. Current work is aimed at functionally characterizing nodule stress-responsive miRNAs in common bean.

Introduction

MicroRNAs (miRNAs), 21 nt non-coding RNAs, regulate gene expression at post-transcriptional level in plants. A precursor miRNA (pre-miRNA) with an imperfect hairpin structure is processed into a mature miRNA and this miRNA is incorporated into an RNA silencing complex which slices the corresponding mRNA target (reviewed by Jones-Rhoades *et al.,* 2006). MiRNAs participate in organ development, nutrional metabolism, hormonal signalling and abiotic or biotic stress responses in plants (reviewed by Sunkar *et al.,* 2007; Chuck *et al.,* 2009). An important number of functional studies of conserved miRNAs have been published for Arabidopsis but much less is known in legumes. Massive sequencing approaches have provided sets of miRNAs of model legumes like *Medicago truncatula* (Szittya *et al.,* 2008; Lelandais-Brière *et al.,* 2009) and soybean (Subramanian *et al.,* 2008; Joshi *et al*., 2010).

Common bean *(Phaseolus vulgaris)* is the most important grain legume for human consumption. In Latin America and Africa, inadequate fertilization and acidity of soils cause nutrient deficiencies or metal toxicities affecting symbiotic nitrogen fixation (SNF) and crop production (Broughton *et al.,* 2003). Complex regulatory mechanisms for adaptation of common bean to abiotic stresses may involve miRNAs along with other regulators. However, the knowledge of miRNAs in common bean is scarce. Arenas-Huertero *et al.* (2009) identified several conserved and six novel miRNAs found in small RNA libraries obtained from common bean seedlings under different treatments. Valdés-López *et al.* (2008) have demonstrated the role of PvmiR399 in P homeostasis in bean roots. Our current research aims to determine the expression profile of selected miRNAs in SNF common bean plants under nutrient deficiency or metal toxicity.

Materials and Methods

Common bean (Mesoamerican variety "Negro Jamapa 81") plants were grown in hydroponic conditions at 25°C using plastic boxes with 8 L of Franco/Munns nutrient solution, pH 6.5 (Franco & Munns, 1982) aerated with aquarium air pumps. Plants were inoculated with *Rhizobium tropici* CIAT 899. Stress treatments were applied 12-15 dpi when nodules were functional. Treatments Pd, Fed, Ac and Mnt have been reported (Valdés-López *et al.,* 2010), whereas for the Alt and Cut toxicity treatments 70 μM Cu and 70 μM Al were used. Total RNA was extracted from 1-1.5 g of nodules, roots and leaves by LiCl precipitation or TRIzol reagent (Invitrogen). Northern blot analysis and miRNA-macroarrays experiments were performed as described by Valdés-López *et al.* (2010).

Results and Discussion

The first step in this study was directed toward determining the expression profile of miRNAs in nodules of common bean plants inoculated with *R. tropici*, using a miRNA-macroarray hybridization approach. Two miRNA-macroarrays were prepared, one contained 24 conserved, 35 from soybean and 9 common bean miRNAs (Valdés-López *et al.,* 2010) and the second contained 20 new Mtr-miRNAs from *M. truncatula* (Lelandais-Brière *et al.*, 2009) and 4 conserved miRNAs as internal controls. In control nodules we detected 62 miRNAs: 24 conserved, 8 from bean, 19 soybean, and 11 from *M. truncatula*.

The second step was to identify miRNAs differentially regulated in nodules of SNF common bean plants subjected to P or iron deficiencies (Pd, Fed), acidic conditions (Ac) and metal toxicities (Alt, Cut and Mnt). We identified a total of 40 miRNAs that were differentially stress-regulated in nodules (induced $R > 2$ or repressed $R < 0.5$). These included 5 miRNAs (gm-miR1511, gm-miR1516, gm-miR1536, mt-miR1509 and mt-miR2590) that were only detected under stress conditions.

We selected some nodule stress-responsive miRNAs to validate their expression ratio observed in miRNA-macroarrays by Northern blot analysis. We observed a general induction of pvu-miR2118 in Fed, Pd, Mnt and Cut stressed nodules, while pvu-miR1511 was repressed in Fed nodules. MiR172, strongly expressed in nodules as compared to roots and leaves, was repressed in Fed, Mnt and Cut. MiR157 was induced in Fed and Mnt nodules. MiR398 was induced in Pd while it is not detected in Cut nodules. Also, novel miRNA target genes were proposed for common bean and the expression of selected targets was evaluated by qRT-PCR.

This work contributes to the discovery of novel nutrient stress-responsive miRNAs in common bean and provides the foundation to evaluate the individual roles of miRNAs in post-transciriptional regulation of developmental processes and stress-responses of this agronomical important legume. Current work from our group is aimed at functional characterization of selected miRNAs in common bean using genetic approaches as previously reported (Valdés-López *et al.,* 2008).

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The *alcohol dehydrogenase* gene family of *Lotus japonicus*

Sainz MM[1], Ramos J[1*], Sato S[2], Tabata S[2], Becana M[1]

[1]*Departamento de Nutrición Vegetal, Estación Experimental de Aula Dei, CSIC, Apartado 13034, 50080 Zaragoza, Spain;* [2]*Kazusa DNA Research Institute, 2-6-7 Kazusa-Kamatari, Kisarazu, Chiba 292-0818, Japan.* **jramos@eead.csic.es*

Summary

Alcohol dehydrogenases (ADHs) are ubiquous in organisms. In plants and animals, they occur as several isoforms, of which at least one of them is also able to metabolize *S*-nitrosoglutathione. Despite the multiple roles played by antioxidants in rhizobia–legume symbioses, little is known about *S*-nitrosoglutathione reductases (GSNOR) in legumes. In this work we report the characterization of five *ADH* genes of *Lotus japonicus*. Expression of *LjADH* genes was analyzed by quantitative PCR in nodules, roots, leaves and flowers. The *LjADH4* gene (putative GSNOR) displayed the lowest expression levels in all plant organs examined.

Introduction

Alcohol dehydrogenases (ADHs) are present in bacteria, archaea, yeast, plants, and animals. They show high substrate specificity for ethanol, aldehyde, and acetaldehyde (Thompson *et al.,* 2010). In plants, reactive nitrogen species, such as nitric oxide (NO) and *S*-nitrosoglutathione (GSNO), are produced in different subcellular compartments (Valderrama *et al.,* 2007). At low concentrations, these molecules fulfil essential functions in growth and redox signaling (Foyer & Noctor, 2005). NO can bind to thiol groups of proteins (modulating enzyme activity by nitrosylation) or of glutathione (forming GSNO), and this binding may affect NO bioactivity. In *Arabidopsis*, a second function has been reported for ADH2: in addition to removing formaldehyde, a toxic metabolite formed *in vivo*, ADH2 can metabolize GSNO acting as nitrosoglutathione reductase (GSNOR; Sakamoto *et al.,* 2002). Interestingly, some other ADHs may participate in GSNO metabolism. For clarity, we will designate the corresponding enzymes of *Lotus japonicus* as ADHs, although the relative ADH/GSNOR catalytic activities will need to be ascertained for each enzyme. Several cDNA clones encoding ADHs have been isolated from plants. There are nine ADHs in *Arabidopsis* but only two of them, ADH1 and ADH2 (GSNOR), have been studied comprehensively (Nordling *et al.,* 2002). Comparable studies of legume ADHs are lacking. In this work, we have characterized five *ADH* genes in *L. japonicus* and quantified their expression levels in plants.

Materials and Methods

Genomic sequences were identified by screening *L. japonicus* transformation-competent artificial chromosome libraries using *Arabidopsis ADH1* and *ADH2* sequences. Complete open reading frames (ORFs) were obtained in the expressed sequence tag (EST) databases or by sequencing using nodule RNA as templates. Comparison of genomic and ORF sequences allowed us to elucidate exon-intron compositions for all genes. Gene-specific primers were designed for real-time quantitative PCR and values were normalized with respect to *ubiquitin*.

Results and Discussion

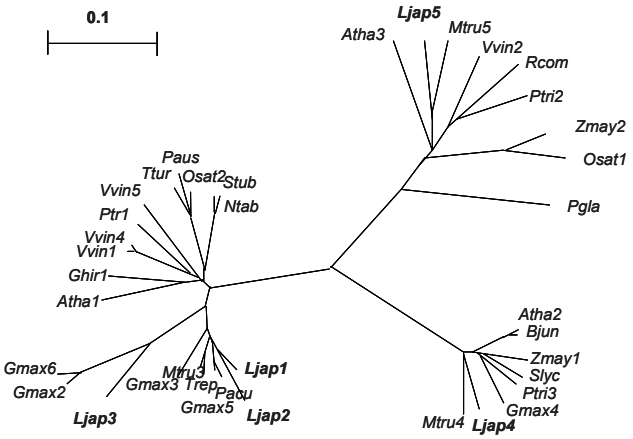
Gene identification and characterization. We have characterized the complete suite of *LjADH* genes (Table). The number of ESTs strongly suggests that the expression of *LjADH1* is much higher than that of the others. Interestingly, *LjADH1* and *LjADH2* are tandemly arranged with the same orientation on chromosome 1, and their ORFs are separated by only 1703 bp. *LjADH1* shows 93% identity with *LjADH2* but 82% with *LjADH3*, an indication that the *LjADH1* and *LjADH2* genes originated from a recent duplication event. All genes contain nine exons, except *LjADH3*, which contains ten exons. The derived proteins show no clear signal peptides for organelle targeting and are putatively localized to the cytosol.

Table. *Alcohol dehydrogenase* genes of *Lotus japonicus*.

LjADH	Genomic clone	Exons	TC	ESTs	ORFs (bp)	Protein (aa)
1	LjT43005	9	TC46080	765	1143	380
2	LjT43005	9	-	-	1143	370
3	LjT18013	10	TC44012	9	1143	380
4	LjT27B01	9	TC48206	4	1143	380
5	LjT16L14	9	TC37561	8	1137	378

Phylogenetic analysis. Based on the alignment of LjADH sequences, three clusters of proteins can be differentiated: *(i)* LjADH1, LjADH2, and LjADH3, which group with classical ADHs; *(ii)* LjADH4, which groups with GSNOR proteins; and *(iii)* LjADH5, which groups with other proteins with unknown enzymatic properties.

Figure. The derived amino acid sequences of the five ADH proteins of *L. japonicus* (***LjapX***) were compared to available plant ADH sequences with Clustal W and a phylogenetic tree was constructed.



Expression analysis. The *LjADH* mRNA levels were determined in nodules, roots, leaves, and flowers. *LjADH1*, *LjADH2*, and *LjADH5* are the most abundantly expressed genes in all plant organs, except in leaves, where most *LjADH3* mRNA is located. *LjADH1* and *LjADH2* have similar expression levels in all tissues. *LjADH4* has the lowest levels of expression.

Taking into account the expression levels in plant organs and the available ORFs for all five genes, we will immunolocalize the proteins and will produce recombinant enzymes to characterize their catalytic activities. Our focus will be on *LjADH4* (putative *LjGSNOR*) and *LjADH5* (unknown enzyme). This is important because GSNOR is involved in plant defense (Rustérucci *et al.,* 2007) and in abiotic stress responses (Corpas *et al.,* 2008).

Acknowledgments

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Understanding how plant-pathogenic *Pseudomonas* resist invertebrate predators

Sanchez-Contreras M[1*], Dorati F[2], Arnold DL[3], Waterfield NR[1], Jackson RW[2]

*[1]Department of Biology and Biochemistry, University of Bath, BA2 7AY Bath, UK; [2]School of Biological Sciences, University of Reading, RG6 6AJ Reading, UK; [3]Centre for Research in Plant Sciences, University of the West of England, BS16 1QY Bristol, UK. *msc23@bath.ac.uk*

Summary

The interactions of pathogenic *Pseudomonas* with its plant host are well studied. However, much less is known about other biotic factors that influence survival in their niche, namely the interaction with invertebrate predators. We have chosen three invertebrate models to characterise their interaction with *Pseudomonas syringae*. Our experiments show that *P. syringae* strains are able to resist predation when compared with *Escherichia coli*. To gain insight to the mechanisms underpinning predation resistance, we have screened genomic libraries from three *P. syringae* strains against the invertebrate models to identify the genes responsible. The use of these approaches to study the environmental performance of other plant-associated *Pseudomonas* is discussed.

Introduction

During their lifecycle, some plant pathogenic bacteria will make contact with bacteriovorous predators such as nematodes and protozoa as well as be ingested by herbivorous insects such as aphids and caterpillars that feed on plant tissue colonised by the pathogen. Some recent studies have shown that *Dickeya*, *Erwinia* and *Pseudomonas* bacteria can kill or colonise aphids and that aphids can actually spread the pathogen from one plant to another. We hypothesised that the frequency of bacterial contact with these predators will have selected for bacterial strains with enhanced survival. To test this hypothesis, we carried out co-inoculation experiments with three pathovars of *Pseudomonas syringae* (pathovars *tomato*, *phaseolicola* and *aesculi*) and the amoeba *Acanthamoeba polyphaga* (a bacteriovorous protozoan), the nematode *Caenorhabditis elegans* (a bacteriovorous worm) and the model insect *Galleria mellonella* (greater wax moth).

Materials and Methods

Assays of *P. syringae* strains survival against *A. polyphaga* or *C. elegans* were carried out by incubating the invertebrates with bacterial mats as described in Waterfield *et al*. (2008). After 7 days, the plates were washed off and the suspension was centrifuged at 800 rpm to separate bacteria from the invertebrates. Serial dilutions were plated to count viable bacterial cells. Toxicity against insects was assessed by injecting 10 µl of 100-fold diluted bacterial overnight culture into *G. mellonella* hemolymph and monitoring the insect condition during 7 days. The rapid virulence annotation of the *P. syringae* cosmid libraries was done according to Waterfield *et al*. (2008) using *G. mellonella* as the insect model.

Results and Discussion

The first aim of this work was to analyse in detail the interaction of *P. syringae* pathovars *tomato* DC3000, *phaseolicola* 1448A and *aesculi* 6617 with the three invertebrate models. Bacterial population numbers over 7 days showed that the *Pseudomonas* bacteria were able to resist amoebal and nematode predation compared to *Escherichia coli*; however, the *Pseudomonas* were only weakly toxic to nematodes and they did not support amoebal growth unlike *E. coli*. In *G. mellonella*, all three pathovars were able kill the insect after injection into the hemolymph, whereas *E. coli* did not. Taken together, we concluded that *P. syringae* has evolved gene systems to enhance survival to these predators. To identify these genes, a rapid virulence annotation screen was carried out using cosmid libraries for each of the three *P.*

syringae pathovars. From 5800 cosmid clones screened in total, we identified 551 cosmid clones that had an effect on the predators; some cosmids were unique to a particular predator, while others had overlapping effects. End sequencing of the positive cosmids was done to allow identification and mapping of the gene regions in the bacterial genome – the newly available genome sequences of *P. syringae* pv. *aesculi* aided this process (Green *et al*., 2010). A variety of gene clusters correlated with motility, type VI protein secretion, insecticidal toxins and hemolysins were among the systems identified.

Figure. Number of positive clones from the genomic libraries of different *P.syringae* pathovars identified in the screens against the invertebrate models tested. The overlaps in the diagrams show the number of positive clones that were common to more than one assay.

Our findings show that the different pathovars of *P. syringae* have evolved both core and novel gene systems to cope with predation pressure, which likely enhance their persistence in the environment. It is very likely that other plant-associated *Pseudomonas*, such as *P. fluorescens*, have evolved similar mechanisms as many of them colonise the rhizosphere where they encounter numerous invertebrates. The future study of *P. fluorescens* F113 using these approaches will reveal whether the similar mechanisms are critical to fight predators in the environment.

Acknowledgments

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Session 3

Plant-microorganism interactions

Control of nodule development and programmed cell death in common bean

Guillén G[1], Rodríguez-Kessler M[1], Ledezma D[1], Flores R[1], Iñiguez LP[1], Zayas AE[1], Hernández AMG[1], Olivares JE[1], Díaz C[1], Bueno O[1], Estrada G[1], Panzeri D[2], Sparvoli F[2], Sanchez F[1*]

*[1]Departamento de Biología Molecular de Plantas, Instituto de Biotecnología. UNAM, Cuernavaca, Mexico; [2]Istituto Biosintesi Vegetali, Milano, Italy. *federico@ibt.unam.mx*

Summary

We have used different genomic approaches to unravel the role of key elements that control root-nodule growth and senescence. Over the last years, our group has characterized a small nodulin gene family (Npv30) in common bean (*Phaseolus vulgaris*). The different members of gene family seem to have anti-cell death properties in bean root nodules, as revealed by loss-of-function and gain-of-function experiments. Here, we present a transcriptome analysis of knockdown Npv30 bean nodules. In addition, the microRNA (miRNA) landscape obtained by deep-sequencing two Illumina miRNA-enriched libraries of young (18 dpi) and senescent (27 dpi) RNAs derived from bean root-nodules induced by *Rhizobium tropici* CIAT899, was obtained.

Introduction

The symbiotic association between beans and rhizobia culminates with the formation of determinate N_2-fixing nodules. Recognition of the symbionts at the root surface leads to plant cell-wall degradation by the rhizobia allowing their penetration through the root hairs up to the nodule primordium, by means of an infection thread. Rhizobia only differentiate into N_2-fixing bacteroids when they are released from infection threads into the infected cells and then become enclosed by the peribacteroid membrane, forming symbiosomes (Cermola *et al.,* 2000; Oldroyd & Downie, 2008). Our main interest is to gain insight into the mechanisms that control nodule development and programmed cell death in bean nodules. High levels of reactive oxygen species (ROS) are generated during the lifespan of nodules (Matamoros *et al.,* 2003). ROS act as signal molecules during the early stages of the bean symbiotic interaction (Cárdenas *et al.,* 2008). However, the decline in antioxidant defenses and the uncontrolled production of ROS trigger nodule senescence, implying that this process is genetically controlled (Becana *et al.,* 2000).

Materials and Methods

Silencing of the *npv30* family was achieved by cloning the open reading frame encoding the Npv30-1 isoform in antisense orientation and by introducing it into bean plants according to the *Agrobacterium rhizogenes*-mediated transformation protocol (Estrada-Navarrete *et al.,* 2007). Total RNA was isolated to make fluorescent amplified RNA and hybridized to the Combimatrix array according to the manufacturer's instructions. The chip contained 32,258 nonredundant probes (35-40-mer oligos) in triplicate. The probes were designed using the tentative consensus sequences (TCs) derived from the TIGR *Phaseolus vulgaris* Gene Index release 3.0 (20,000 probes). The remaining probes are from soybean (*Glycine max*).

Young and senescent nodules were harvested for RNA isolation. RNA was size-fractionated and the 19-30 nt fraction was collected. Adapters were ligated to the RNA pool with T4 RNA ligase. Ligated RNA was fractionated on 10% agarose gels, and the 70-90 nt fraction was excised. After RT-PCR amplification, products were purified and small RNA libraries were sequenced using Illumina technology.

Results and Discussion

The *npv30* nodulin family of common bean is formed by four members (*npv30-1*, *npv30-2*, *npv30-3*, *npv30-4*) that are highly transcribed in root nodules. Composite *npv30* silenced bean roots inoculated with *R. tropici* rendered nodules similar to those from wild-type plants. Microscopy analysis showed a phenotype that could resemble an early senescence process (Cermola *et al.,* 2000) and the onset of autophagy, as evidenced by the loss of nuclei integrity, increased number of peroxisomes, and double membrane vesicles resembling autophagosomes (Hayward *et al.,* 2009). This phenotype is correlated with the transcriptome analysis (transcriptome/CustomArray microarray, analyzed through the MapMan ontology software) (htpp://gabi.rzpd.de/projects/MapMan), since transcript levels of some of the autophagy and proteasome pathways are increased.

Additionally, we sequenced two Illumina/Solexa miRNA-enriched libraries of young (18 dpi) and senescent (27 dpi) nodules of common bean cv. Negro Jamapa plants inoculated with *R. tropici* CIAT899 rendering 14,044,033 and 13,526,845 reads, respectively. Because of the lack of the complete genomic sequence for common bean, reads were aligned against soybean genomic sequences, which diverged from bean by *c.* 20 Mya. The 914,090 and 341,778 reads, for young and senescent nodules respectively, had at least one hit in the genome. In addition, alignments against miRBase and PMRD databases were run for identification of miRNAs. Only reads found in both databases were further characterized which comprised 180,144 and 110,231 reads, representing 109 and 101 reported miRNAs (Figure). After normalization, with a cut-off differential expression ratio of 1.5X, 15 miRNAs were classified as over-represented and 10 miRNAs as under-represented in senescent nodules with respect to young nodules, suggesting that these miRNAs and their targets might be involved in nodule senescence.

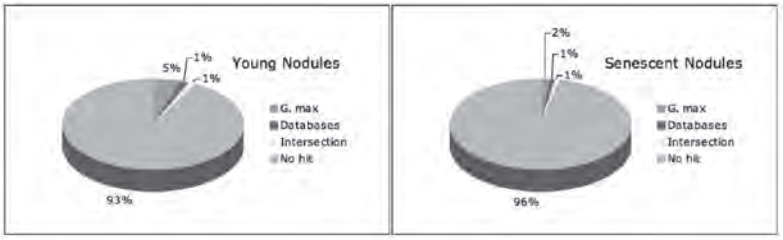

Figure. Identification of miRNAs from a large pool of bean small RNAs sequenced from a single Illumina/Solexa run. Reads found in soybean but without database match are being analyzed as bean potential not previously reported miRNAs.

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Role of the secretory pathway and antimicrobial peptides in the accommodation and differentiation of endosymbiotic *Rhizobium* bacteria in *Medicago* nodules

Mergaert P[1*], Kondorosi E[1,2]

*[1]Institut des Sciences du Végétal, CNRS, Gif-sur-Yvette, France; [2]BayGen Institute, Szeged, Hungary. *peter.mergaert@isv.cnrs-gif.fr*

Summary

In *Medicago truncatula* and related legumes, endosymbiotic rhizobia (bacteroids) in symbiotic nodule cells are strongly elongated, display genome amplification, are incapable to reproduce and are thus terminally differentiated. This differentiation pathway is determined by plant factors which were identified as antimicrobial peptides belonging to the very large NCR family. NCRs are transported to the bacteroids. They can induce bacteroid differentiation *in planta* when expressed in legumes without terminal bacteroid differentiation and synthetic peptides can induce *in vitro* features of bacteroids in rhizobia. Genetic analysis showed that, in a mutant of a nodule-specific signal peptidase complex (a component of the secretory pathway), the transportation of NCR peptides to the symbiosomes is blocked, and this is correlated with the absence of bacteroid differentiation. In addition, transcriptome analysis has shown that the secretory pathway is a dominant feature of symbiotic cells which dispose of a specific pathway for protein transport to symbiosomes. Our findings together with the far-stretched metabolic integration of the endosymbiont and its host cell suggest that symbiosomes in symbiotic nodule cells are organelle-like compartments that represent an intermediate step in organellogenesis.

Bacteroid differentiation is determined by plant factors

Bacteroids are differentiated bacteria with a metabolism that is adapted to symbiotic life and nitrogen fixation and that is unlike the metabolism in free-living rhizobia in the rhizosphere. In addition, bacteroids can take diverse morphologies in different legume species. In many legumes (e.g. *Lotus japonicus*, *Glycine max*, *Phaseolus vulgaris*, etc), bacteroids are rod shaped, not different from the free-living rhizobia. These bacteroids can resume growth if they are released from nodules. However, in other legumes, bacteroids differentiate into strongly elongated and branched cells which have undergone amplification of the bacterial genome leading to polyploid bacteria. In addition, their membranes are fragilized. This extreme differentiation is terminal because it is accompanied with a loss of reproductive capacity. This is the case for legumes of the Inverted Repeat Lacking Clade (IRLC) such as *Medicago*, *Vicia*, *Galega*, *Trifolium* and *Astragalus* species (Mergaert *et al.,* 2006). In other legumes of the genera *Arachis* and *Aeschynomene* (Dalbergioid legumes), bacteroids are enlarged and spherical and have low reproductive capacity (Oono *et al.,* 2010).

Broad host-range natural *Rhizobium* strains or transgenic strains crossing the IRLC/non-IRLC host-range barrier display elongated bacteroid morphology in IRLC legumes but remain rod shaped in the other hosts (Mergaert *et al.,* 2006; unpublished data). Similarly, a strain that nodulates both *Arachis* and *Vigna* transformed to spherical bacteroids in the first host but remained rod shaped in the second (Sen *et al.,* 1986). Thus, morphological differentiation of bacteroids is not encoded by the bacterial genome but is controlled by factors from the host plant in both IRLC legumes and the Dalbergioid legumes. The elongated, polyploid state of bacteroids in the IRLC legumes and the induction of bacteroid-like cells by genetic or pharmacological interference with the bacterial cell cycle suggest that these factors in IRLC legumes might alter the bacterial cell cycle. On the other hand, spherical bacteroids in Dalbergioids could be the result of interference with the synthesis of the bacterial peptidoglycan polymer of the cell wall.

Antimicrobial peptides govern bacteroid differentiation in *Medicago truncatula* nodules

In order to identify the plant factors that mediate bacteroid differentiation in *M. truncatula*, we made the following assumptions: The genes coding for those factors might be induced during nodule formation, they should be expressed in symbiotic cells and finally, corresponding genes should not be found in legumes without terminal bacteroid differentiation. We analyzed EST and microarray transcriptome data from *M. truncatula* wild type and mutant nodules as well as from *L. japonicus*, *G. max* and *P. vulgaris* nodules. From these comparisons, we identified the nodule-specific cysteine-rich (NCR) peptides as likely candidates for these factors. The *NCR* gene family in *M. truncatula* encodes more than 300 highly divergent peptides, which are most similar to antimicrobial peptides (AMP) (Mergaert *et al.,* 2003; Alunni *et al.,* 2007). The *NCR* gene family is exclusively expressed in nodules. Transcriptome analysis of non-functional nodules obtained with a large collection of symbiotic mutants of *M. truncatula* or its symbiont *Sinorhizobium meliloti* revealed that the *NCR* genes were only expressed when symbiotic cells are formed (Maunoury *et al.,* 2010). Moreover, the expression of the tested *NCR* genes, detected by *in situ* hybridization or promoter-*GUS* fusions, was restricted to the symbiotic cells and different subsets of *NCR* genes were activated during distinct developmental stages of the symbiotic cells.

Therefore, we postulated that NCRs could be the plant factors that mediate terminal bacteroid differentiation. Cell fractionation, Western and peptidome analysis of purified bacteroids as well as *in situ* detection of peptides with antibodies or as fused fluorescent proteins in transgenic nodules showed that NCR peptides are transported to bacteroids, in agreement with the predicted role of NCRs. Expression of *NCR* genes in *L. japonicus* nodules, where terminal bacteroid differentiation does not occur and the *NCR* genes are absent, inhibited multiplication of bacteroids in the symbiosomes resulting in single, remarkably elongated bacteroids as in *M. truncatula* nodules and unlike the normal symbiosomes and bacteroids in *L. japonicus*. This indicates that expression of *NCR* genes in *L. japonicus* is sufficient to inhibit division of bacteroids within symbiosomes and to induce morphologies reminiscent of terminally differentiated bacteroids. To further support the role of NCRs in terminal bacteroid differentiation, synthetic NCR peptides were applied to *S. meliloti* cultures. They induced modifications of the bacterial membrane and inhibition of bacterial reproduction. It also led to a higher DNA content and the elongation of *S. meliloti* cells. Taken together, the *in vitro* response of *S. meliloti* to NCR peptides partially mimicked characteristics of terminally differentiated bacteroids.

Protein transport to symbiosomes by a nodule-specific secretory pathway

Bacteroids in symbiotic nodule cells are not free in the cytoplasm of the host cell but they are surrounded by a plant-derived membrane forming the symbiosome compartment. Many symbiosome-proteins are encoded by the host cell and thus symbiotic cells require a machinery to traffic proteins to the symbiosomes. Because most known symbiosome-proteins, including the NCR peptides, have a characteristic signal peptide, this machinery is likely derived from the secretory pathway. The *M. truncatula dnf1* mutant, which forms non-functional nodules, is deficient in a nodule-specific subunit of the signal peptidase complex (SPC) of the secretory pathway (Wang *et al.,* 2010). In this mutant, NCR trafficking to bacteroids is impaired and this is correlated with the absence of bacteroid differentiation (Van de Velde *et al.,* 2010). The transcriptome in *M. truncatula* nodules as well as microscopic analysis also pointed to a dominant role of the secretory pathway in symbiotic cells (Maunoury *et al.,* 2010). Half of the nodule-specific genes in *M. truncatula* are secretory proteins. Moreover, many components of the secretory pathway are induced in the symbiotic cells. Some have acquired a nodule enhanced expression besides an otherwise ubiquitous

subunits. One copy has a nodule-specific expression while the second has retained the ubiquitous expression. Finally, an alternative nodule-specific splice variant was observed for the gene encoding the SYP132 SNARE protein. In addition, the prominent role of the secretory pathway in symbiotic cells was also shown by an endoplasmic reticulum (ER) specific antibody that revealed by *in situ* immunolocalization that the ER is particularly well developed in the young symbiotic cells. Thus taken together, one of the specialized functions of symbiotic cells is to coordinate protein secretion to the symbiosomes which is essential for proper differentiation of the bacteroids.

Conclusions

It is well established that mitochondria and plastids are of endosymbiotic origin (Dyall *et al.,* 2004). Gram-negative bacteria that once resided inside a primitive eukaryotic host cell as endosymbionts were converted over time into organelles. Among the key steps in organellogenesis are metabolic integration, control of the host over the division of the organelle and the acquisition of a specific machinery for protein targeting to the organelle (Bhattacharya *et al.,* 2007). The bacteroids and the symbiotic cells show a high degree of metabolic interdependence. The symbiosome and bacteroid membranes contain a large number of symbiosis-specific transporters for nutrient exchange which are essential for a functional symbiosis (White *et al.,* 2007). Ammonium production by bacteroids is uncoupled from ammonium assimilation and nitrogen stress metabolism in such a way that enough ammonium is generated for the bacterial needs as well as for the much larger needs of the plant (Yurgel & Kahn, 2008). Other striking examples of metabolic integration are the host-dependence of bacteroids for branched-chain amino acids (despite that the free rhizobia are nonauxotrophic; they become symbiotic auxotrophs) (Prell *et al.,* 2009) and for the production of the Fe-Mo-cofactor of the bacterial nitrogenase that *Rhizobium* is unable to produce (Hakoyama *et al.,* 2009). The production of NCR peptides is a mechanism for the host cell to control the bacterial replication and the optimal accommodation of endosymbionts. Finally, the symbiotic cells dispose of a specific machinery that is derived from the secretory pathway for protein transport to the symbiosomes. It is interesting to note that recent evidence suggests that also the ancestral route of proteins to the plastids was the secretory pathway (Bhattacharya *et al.,* 2007). Taken together, these facts advocate the hypothesis that symbiosomes are organelle-like compartments which may have resemblance to an early stage of the conversion of endosymbionts to *bona fide* organelles.

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Deciphering the role of NADPH oxidases in bean roots after rhizobial infection

Montiel J, Santana O, Guillén G, Nava N, Cárdenas L, Quinto C*

*Departamento de Biología Molecular de Plantas, Instituto de Biotecnologia,Universidad Nacional Autónoma de México, Av. Universidad 2001, Chamilpa, Cuernavaca, México 62210. *quinto@ibt.unam.mx*

Summary

Reactive oxygen species (ROS) generated by plant NADPH oxidases (RBOH, Respiratory Burst Oxidase Homolog) have been shown to play numerous important roles in signaling and development in plants. Experimental evidence obtained in our group suggests the dynamic participation of RBOHs in ROS generation at early events of the *Phaseolus vulgaris-Rhizobium etli* symbiotic interaction. The role of RBOH in the bean-rhizobia symbiosis was further analyzed using biochemical and molecular biology approaches. An increase in H_2O_2 levels was detected in bean roots 5 days after rhizobial inoculation (dpi); superoxide anion production was also observed in nodule primordia (5dpi), which was prevented by diphenyl iodonium (DPI; an NADPH oxidase inhibitor). On the other hand, three *Rboh* genes were identified in the *P. vulgaris* genome: *PvRboh1, PvRboh2* and *PvRboh3*. The three *PvRboh* transcripts were found in stems, roots and nodules; no evident changes in their accumulation profiles were observed at different stages after bacterial inoculation. Nevertheless, an increased NADPH oxidase activity (prevented by DPI) was observed in microsomal fractions from bean roots (5 dpi), when compared to non-inoculated roots. To explore the participation of *PvRboh1, PvRboh2* and *PvRboh3* at early stages of the interaction, a reverse genetics approach was pursued.

Introduction

Increased ROS levels have been found at the tip of growing plant cells such as pollen tubes and root hair cells, as well as Fucus and fungal hyphae (Foreman *et al.,* 2003; Cárdenas *et al.,* 2006; Monshausen *et al.,* 2007; Coelho *et al.,* 2008). In *Arabidopsis thaliana* a mutant in *AtrbohC (RHD2)* is characterized by the absence of root hairs (or underdeveloped) and do not display a tip-localized ROS gradient (Foreman *et al.,* 2003).

Recently, we found that there is a fast and transient ROS increase at the tip of actively growing bean root hair cells after seconds of treatment with specific Nod factors (NFs). This response is inhibited by diphenylene iodonium (DPI), an NADPH oxidase inhibitor (Cárdenas *et al.,* 2008). These and other results regarding the involvement of RBOHs in the nodulation process (Shaw & Long, 2003; Rubio *et al.,* 2004; Lohar *et al.,* 2007) prompted us to analyze by reverse genetics, the participation of RBOHs as well as ROS generation, in the early stages of the bean-rhizobia symbiotic interaction.

Material and Methods

Bean roots were inoculated with *R. tropici* (CIAT899) and harvested at 3 and 5 days post-inoculation (dpi). The total content of H_2O_2 was quantified with a colorimetric method using titanium sulfate (0.1%). RNA was isolated with hot-phenol; qRT-PCR assays were performed according to the SYBR Green protocol (Bio-Rad). Microsomal fractions from bean roots were obtained to assay for NADPH oxidase activity. Superoxide anion was detected incubating root segments in 10mM sodium phosphate and nitroblue tetrazolium (NBT; 0.1%). DPI (50 μM), sodium azide (10 μM) and imidazole (50 mM) inhibitors were preincubated for 15 min before the tests were accomplished.

Results and Discussion

To address the participation of RBOHs at the early stages of the bean-rhizobia symbiotic process, first the total amount of hydrogen peroxide was monitored in bean roots at 3 and 5 days post-inoculation (dpi). H_2O_2 levels at 3dpi were comparable to the control (uninoculated roots); nonetheless, a 40% increase was observed 5dpi. Likewise, a high accumulation of superoxide was found in nodule primordia 5 dpi, which was prevented by DPI but not by sodium azide (peroxidase apoplastic inhibitor). These results highlight the involvement of flavoenzymes, such as RBOHs, in ROS production during nodule primordia development. Using different strategies such as searching in bean databases, Southern genomic blot assays and PCR using degenerate and specific oligonucleotides, three different *Rbohs* encoding partial sequences in the *P. vulgaris* genome (*PvRboh1, PvRboh2* and *PvRboh3*) were identified. Nearly all the features of RBOHs, such as transmembrane domains, NADPH and FAD binding-motives and histidine residues of the catalytic site, were identified in the amino acid derived sequences. The highest percentage of identity was found between PvRBOH1 and PvRBOH3 (76%). The three *PvRboh* transcripts were detected in non-inoculated root hairs (3 d); data obtained by semi-quantitative RT-PCR indicated that *PvRboh1, PvRboh2* and *PvRboh3* are differentially expressed in roots, stems and nodules (15 dpi). No evident changes in transcript accumulation profile were observed following bacterial inoculation. Nevertheless, a higher NADPH oxidase activity in microsomal fractions from 5 dpi bean roots was found, which is inhibited with DPI and imidazole (NADPH oxidase inhibitor). The increase in NADPH oxidase activity at 5 dpi correlates with the rise in H_2O_2 content in bean roots and the high accumulation of superoxide in nodule primordia. To gain insight into the function of each of the three *PvRboh* identified in this work, transgenic *P. vulgaris* roots with different down-regulated levels, were generated by using RNAi or microRNAs. *PvRboh2* RNA interference (RNAi) transgenic roots showed an evident reduction in size and in number of nodule primordia; these data correlate with a substantial decrease in transcript accumulation detected by qRT-PCR analysis. Hence, PvRBOH2 is essential in the legume-rhizobia symbiosis. Experiments are in progress for the functional analysis of *Pvrboh1* and *Pvrboh3* using microRNAs.

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Infection thread development requires borate dependent *Rhizobium* polysaccharide capsule production and formation of a legume-AGP extensin-Rhamnogalacturonan II complex

Reguera M[1], Abreu I[1*], Brewin NJ[2], Bonilla I[1], Bolaños L[1]

*[1]Departamento de Biología, Facultad de Ciencias, Universidad Autónoma de Madrid, Darwin 2, 28049, Madrid, Spain; [2]Department of Molecular Microbiology, John Innes Centre, Norwich Research Park, Colney, Norwich NR4 7UH, UK . *isidro.abreu@estudiante.uam.es*

Summary

The essential role of boron (B) in infection thread development in pea (*Pisum sativum*) nodules was investigated using cytological and immunochemical techniques. Under low B (-B) conditions, infection threads aborted prematurely and contained reduced numbers of bacteria that appear to be devoid of a polysaccharide capsule. Rhizobia grown on –B solid media developed colonies with low production of exopolysaccharide (EPS), being similar to EPS-deficient mutants. MAC265 immunostaining revealed that legume AGP-extensin (AGPE) was closely associated with the cell surface of rhizobia in the lumen of threads in -B nodules, but it was separated from the bacterial surface by the capsular sheath in +B nodules. Legume-AGPE could be immunopurified with anti-rhamnogalacturonan (RGII) from +B nodule extracts but only weakly from –B extracts, suggesting that this association resulted from a molecular complex involving AGPE and RGII. These observations lead to a refined model for infection thread growth involving a B-dependent cell surface interaction between capsular polysaccharide and AGPE and a B-dependent interaction involving AGPE and RGII at the interface between the infection thread matrix and the infection thread wall.

Introduction

Rhizobial infection of legume host cells is typically a transcellular process that involves the formation of an infection thread within the host cell that grows coordinately with bacterial division and advance (Fournier *et al.,* 2008). Inside threads, rhizobia are embedded in intercellular plant-derived matrix material, including a legume-specific glycoprotein that has been termed legume AGP-extensin (AGPE) because it is a heteropolymer comprising glycomotifs for both arabinogalactan protein and extensin (Brewin, 2004). This AGPE component, recognized by MAC265 antibody, has recently been identified as a potential ligand of B (Reguera *et al.,* 2010) and its interaction with the pectin network and with the cell surface of rhizobia could be important for apical growth of the infection thread wall. Previous *in vitro* studies (Bolaños *et al.,* 1996) have shown that AGPE and other nodule glycoproteins were capable of binding to the surface of bacterial cells, and that this interaction was prevented by borate and by the presence of a barrier of capsular polysaccharide (EPS) surrounding the bacterial cells. Because infection threads abort prematurely in B-deficient nodules, we used immunological techniques to investigate the effects of B deprivation on potential *in vivo* interactions involving components of the infection thread matrix.

Materials and Methods

Pea (*Pisum sativum* cv. Lincoln) plants used for inoculation with *Rhizobium leguminosarum* bv. *viciae* strain 3841 or with the EPS defective B551 mutant were grown with B or B-free conditions as described (Bolaños *et al.,* 2001). Three-week-old nodules were harvested, homogenized, and fractionated for immunoelectrophoresis assays or processed for immunogold staining and electron microscopy (Rae *et al.,* 1991). MAC 265 (anti-legume AGPE) and anti-RGII were used for immunological analyses and for immunopurification assays.

Results and Discussion

Electron microscopy on ultra-thin nodule sections revealed that infection threads from –B nodules showed a wall disorganized. Moreover, bacteria inside –B infection threads showed a

degraded appearance and were not surrounded by a capsular polysaccharide that was clearly observable in +B infection threads (Figure, **a-b**). Consequently, matrix material, including a legume AGP-Extensin identified with the MAC265 antibody, was in direct contact with the cell surface of rhizobia inside –B infection threads, whereas most of the bacteria inside +B threads were separated from the matrix material by its capsule (Figure, **c-d**).

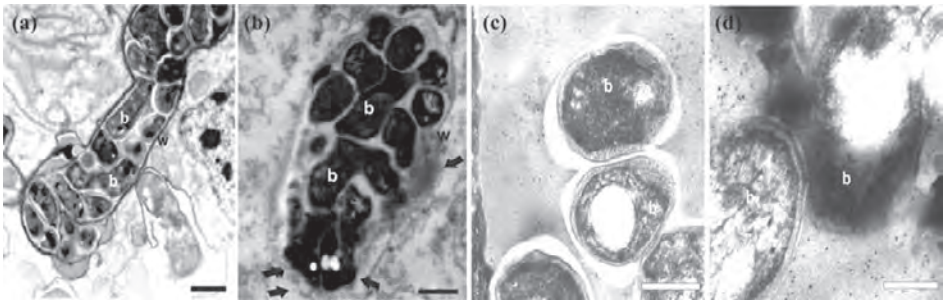

Figure. Electron micrographs of +B (**a, c**) or –B (**b, d**) pea nodule sections, three weeks after inoculation with *R. leguminosarum* 3841, showing the structure of infection threads (**a, b**) and immunogold localization of MAC265 antigens (legume AGP-Extensin) (**c, d**). Bacterial cells (b) in +B threads are embedded in an amorphous matrix, but most of them are surrounded by a polysaccharide capsule clear halo without any apparent physical association between the bacterial cell surface and the matrix material. The thread matrix material in –B nodules is more heterogeneous and comes into direct contact with the surface of bacterial cells, some of which are at an advanced stage of degradation. Arrows in (**d**) indicate breaks of a highly disorganized infection thread wall (w). Bars: 0.3 μm (**a**, **b**); 0.1 μm (**c**, **d**).

When grown in solid –B media, *R. leguminosarum* 3841 did not produce capsule, resembling an EPS-defective mutant. Following nodule fractionation, most of the MAC265 antigens were retained in the soluble fraction derived from +B nodules. Conversely, for homogenates derived from –B nodules, most MAC265 labelling was associated with the bacterial pellet and could be released when nodules were fractionated in the presence of borate. Therefore, B not only affected capsule production but could directly modify the interaction between AGPE and the bacterial cell surface. Furthermore, it could be demonstrated by immunoprecipitation assays that borate promoted formation of a complex involving RGII and infection thread matrix glycoproteins that could be important for the apical growth of the thread wall.

Our new results suggest two refinements to the mechanism of infection thread growth: (i) in the thread lumen, B is necessary to maintain a bacterial capsule and to prevent attachment of matrix glycoproteins to the rhizobial surface, so that bacterial cells can advance without entrapment in the luminal matrix; and (ii) that AGPE can interact with cell wall- associated pectin through RGII-borate cross-linking, and that this process might serve to strengthen the infection thread wall and promote apical growth of the thread cylinder. Finally, it is important to highlight that the B requirement for capsule production makes this element essential for the infection capacity of *Rhizobium*. To our knowledge, this is the first evidence for a role of B in rhizobia, which in addition increases the list of roles of this micronutrient in prokaryotes.

Acknowledgments

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Pseudomonas fluorescens F113 can produce a second flagellum important for rhizosphere colonization

Barahona E*, Navazo A, Redondo-Nieto M, Martínez-Granero F, Martín M, Rivilla R

Departamento de Biología, Universidad Autónoma de Madrid, 28049 Madrid, Spain.
**emma.barahona@uam.es*

Summary

The chromosome of *Pseudomonas fluorescens* F113 contain genes encoding a second flagellar apparatus related to the flagella of *Azotobacter vinelandii*. Although this flagellum is not produced by the wt strain, several mutants can produce it. Production of this second flagellum fully explains the swimming phenotype of a *kinB* mutant. Ectopic expression of the master regulatory operon *flhDC* is sufficient for production of this flagellum that is also produced by phenotypic variants isolated from the rhizosphere and by strain KSW a hypercompetitive mutant that has enhanced biocontrol ability.

Introducción

En los últimos años hemos estudiado genes de *Pseudomonas fluorescens* F113 que estén implicados tanto en la represión, como en la activación de la movilidad. Mutantes más móviles que la cepa silvestre, así como variantes fenotípicos aislados de la rizosfera, son capaces de desplazar a ésta en estudios de colonización competitiva (Martínez.Granero *et al.,* 2006). Por este motivo, hemos construido una batería de mutantes simples, dobles y triples en genes que están reprimiendo la movilidad y que fueron detectados por medio de mutagénesis por inserción al azar de un transposón (Navazo *et al.,* 2009). Entre estos genes, se encuentran, *kinB* (KinB está insertada en la membrana interna de la bacteria, y forma un sistema de dos componentes junto con AlgB; ambas implicadas en la síntesis del exopolisacárido alginato), *sadB* (SadB es una proteína citoplasmática con un dominio HD modificado) y *wspR* (cuyo producto presenta un dominio GGDEF con actividad diguanilato ciclasa). Todos estos mutantes son hipermóviles, y además están afectados en la formación de biopelículas en distintos tipos de plásticos, vidrio y en sistemas de flujo continuo. Análisis a nivel de expresión y de proteína han demostrado que tanto SadB (actúa reprimiendo la movilidad a través de FleQ, regulador principal de la síntesis del primer flagelo), como WspR (pudiera estar actuando a nivel del motor del primer flagelo), definen dos rutas independientes que regulan la movilidad en *Pseudomonas fluorescens* F113 (Navazo *et al.,* 2009). KinB no forma parte de ninguna de estas rutas, y hemos comprobado, que un mutante presenta sobreexpresión del master regulador, así como de la proteína estructural del segundo flagelo. Por este motivo, parece interesante conocer la regulación de este nuevo sistema flagelar de nuestra cepa, ya que esta expresándose en mutantes naturales aislados de la raíz, así como en el triple mutante *kinB*$^-$*sadB*$^-$*wspR*$^-$, excelente colonizador de la rizosfera y biocontrolador de hongos patógenos.

Materiales y Métodos

La totalidad de los mutantes ensayados en este trabajo fueron construidos mediante mutagénesis dirigida. Los dobles y triples mutantes se generaron por medio de conjugaciones triparentales. En los ensayos de movilidad tipo *swimming,* se inocularon las cepas en placas con medio SA al 0.3% de agar purificado y se midieron los halos de movilidad a las 18 horas de su inoculación. Cada cepa fue inoculada por cuadriplicado. Los estudios del proteoma comparado entre wt y triple mutante se realizaron utilizando la técnica electroforesis bidimensionales en geles de poliacrilamida. Los análisis de expresión se hicieron por PCR cuantitativa y semicuantitativa a tiempo real. El análisis de secuencias se realizó utilizando el programa BLAST.

Resultados y Discusión

Analysis of the genomic sequence of *Pseudomonas fluorescens* F113 has shown the presence of genes encoding a cryptic second flagellar apparatus that is not present in any sequenced genome of pseudomonads. The flagellar genes show maximum homology with the genes encoding the flagellar apparatus of *Azotobacter vinelandii*, a soil bacterium closely related to the genus *Pseudomonas* that produces flagella related to the Enterobacterial flagella. The flagellar genes in F113 showed the same syntheny than in *A. vinelandii*, except that in this bacterium genes are located in two genetic regions, whereas in F113 the genes are contiguous and flanked by insertion sequences, suggesting a horizontal transfer event. Genes encoding the second flagellum are not expressed in wild-type F113 but expression of the master operon *flhDC* and the gene encoding flagellin, *fliC2* was observed in several motility mutants such as *kinB* and *AlgT*. Since AlgT is a sigma factor required for the expression of *kinB*, these results indicate that the production of the second flagellum is repressed directly or indirectly by KinB. In *A. vinelandii* it has been shown repression of the flagellum through AlgT , indicating a common mechanism of regulation. Mutation of the *fliC2* gene in a *kinB* mutant background resulted in the restoration of wild-type motility, showing that the motility phenotype of a *kinB* mutant can be fully explained by the production of the second flagellum.

In order to test the functionality of the second flagellum, we have expressed the *flhDC* operon from a heterologous promoter in F113 and a non-motile *fliC* mutant. Ectopic expression of *flhDC* was sufficient to increase the swimming motility of F113, indicating that it was producing the second flagellum. In the case of the *fliC* mutant, although expression of the master operon resulted in a gain of function of motility, this was transitory and was lost very soon. These results could be explained by the fact that the gene *flgM2* of F113 has a frameshift mutation inactivating it. It is therefore likely that the second flagellum requires *flgM* and a first flagellar apparatus for its synthesis and functioning. In order to test this hypothesis, we mutated *flgM* in a *kinB* mutant background resulting in a total loss of motility, confirming the requirement of *flgM* for the synthesis of both flagella. The importance of this second flagellum for rhizosphere colonization is highlighted by the fact that it is expressed in phenotypic variants F and S isolated from the rhizosphere. It is also interesting that the KSW mutant that shows enhanced competitive colonization and a better biocontrol activity than the wild-type F113 also expresses the second flagellum.

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Identification and characterization of a cambialistic superoxide dismutase from bacteroids in the cytosol of pea nodules

Asensio AC[1], Marino D[1], James EK[2], Ariz I[1], Bustos G[2], Aparicio-Tejo PM[1], Arredondo-Peter R[3], Moran JF[1]

[1]Institute of Agri-Biotechnology, UPNa-CSIC-GN, Universidad Pública de Navarra, Spain; [2]Scottish Crop Research Institute, Invergowrie, Dundee DD2 5DA, UK; [3]Laboratorio de Biofísica y Biología Molecular, Facultad de Ciencias, Universidad Autónoma del Estado de Morelos, Cuernavaca, Morelos, México.

Summary

Superoxide dismutase (SOD) isoenzymes are an essential part of the defense system against reactive oxygen species (ROS) in legume nodules. We have used the ROS-generating herbicide paraquat (PQ) to study SOD expression in the indeterminate nodule-forming legume pea (*Pisum sativum* L. cv. Sugar-snap). Immunoblot techniques, *in-gel* SOD activity assays, and bioinformatics tools were used to achieve this aim. Application of 10 µM PQ in the hydroponic solution caused severe effects on shoot and nodule physiology parameters as well as an increase of the *in-gel* activity of a SOD isozyme that was later identified by N-terminal sequencing as *Rhizobium leguminosarum* cambialistic SOD (CamSOD). An antibody was raised based on the deposited sequence of the *R. leguminosarum* CamSOD and on the 3-D model of the protein, and after testing its specificity, immunoblotting and immunoelectron microscopy (EM) were performed on nodules. The latter showed that the CamSOD protein was not only localized in the bacteroids, but also appeared to be in the cytosol and organelles of both infected and uninfected cells. In summary, a new bacteroidal CamSOD protein, located within the cytosol of pea nodules, is described, and also a possible role for cytosolic SODs in legume nodules is proposed.

Introduction

Reactive oxygen species (ROS) are important intermediates in plant physiological processes. They originate under several conditions *in planta*, but most especially during stress. Legume root nodules are particularly sensitive to ROS and thus contain a high concentration and diversity of antioxidants. They are, therefore, an important model to study antioxidant systems, and the SOD family represents the first line of defense against ROS.

Materials and Methods

Pea (*Pisum sativum* L. cv. Sugar-snap) seeds were inoculated with *Rhizobium leguminosarum* bv. *viciae* strain NLV8 in 1 L pots 1:1 (v:v) perlite:vermiculite mixture, under controlled environment and watered with a N-free nutrient solution. Four week-old plants were transferred to hydroponic tanks of 1 L four days prior to treatments, which were 0 (controls), 10 µM PQ, or Fe-excess. Physiological parameters and nitrogen fixation capacity were measured, as well the contents of ascorbate (ASC), dehydroascorbate (DHA), reduced and oxidised glutathione (GSH and GSSG, respectively), total protein and leghemoglobin (Lb), as described by Marino *et al.* (2008). For SOD purification, nodules were homogenized and processed in two sequential ion exchange chromatograpy steps. Fractions after chromatography were assayed by the *in-gel* SOD activity method (Beauchamp & Fridovich, 1971). Native SOD bands were excised and purified as described by Moran *et al.* (2003). N-terminal sequencing was performed using Edman degradation. For the immunoblotting assay a rabbit antibody was raised based on the sequence of the protein and the 3-D structure of the protein. For determination of CamSOD localization, nodule extract fractionation was done according to Moran *et al.* (2003). Swiss-Model, Signal P, Tap T, and Secretome P 2.0 software were used.

Results and Discussion

The application of 10 µM PQ in the nutrient solution provoked severe effects on shoot and nodule physiology parameters. Plant dry weight, photosynthesis, and chlorophyll content were affected indicating that the herbicide was translocated from roots to shoots. In nodules, the PQ treatment generates an evident cell redox state alteration. ASC and GSH were highly

oxidized as indicated by a striking 75% decline in both ASC/DHA and GSH/GSSG ratios. PQ treatment markedly decreased Lb contents, and nodule and plant-fraction protein contents. The N_2 fixation process was also halted, as can be seen from the apparent nitrogenase activity values. PQ application also induced the response of most SOD isoenzymes. MnSOD, cytosolic and plastidial CuZnSODs, and a novel SOD band were observed in the nodule cytosol, but not in the leaves. The novel SOD had the mobility of a FeSOD, but resisted cyanide and H_2O_2 treatments which characterize MnSODs and CamSODs. This enzyme was purified after sequential anion exchange chromatography, native gel PAGE and SDS-PAGE gel. Further analysis by N-terminal sequencing allowed us to assign the N-terminal sequence of this CamSOD to the *sodA* gene from *R. leguminosarum*. The anti-CamSOD antibody (IgG) was checked under native and denaturing conditions. In order to unambiguously assign localization of SODs within pea, nodule extracts were fractionated using differential centrifugation, and *in-gel* SOD activity was assayed from these fractions. The results indicated that the CamSOD enzyme is present in free-living bacteria, bacteroids and in plant cell cytosol (Fig 1) even under mild extraction conditions. A western blot using an anti-nitrogenase antibody showed that the nodule cytosol fraction is free of bacteroids (Fig. 1B). This extended localization was also confirmed by EM, and was evident even in the absence of PQ-induced stress (Fig 2).

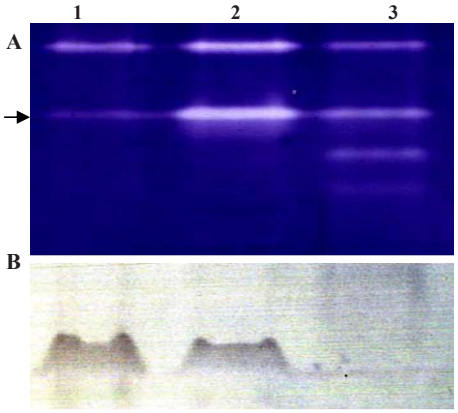

Figure 1. SOD activity in native gel (**A**) and western blot with anti-nitrogenase (anti-nif DK) as a bacteroidal marker (**B**). Lanes are: 1) free living *R. leguminosarum* crude extract, 2) Pea nodule bacteroidal fraction and 3) pea nodule cytosol crude extract. Arrow: CamSOD band.

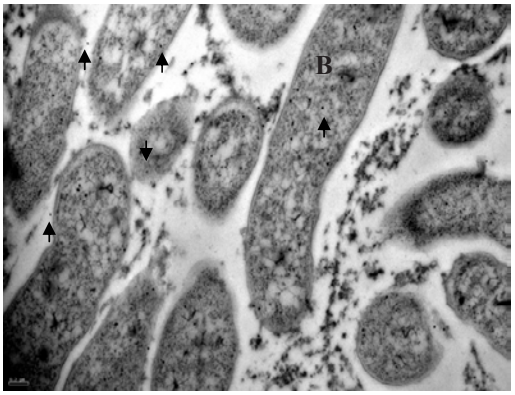

Figure 2. Electron micrograph from non-PQ-treated nodules. Bacteroids (B) are immunogold labeled with an antibody against the CamSOD protein (arrows). Labelling was also present within the cytosol of the infected cell.

In conclusion, application of PQ in the nutrient solution induced severe effects on shoot and nodule physiology parameters, as well as a decrease in the cytosolic content of Lb. An important induction of CamSOD in nodule under oxidative stress condition was also detected, and immunolocalization with EM showed that this bacteroidal protein is also present in the plant cell cytosol.

Acknowledgments

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Copper tolerance and antioxidant responses of a metallophyte plant by the colonization of arbuscular mycorrhizal copper-adapted fungi and biotransformed agrowaste residue

Meier S[1], Cornejo P[1], Azcón R[2*]
[1]*Universidad de La Frontera, Departamento de Ciencias Químicas, Temuco, Chile;* [2]*Departamento de Microbiología del Suelo y Sistemas Simbióticos, Estación Experimental del Zaidín (CSIC), Apartado 419, 18080 Granada, Spain. *rosario.azcon@eez.csic.es*

Summary
In this study we determine, under copper (Cu) contamination (500 ppm) conditions, the effectiveness on plant development of arbuscular mycorrhizal (AM) colonization by Cu-adapted AM fungi with or without *Aspergillus niger*-treated sugarbeet waste (SBW). The two treatments applied (SBW and AM inoculation) elicited alternative strategies for plant survival under 500 ppm Cu contamination. This Cu level in the soil decreased shoot biomass by 237% in non-mycorrhizal plants and by 162% in AM-colonized plants. Concomitantly, AM-colonized plants accumulated 76% less Cu than non-AM plants. As a result, only plants colonized by this adapted AM inoculum may survive under the high amount of Cu without residue. No negative effect of high Cu concentration on mycorrhizal development was observed. Results suggest that an important function of AM-colonized plants was to cope with the most toxic Cu levels that induced oxidative stress. Glutathione reductase and ascorbate peroxidase activities were lowest in AM-colonized plants. The high efficiency of Cu-adapted mycorrhizal inoculum to decrease Cu in plants under heavy Cu contamination resulted in alleviation of Cu toxicity and an increase in the plant tolerance of Cu pollution. To successfully remediate Cu polluted sites, it is essential to colonize plants with efficient and adapted AM fungi.

Introduction
Arbuscular-mycorrhizal (AM) fungi colonization may reduce plant stress and affect metal uptake by the host plant (Vivas *et al.,* 2003). AM fungi contribute to establishment of plants by providing a metal excluder barrier and by improving their nutritional status (González-Chávez *et al.,* 2004). Degenerative reactions associated with heavy metal stress are mediated by reactive oxygen species. Results by Ouziad *et al.* (2005) suggested that a primary function of the fungal cells in symbiosis was to cope with heavy metal-induced oxidative stress. Antioxidant systems, such as glutathione reductase (GR), ascorbate peroxidase (APX), catalase (CAT) and superoxide dismutase (SOD), are involved in strategies for metal tolerance (Smirnoff, 1993).

In this study we have investigated the changes in Cu concentration in a metallophyte plant (*Oenothera affinis*) growing in a non-contaminated or in a Cu-polluted soil, and the relevance for plant development of being colonized by Cu-adapted AM fungal strains. The role of *A. niger* treated residue of sugarbeet agrowaste was also tested. Changes in antioxidant enzyme activities, including SOD, CAT, GR and APX, were also evaluated in response to the various treatments applied. These activities can be considered as markers of heavy metal tolerance.

Materials and Methods
The soil was mixed with quartz-sand (<1mm) (2:1 soil:sand, v/v). After sterilization the soil/sand mixture was supplemented or not with 500 ppm of Cu by adding the adequate amount of $CuCl_2$. The agrowaste residue used here as amendment was *A. niger*-treated sugar beet waste (SBW) supplemented with rock phosphate (RP) (Medina *et al.,* 2005). The metallophyte *Oenothera affinis*, generally reported as tolerant to high Cu concentration, was used for this bioassay. AM inocula consisted of thoroughly mixed rhizosphere soil containing spores, mycelium and mycorrhizal root fragments from rhizosphere of *O. affinis* after multiplication. Plants were grown for three months under greenhouse controlled conditions. At harvest, growth, nutrition, Cu accumulation and antioxidant enzyme activities (SOD, CAT, GR and APX) were measured (Azcón *et al.,* 2009).

Results and Discussion

The colonization by AM (Cu adapted) fungi was essential for plant survival under 500 ppm Cu and the SBW was relevant for growth, particularly for non-AM plants when soil was contaminated with high Cu. In SBW-amended plants, increasing Cu levels decreased shoot biomass. This detrimental effect of Cu on shoot growth ranged from 237% in plants without mycorrhiza to 162% in plants colonized by AM fungi. The application of agrowaste residue was highly effective in enhancing plant growth, and, under high Cu polluted conditions plus SBW, control non-mycorrhizal plants accumulated 76% more Cu than did AM-colonized plants. The extraradical mycelium may selective exclude toxic elements by intracellular precipitation, extracellular glycoprotein or chitin-containing cell walls (Zhou, 1999).

Treatment with Cu increased the antioxidant activities in amended plants (except CAT), but plants colonized by AM showed the lowest APX and GR activities. The efficient destruction of reactive oxygen species generated under Cu stress requires the action of several antioxidant enzymes acting coordinately. Plant antioxidant enzymes would be useful markers of the AM-colonized plant strategies for metal tolerance.

The availability of Cu to the plant, its toxicity and antioxidant responses depend on exchange processes among soil, plants and the microorganisms colonizing the roots (Azcón *et al.,* 2009). Selection of the AM fungi should be assisted by knowledge of metal-tolerance fungal species that are capable of growing and functioning on toxic soils and that may adapt to nutrient limited and poor soils. The role of amendment and/or AM fungi is important in phytoremediation programmes to reduce Cu toxicity.

Table. Shoot dry weight, plant Cu concentration and antioxidant activities of mycorrhizal (AM) and non-mycorrhizal (C) colonized plants grown with and without treated sugar beet waste (SBW) in non-contaminated or Cu (500 ppm) contaminated soil.

Cu (ppm)	**AM inoculum**	Shoot dw (mg)		Plant Cu content		Antioxidant activities + SBW (mg^{-1} prot)			
		-	SBW	-	SBW	CAT	APX	GR	SOD
						($\mu mol\ min^{-1}$)			(Units)
0	C	1,250c	6,230a	67.1d	79.1d	5.37a	15.4c	7.5c	5.8b
	AM	1,075c	5,625a	80.2d	68.0d	5.75a	8.9d	6.0c	3.8b
500	C	-	2,630b	-	911.8a	2.31a	105.0a	26.3a	12.6a
	AM	1,473c	3,463b	523.2b	696.2b	4.66a	51.1b	10.8b	12.0a

Means followed by different letters are significantly different ($p<0.05$) using an orthogonal contrast test.

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Development of the arbuscular mycorrhizal symbiosis specifically induces the expression of genes encoding proteins involved in ammonium transport and assimilation

Pérez-Tienda J, Corrêa A, Azcón-Aguilar C, Ferrol N

Departamento de Microbiología del Suelo y Sistemas Simbióticos, Estación Experimental del Zaidín, CSIC, Apartado 419, 18080 Granada, Spain.

Summary

Under natural conditions, the roots of most plant species are colonized by arbuscular mycorrhizal (AM) fungi to form mutualistic symbioses called arbuscular mycorrhizas. It is generally considered that the main benefit for the plant is an improved phosphorus nutrition. However, AM fungi are also able to take up and transfer nitrogen to their hosts. The aim of this work was to analyze the mechanisms of nitrogen transport and metabolism in the AM symbiosis. Rice (*Oryza sativa*) and the AM fungus *Glomus intraradices* were used as symbiotic partners. Gene expression of rice ammonium transporters (AMT/MEP), and of a key enzyme of the ammonium assimilation pathway (glutamate synthase; GOGAT), were analyzed by real-time RT-PCR in mycorrhizal and non-symbiotic plants subjected to different nitrogen treatments. The establishment of the AM symbiosis significantly induced the expression of *OsAMT2;2*. However, the expression levels of *OsAMT1;1* and *OsAMT1;3* were repressed in mycorrhizal plants when growing at N-limiting conditions. Expression of *OsGOGAT2* was only detected in mycorrhizal plants. The physiological role of these proteins in the symbiosis will be discussed.

Introducción

La mayoría de las plantas terrestres viven asociadas con hongos microscópicos del suelo formando simbiosis mutualistas denominadas micorrizas arbusculares (MA). El principal beneficio de la formación de la simbiosis es una mejora de la nutrición fosforada; sin embargo, los hongos micorrícicos son capaces de absorber del suelo distintos compuestos nitrogenados, tanto inorgánicos (NH_4^+, NO_3^-), como orgánicos (aminoácidos) y transferir el N a la planta. De hecho, las plantas micorrizadas presentan dos vías de absorción de nutrientes: la vía directa, a través de las células de la epidermis, y la vía micorrícica, que ocurre a través de las células corticales colonizadas por arbúsculos (Figura).

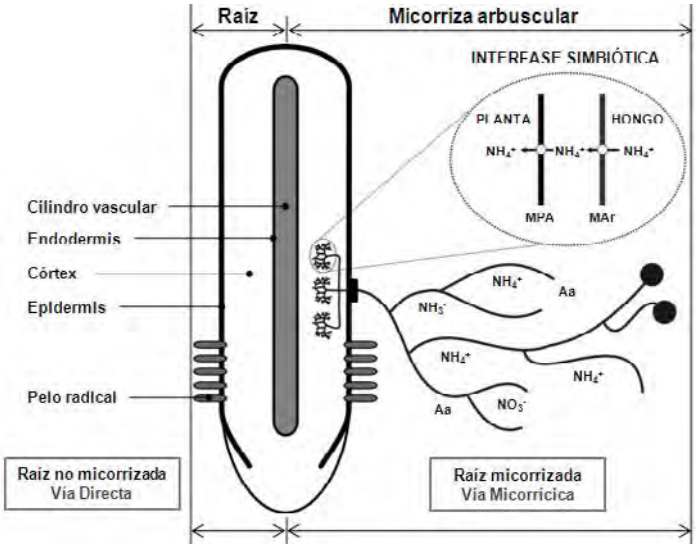

Figura. Modelo propuesto para la absorción de nitrógeno en plantas micorrizadas. Las plantas micorrizadas presentan dos vías de absorción de nutrientes: la vía directa, a través de las células epidérmicas, y la vía micorrícica, que ocurre a través de las células corticales colonizadas con arbúsculos. El N absorbido por el micelio extrarradical es translocado hacia el micelio intrarradical en forma de arginina, aunque la forma en la que el N es transferido a la planta es el NH_4^+. Una vez en la interfase simbiótica el NH_4^+ atraviesa la membrana plasmática periarbuscular y, una vez en el interior de la célula vegetal, es asimilado. MPA = Membrana periarbuscular; MAr = Membrana arbuscular.

Aunque los conocimientos actuales sobre los mecanismos implicados en el transporte de N en MA son bastante limitados, existen evidencias de que la forma molecular en la que el N se transfiere del hongo a la planta es el NH_4^+ (Ferrol y Pérez-Tienda, 2009). El objetivo de este trabajo ha consistido en profundizar en el estudio de los mecanismos implicados en el transporte y metabolismo del N en MA mediante el análisis de la expresión de genes de la planta que codifican diferentes transportadores de amonio y una enzima, la glutamato sintasa (GOGAT), que es clave en la asimilación del N por la planta.

Materiales y Métodos

Como modelo experimental se utilizó la asociación micorrícica establecida entre plantas de arroz (*Oryza sativa* L.) y el hongo formador de micorrizas *Glomus intraradices* Smith & Schenck. Las plantas se fertilizaron con solución de Hoagland modificada en la que el P se reducía al 25% y el N se mantenía a la concentración estándar (15 mM NO_3^-) o se reducía también al 25% (3.75 mM NO_3^-). Tras 8 semanas de crecimiento, la mitad de las plantas de cada tratamiento se sometieron a un período de ayuno de N de una semana, tras la cual se les aplicó 15 mM de NO_3^- o de NH_4^+ durante 3 días, o se mantuvieron en ayuno de N. Transcurrido este tiempo se cosecharon las plantas y se extrajo el ARN de las raíces usando el método del LiCl. La cuantificación de la expresión génica se realizó mediante técnicas de RT-PCR a tiempo real usando el iQTM5 Multicolor Real-Time PCR Detection System (Bio-Rad). Se determinaron los niveles de expresión relativa de los genes *OsAMT1;1*, *OsAMT1;3*, *OsAMT2;2* y *OsGOGAT2* usando como referencia los niveles de expresión del gen *OsTEF*.

Resultados y Discusión

El establecimiento de la simbiosis en condiciones limitantes de N reprimió la expresión de los genes *OsAMT1;1* y *OsAMT1;3*, miembros de la subfamilia I de los transportadores de amonio (AMTs), mientras que indujo de forma destacada la expresión del gen *OsAMT2;2*, el cual pertenece a la subfamilia II de dichos transportadores. Además, en las plantas control, en mayor medida que en las micorrizadas, la expresión de *OsAMT1;1* y *OsAMT1;3* se inducía por ayuno de N.Esta inducción, sin embargo, se reprimía tras la aplicación de un pulso de NH_4^+. A diferencia de *OsAMT1;1* y *OsAMT1;3*, la expresión del gen *OsAMT2;2* no se alteraba por los diferentes tratamientos de N.Por otro lado, se observó que en las raíces de las plantas micorrizadas se activaba de forma específica la expresión del gen *OsGOGAT2*. La consideración de que los AMTs de la subfamilia I son los responsables de la absorción del N a través de las células de la epidermis (Suenaga *et al.,* 2003), junto con el hecho de que la expresión de dichos transportadores se induce en condiciones de deficiencia de N y se reprime en las raíces de las plantas micorrizadas, sugiere que el desarrollo de la simbiosis provoca una inhibición de la vía directa de absorción de amonio a través de las células de la epidermis. Por otro lado, la activación específica del gen *OsAMT2;2* en las raíces micorrizadas apoya la hipótesis de que el amonio es la forma molecular en la que se transfiere el nitrógeno a la planta (Bago *et al.,* 2001; Govindarajulu *et al.,* 2005), mediante la actividad de dicho gen, y que el amonio sería asimilado por la planta mediante la ruta glutamina sintetasa (GS)/GOGAT en la cual jugaría un papel destacado *OsGOGAT2*.

Agradecimientos

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Arbuscular mycorrhizal structures and fungi associated with endemic and endangered plant species from Sierra Nevada National Park (southeastern Spain)

Palenzuela J[1], Ruíz-Girela M[2], Barea JM[1], Azcón-Aguilar C[1]

[1]Departamento de Microbiología del Suelo y Sistemas Simbióticos, Estación Experimental del Zaidín, CSIC, Apartado 149, 18080 Granada, Spain; [2]Jardín Botánico Hoya de Pedraza, EGMASA-Consejería de Medio Ambiente-Junta de Andalucía, 18191 Pinos Genil (Granada), Spain.

Summary

Most plants depend on mycorrhizas to thrive, particularly in fragile and stressed environments, as those in certain areas of the high Mediterranean mountains of Sierra Nevada. This protected environment constitutes an exceptional refuge for the flora, being one of the enclaves with higher biodiversity levels of the European continent. The objectives of this study were to determine the mycorrhizal status of selected species of the endangered flora of Sierra Nevada, including endemic species in serious extinction danger, and to analyze the diversity of their associated mycorrhizal fungi. Most of the target plant species (79%) form arbuscular mycorrhizas as shown by their typical symbiotic structures, i.e. arbuscules, vesicles and hyphal coils. Different colonization patterns (*Arum* and *Paris*) were observed, as well as coarse and fine endophytes. The diversity of mycorrhizal fungi associated to the selected species was also high. Some of the isolated fungi do not correspond to any of the species described up to now. The results will be discussed in the context of using mycorrhizal technology as a tool for the propagation, conservation and reintroduction of threatened plant species in their natural environments in Sierra Nevada National Park.

Introducción

La mayoría de las plantas desarrollan en sus ambientes naturales micorrizas, asociaciones simbióticas mutualistas entre hongos del suelo y raíces. Las micorrizas han desempeñado un papel clave en la evolución de las plantas sobre la superficie terrestre (Simon *et al*., 1993), debido, fundamentalmente, a la capacidad que confieren a las plantas para desarrollarse en ambientes extremos. Particularmente, las micorrizas son importantes para el establecimiento y desarrollo de las plantas en ecosistemas frágiles y amenazados, como son los característicos de las altas montañas Mediterráneas. Sierra Nevada constituye un refugio excepcional para la flora y uno de los enclaves con mayor índice de biodiversidad del continente europeo. Presenta 66 endemismos exclusivos, algunos de ellos en peligro de extinción (Blanca *et al.,* 2002). Es conocido que en los hábitats de alta montaña, la flora desarrolla numerosas adaptaciones impuestas por las rigurosas condiciones ambientales, entre las cuales está la capacidad para formar micorrizas (Körner, 1999). Por ello, en el, presente estudio se propuso determinar el estado micorrícico de especies de la flora amenazada de Sierra Nevada, así como analizar la diversidad de hongos micorrícicos asociados con dichas plantas, con la finalidad de incorporar la tecnología de la micorrización dirigida para facilitar la propagación y reintroducción de dicha flora en sus ambientes naturales.

Materiales y Métodos

Se seleccionaron 34 especies amenazadas y/o endémicas de Sierra Nevada de acuerdo a los criterios de los técnicos del Parque Nacional, y se procedió al muestreo de raíces y suelo asociado de las especies seleccionadas. Parte de las raíces recogidas se tiñeron y se observaron al microcopio para determinar la presencia o ausencia de micorrizas, mientras que otra parte se congeló a -80ºC para el posterior análisis molecular de los hongos micorrícicos presentes. A partir del suelo rizosférico muestreado se procedió al aislamiento y multiplicación de los hongos micorrícicos asociados a las plantas objeto de estudio. Las esporas de los hongos se aislaron y se caracterizaron morfológicamente. Finalmente, y con el propósito de caracterizar genéticamente los hongos encontrados se aplicaron técnicas de biología molecular basadas en la amplificación mediante PCR y posterior secuenciación de un fragmento de la subunidad 18S del ARNr (Ferrol *et al.,* 2004).

Resultados y Discusión

La mayoría de las especies investigadas, el 79%, presentan micorrizas arbusculares en las que se podían observar las estructuras típicas que caracterizan la simbiosis, fundamentalmente arbúsculos y vesículas. Algunas especies, como *Ilex aquifolium*, *Laserpitium longiradium* y el pteridofito *Dryopteris tyrrhena*, forman micorrizas arbusculares tipo *Paris*, caracterizadas por la ausencia de hifas intercelulares, abundancia de hifas que forman lazadas dentro de las células (ovillos) y arbúsculos que se originan a partir de los ovillos. Los dos pteridofitos estudiados, *Ophioglossum vulgatum* y *D. tyrrhena* presentan micorrizas arbusculares con una estructura muy peculiar, como se ha descrito previamente para otros helechos (Zhang *et al.,* 2004). En especial *D. tyrrhena*, que presentaba células frecuentemente colonizadas con vesículas y arbúsculos o vesículas y ovillos, simultáneamente. En bastantes de las especies estudiadas se detectó la presencia de endofitos finos junto a endofitos mas gruesos, que son los mas frecuentes en la mayoría de los ecosistemas. Los endofitos finos parecen estar bien adaptados a ambientes muy fríos con condiciones muy adversas habiendose descrito un incremento de su presencia relativa en gradientes altitudinales (Ruotsalainen *et al.,* 2004). En una de las especies investigadas (*Salix hastata*) se detectó la presencia de ectomicorrizas y en otras seis especies (*Papaver lapeyrousianum*, *Sarcocapnos speciosa*, *Arenaria nevadensis*, *Sempervivum tectorum*, *Pinguicola grandiflora* y *Pinguicola nevadensis*) no se detectó ningún tipo de micorriza. Aproximadamente la mitad de las especies muestreadas presentaba endofitos septados oscuros. Estos endofitos se han descrito con frecuencia en plantas de ecosistemas alpinos (Schmidt *et al.,* 2008) y en los polos (Newsham *et al.,* 2009). Aunque su función aún se desconoce, algunos estudios sugieren que ejercen un efecto beneficioso sobre el crecimiento de la planta en determinadas condiciones (Newsham *et al.,* 2009).

La diversidad de especies de hongos micorrícicos asociados con las especies estudiadas fue muy elevada. Se detectaron más de 50 morfotipos diferentes. Mediante aproximaciones moleculares, complementarias a las morfológicas, algunos de estos hongos se han identificado a nivel de especie. *Glomus*, con casi 30 especies distintas, es el género mejor representado, seguido de *Acaulospora*. Algunas especies no se corresponden con ninguna de las descritas hasta la fecha, por lo que se trata de especies nuevas. Uno de estos hongos se ha denominado *Entrophospora nevadensis* (Palenzuela *et al.,* 2010). Presumiblemente los hongos micorrícicos aislados juegan un papel importante en la supervivencia y desarrollo de estas plantas amenazadas en sus hábitats naturales. Por ello, se propone implementar las estrategias adecuadas para optimizar la micorrización de las especies micotrofas amenazadas, en el contexto de los programas de conservación de flora amenazada de Sierra Nevada.

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Rhizobial and arbuscular mycorrhizal fungal symbioses in *Dimorphandra wilsonii,* a threatened caesalpinioid legume native to the Brazilian Cerrado

Fonseca MB[1], Simões JL[2], Isaías RMS[1], França MG[1], Cruz C[3], Scotti MR[1], James EK[4], Sprent JI[5]

[1]Universidade Federal de Minas Gerais. Depto Botânica / ICB,.Belo Horizonte, Minas Gerais, Brasil; [2]Empresa Brasileira de Agropecuária –Agrobiologia. Seropédica, Rio de Janeiro, Brasil; [3]Universidade de Lisboa, Faculdade de Ciências, Centro de Biologia Ambiental (CBA), Lisboa, Portugal; [4]Scottish Crop Research Institute, Dundee DD2 5DA, UK; [5]Division of Plant Sciences, University of Dundee, Dundee DD2 5DA, UK.

Summary

Dimorphandra wilsonii is a caesalpinoid leguminous tree from the Brazilian Cerrado biome (savannah) that is threatened with extinction e.g. only twenty one individuals can currently be found in the state of Minas Gerais. The species forms two types of symbiosis: nitrogen fixing root nodules and arbuscular mycorrhizas, both of which help it to thrive in the Cerrado soils, which are typically low in nitrogen and available phosphorus. We have studied both these symbioses using molecular techniques and light and transmission electron microscopy. Nodules were shown to be indeterminate in structure with an apical meristem, and central tissue containing both infected and uninfected cells. The symbiosomes in the infected cells contained bacteroids, some of which were surrounded by a membrane and some also with cell wall material ('persistent infection threads' or PITs). Peripheral vascular bundles, an outer endodermis and a cortex were also present. Bacteria isolated from the nodules were able to nodulate and fix N_2 in association with *D. wilsonii*; which were identified as *Bradyrhizobium japonicum, B. elkanii* and *B. canariense* via their16S rRNA sequences. A significant arbuscular mycorrhizal fungal root colonization was also found. High spore density was found in the rhizosphere and the acaulosporoid morphotype was dominant followed by the glomoid type independently of the root zone. This is the first report of the occurence of *Bradyrhizobium* spp and AMF symbiotic associations in *D. wilsonii.*

Introduction

Dimorphandra wilsonii is a caesalpinoid leguminous tree, belonging to tribe Caesalpinieae. Nowadays, *D. wilsonii* is found in a restricted area in the state of Minas Gerais, southeastern Brazil (Rizzini, 1997) and at the time, only 21 individuals are known in the wild, distributed in three localities. Because of its endangered status, *D. wilsonii* is listed in the IUCN Red List of Threatened Plants 2006 as critically endangered. It has undergone demographic decline mainly due to habitat destruction by anthropogenic pressure: agricultural development and clearing of areas for cattle-pasture and production of charcoal (http://www.iucnredlist.org). Although the genus *Dimorphandra* is confirmed to nodulate (Sprent, 2007**),** to the best of our knowledge, there are no reports on nodulation for this species. Legumes can also form both ecto and endomycorrhizas (Lavin *et al.,* 2005; Sprent, 2007) and the latter are especially found among tropical species (Sprent, 2001). The aim of this study is to describe both the rhizobial and arbuscular mycorrhizal symbiosis in this threatened species.

Material and Methods

Dimorphandra wilsonii was grown in pots with cerrado soils without or with urea (0.1 g/ kg soil) using 10 replicates. After 120 days, the plants were harvested for measurement of biomass and bacteria strains were isolated from nodules. *D. wilsonii* plants were inoculated with isolated strains under Leonard jars conditions using reduced N fertization (0.025% KNO_3). After 120 days the nodules were collected and prepared for light and transmission electron microscopy essentially according to James *et al.* (2001). The DNA of 5 isolates were extracted and specific primers for the 16S rDNA region (F968CG 5' AACGAAGAAQCCTTAC 3' and R1401 5' CGGTGTGTACAAGACCC 3') with a GC clamp attached to the 5´end were used for the PCR reactions. The PCR products were sequenced and sequences from GenBank. Root samples from pot experiment were harvested and assessed for mycorrhizal infection (McGonigle *et al.,* 1990), Arbuscular mycorrhizal spores were recovered

from rhizosphere soil and separated by wet sieving (Gerdemann & Nicolson, 1963), and the analyzed data were expressed as the number of spores/100 g of dry soil.

Results and Discussion

Growth of *D. wilsonii* plants in pots filled with cerrado soils was improved when fertilized and all the plants were also found to be nodulated. Under Leonard Jars conditions nodules plants developed on lateral roots and were spherical, cylindrical or branshed with a thin periderm, and vascular bundles in the outer cortex. Under light microscopy, the nodules were shown to be indeterminate in structure with an apical meristem, and central tissue containing both infected and uninfected cells. Peripheral vascular bundles, an endodermis and a cortex were also present (Figure). Transmission electron microscopy showed that the symbiosomes in the infected cells contained bacteroids, some of which were surrounded by a membrane and some with cell wall material ('persistent infection threads' or PITs).

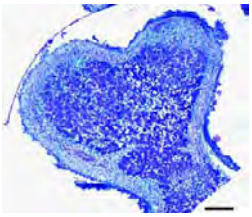

Figure. Light micrograph of a section of an indeterminate *Dimorphandra wilsonii* nodule.

Based on their 16S r DNA sequence, some of the isolated bacteria were identified as *Bradyrhizobium japonicum*, *Bradyrhizobium* sp, *B. elkanii* and *B. canariense*. While the *Bradyrhizonium* genus was the preferential bacterial symbiont selected by *D. wilsonii*, it was colonized by different arbuscular mycorrhizal fungi (AMF) species, especially glomoids and acaulosporoids morphotypes, with a dominance of the latter. A similar dominance profile was found among the AMF population in the rhizosphere (Table). In fact, nodulation has a significant requirement for phosphorus (P) (Sprent & James, 2007) and rhizobial and arbuscular mycorrhizal symbioses share common signaling components from the common symbiosis (SYM) genes (Chen *et al.,* 2007). These considerations allow us to infer that nodulation and AMF colonization in *D. wilsonii* plants may have a synergenistic role as a strategy for surviving. Therefore, a better understanding of such symbiotic relationships may contribute to the establishment of effective conservation and management measures for *D. wilsonii*.

AMF morphotypes / root zones	Uproot zone	Branch zone	Distal zone
Glomoid type	247.8bA	217.4bA	250.1bA
Acaulosporoid type	431aA	407.9aA	368.1aA
Gigasporoid type	14.9cA	30.2cA	17.7cA

Table. Spore density (spres/100g dry soil) of AMF morphotypes found in rhizophere of *D. wilsonii* plants. Means denoted by different letter(s) within each row (capital letter) or column (lowercase) are statistically significant at $p<0.05$ (Tukey's multiple-range test).

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Colonization of soybean roots by *Bradyrhizobium japonicum* expressing each one of its flagellar systems

Althabegoiti MJ[1*], Covelli JM[1], Rivilla R[2], Martín M[2], Lodeiro AR[1]

[1]*IBBM, Universidad Nacional de La Plata-CONICET, Calles 47 y 115 (1900) La Plata, Argentina;* [2]*Departamento de Biología, Universidad Autónoma de Madrid, Campus de Cantoblanco, 28049 Madrid, Spain.*

Summary

Bradyrhizobium japonicum, the symbiont of soybean, has two flagellar systems. One of them possesses a flagellin of 33 kDa and has a thin filament, while the other, with a flagellin of 65 kDa has a thick filament. We obtained mutants in each of the flagellins, as well as a double mutant, in the background of the type strain USDA 110. We observed that the mutant that expressed the thick flagellum only was equally competitive as the wild type for soybean roots colonization, whereas all other mutants were less competitive. Since it is believed that the thin flagellum is required for swarming motility, our results suggest that this type of motility is not required for roots colonization.

Introducción

Se conocen distintos tipos de movilidad en las bacterias, algunas de los cuales requieren de los flagelos. La mayoría de las especies bacterianas poseen un solo sistema flagelar, pero otras, como *Aeromonas* sp., *Azospirillum* sp., *Vibrio alginolyticus* y *Bradyrhizobium japonicum* poseen dos sistemas de flagelos. Uno de los sistemas de flagelos de *B. japonicum* está compuesto por flagelinas de 33 kDa (filamento fino) y el otro está constituido por flagelinas de 65 kDa (filamento grueso), y ha sido propuesto que cada uno de ellos jugaría un papel diferente (Kanbe *et al.,* 2007). *B. japonicum* es capaz de fijar N_2 en simbiosis con soja, para lo cual debe colonizar las raíces como primer paso de la simbiosis. Nuestra propuesta es observar cuál es la influencia de cada uno de de estos sistemas de flagelos sobre la colonización radical.

Materiales y Métodos

Las cepas mutantes LP 6865, LP 5843 y LP 6543 se obtuvieron con la estrategia ya descrita (Kanbe *et al.,* 2007). Las microscopías electrónicas de transmisión (MET) se realizaron a partir de cultivos líquidos en medio HM como se describió (Althabegoiti *et al.*, 2008). Para los ensayos de colonización de raíces de soja se siguió la metodología descrita por Martinez-Granero *et al.* (2006), pero con 4 plantas de soja por maceta y un período de 16 h de luz a 25 ºC y 8 h en oscuridad a 18 ºC. Cada experimento se realizó por triplicado. Los inóculos fueron cultivos en fase exponencial del orden de 10^8 UFC/ml de cada cepa de *B. japonicum* en el medio de Götz (Althabegoiti *et al.,* 2008).

Resultados y Discusión

A partir de la cepa LP 3004 (derivada de USDA 110, resistente a Sm) se obtuvieron las mutantes denominadas LP 6865 (carece de flagelo fino), LP 5843 (carece de flagelo grueso) y LP 6543 (no posee flagelo). Las cepas se observaron al MET y se corroboraron las mutaciones y la morfología de cada uno de los flagelos (Figura). Posteriormente observamos al microscopio óptico cultivos líquidos de cada uno de los mutantes. La única cepa capaz de desplazarse en una trayectoria rectilínea fue la que tiene el flagelo grueso (LP 6865), mientras que la que tiene el fino (LP 5843) daba tumbos con mucha frecuencia.

Posteriormente evaluamos la capacidad de cada cepa para colonizar la raíz de soja en presencia de la cepa salvaje como competidora. Estos ensayos se realizaron en macetas Riviera, donde cabe destacar que las macetas poseen un sistema donde el riego llega por capilaridad al sustrato (perlita). Los resultados se muestran en la Tabla, donde se puede ver que tanto la cepa LP 5843 como la LP 6543 fueron menos competitivas que la salvaje.

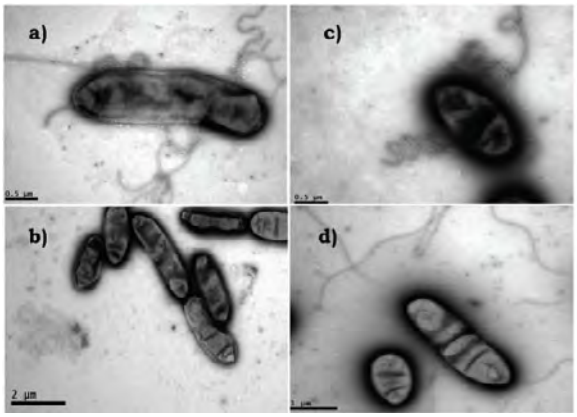

Figura. MET. **a)** LP 3004, **b)** LP 6543 (no tiene flagelo), **c)** LP 6865 (sólo tiene flagelo grueso) y **d)** LP 5843 (sólo tiene flagelo fino).

Tabla. Ensayo de colonización. Los cultivos bacterianos fueron inoculados sobre las semillas en una proporción 1:1 con aprox. 5.10^7 UFC ml^{-1} de cada cepa. El recuento de bacterias se realizó al cabo de tres semanas en el cm apical de cada una de las raíces.

Cepas competidoras	log nro. bacterias/g de raíz	
	3004	mutante
3004 vs 6865	7,77 ± 0,61	8,10 ± 0,55
3004 vs 5843	8,66 ± 0,16	6,96 ± 0,23
3004 vs 6543	8,83 ± 0,10	7,47 ± 0,27

Sin embargo, la cepa LP 6865 fue igualmente competitiva que la salvaje, a pesar de tener una movilidad disminuida. Se cree que las especies que poseen dos sistemas flagelares utilizan uno (polar) para nadar, el cual se expresaría de manera constitutiva; y el otro (lateral) para el movimiento de *swarming,* que puede ser inducido en medios viscosos, limitaciones de hierro y otros factores desconocidos (Atsumi *et al*., 1996, Merino *et al.*, 2006). *B. japonicum* utilizaría el flagelo fino para el *swarming* [Althabegoiti *et al*. (2008) y datos no publicados], por lo cual nuestros resultados indican que este tipo de movimiento no es requerido para la colonización rizosférica.

Agradecimientos

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FlgZ: a sensor protein which integrates c-di-GMP signals from two independent motility regulation pathways in *Pseudomonas fluorescens* F113

Navazo A*, Barahona E, Martinez-Granero F, Redondo-Nieto M, Rivilla R, Martín M

*Departamento de Biología, Universidad Autónoma de Madrid, 28049 Madrid, Spain .*ana.navazo@uam.es*

Summary

(2',5')-ciclic di-guanosin monophosphate (c-di-GMP), a second messenger very important in microorganisms, regulates two differences lifestyles: motile (lower levels of c-di-GMP) or sessile (with higher levels). These levels are modulated by proteins with guanidilate ciclase or phospodiesterase activities. It has been described that pilZ domains could sense c-di-GMP. A protein with a PilZ domain is encoded by a gene located in a flagellar genes operon in *P. fluorescens*. Since an homologous protein in *E. coli* interferes with the motor function of flagella altering flagellar rotational pattern, we decided to investigate its possible function in motility regulation. This protein has orthologues in all sequenced pseudomonads genomes, with the same syntheny and we have called it FlgZ. A *flgZ* mutant showed increased *swimming* motility phenotype, indicating its role in motility regulation. Since c-di-GMP levels could be regulated by the Wsp and SadC/BifA systems, we generated double mutants in both of these genes, and we observed that *sadCflgZ* and *wspRflgZ* mutants showed the same motility phenotype than *sadC* and *wspR* mutants respectively, indicating that FlgZ participates in both pathways. Conversely, a *sadCwspR* double mutant was more motile than any of these singles mutants, indicating that the Wsp system and SadC are implicated in independent pathways. Therefore, FlgZ seems to be the sensor protein of these independent motility regulation pathways in *Pseudomonas fluorescens* F113.

Introducción

En nuestro laboratorio se han descrito tres rutas independientes de regulación de la movilidad en la cepa *Pseudomonas fluorescens* F113, en las que participan: por un lado, el sistema Wsp, por otro la proteína soluble SadB, y por último el sistema de dos componentes GacA/GacS; Estas dos últimas rutas, regulan la movilidad de forma indirecta a través de la represión del regulador principal de la síntesis de flagelo (*fleQ*) (Navazo *et al.,* 2009). Por otro lado, Merrit *et al.* (2007), propusieron a SadB como la proteína sensora del sistema formado por SadC y BifA, proteínas con actividad diguanilato ciclasa y fosfodiesterasa respectivamente, y que regulan los niveles de c-di-GMP, un segundo mensajero muy importante en bacterias que determina el estilo de vida de estas: sésil o móvil. Además, está descrito que proteínas con dominios PilZ son capaces de sensar c-di-GMP (Benach *et al.,* 2007) y afectar a la velocidad de rotación del flagelo (Girgis *et al.,* 2007). Nuestra cepa presenta varias proteínas con este tipo de dominio, pero en concreto nos centramos en el estudio de la proteína FlgZ. El objetivo de este trabajo es averiguar cuál es la relación entre la proteína FlgZ y otros dos sistemas implicados en la regulación de los niveles de c-di-GMP en la cepa *P. fluorescens* F113: el sistema Wsp y el sistema SadC/BifA.

Materiales y Métodos

Todos los mutantes utilizados se construyeron mediante mutagénesis dirigida y se comprobaron por *Southern-blot*. Para los ensayos de movilidad tipo *swimming,* se inocularon las cepas correspondientes utilizando un palillo dental en placas con medio SA al 0.3% de agar purificado y se midieron los halos de movilidad a las 18 h de su inoculación. Cada cepa fue inoculada por cuadriplicado.

Resultados y Discusión

Para el estudio de la ruta en la que participa el sistema *sadC/bifA* construimos mutantes en estos genes, observando que la movilidad tipo *swimming* era superior a la de la estirpe silvestre en el mutante *sadC*, menor en el caso de *bifA*, e idéntica al fenotipo de la estirpe

silvestre para el doble mutante *sadCbifA*, lo que permite deducir que existe una única ruta de regulación de la movilidad. Por otro lado, el doble mutante *sadBsadC* es más móvil que cualquiera de los dos mutantes simples, mientras que *sadBbifA* no restablece el fenotipo móvil de la estirpe silvestre, indicándonos que SadB no forma parte de esta nueva ruta.

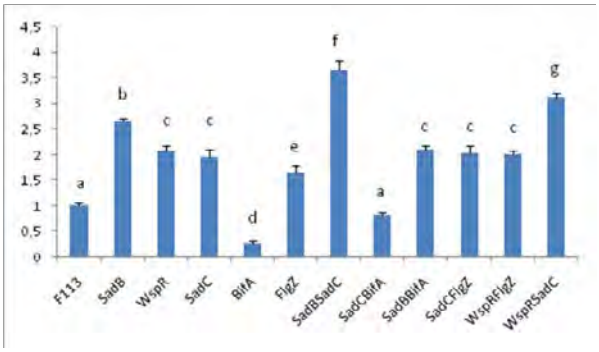

Figure. Fenotipo de movilidad tipo swimming de F113 y mutantes (porcentaje referido a la movilidad de F113).

Además, mutantes en *flgZ* presentan incrementado su fenotipo de movilidad tipo *swimming*. Sabiendo que tanto el sistema Wsp (Bantinaki *et al.,* 2007) como el formado por SadC/BifA (Merrit *et al.,* 2007) son capaces de modular los niveles de c-di-GMP construimos dobles mutantes en estos genes y observamos que tanto el doble mutante *sadCflgZ* como el *wspRflgZ* presentaban el fenotipo de movilidad del mutante *sadC* y *wspR* respectivamente; mientras que el doble mutante *sadCwspR* presentaba mayor movilidad que la de cualquiera de los dos mutantes simples. Estos resultados indican que las rutas en las que participa el sistema Wsp y el sistema SadC/BifA son independientes entre sí, siendo la proteína FlgZ la que integra las señales de estas dos rutas de regulación de la movilidad en *Pseudomonas fluorescens* F113.

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Repression of motility in *Pseudomonas fluorescens* F113 by the GacAS and SadB pathways occurs through activation of the production of the AlgT sigma factor

Martínez-Granero F, Navazo A, Barahona E, Redondo-Nieto M, Martín M, Rivilla R*

Departamento de Biología, Universidad Autónoma de Madrid, Campus de Cantoblanco, 28049 Madrid, Spain.
**rafael.rivilla@uam.es*

Summary
Motility is one of the most important traits for rhizosphere colonization. In *Pseudomonas fluorescens* F113, at least three independent pathways repress motility in response to environmental signals. Two of these pathways regulate motility through repression of the master regulatory gene *fleQ*, resulting in a lower production of flagellin. These pathways are represented by the GacAS system and the SadB protein. Here we show that these two pathways are convergent, and the GacAS system and SadB activate the expression of the *algT* gene, encoding an alternative sigma factor. AlgT is in turn required for the expression and production of AmrZ, which is the actual repressor of *fleQ* expression.

Introduction

Motility is required for efficient rhizosphere colonization by *Pseudomonas fluorescens* and mutants affected in motility and/or chemotaxis are severely impaired for competitive colonization. Furthermore, the rhizospheric environment selects for hypermotile, highly competitive phenotypic variants (Martinez-Granero *et al.,* 2006). Motility is a polygenic trait, and at least three independent signalling pathways co-operate in motility repression in response to environmental conditions or signals (Navazo *et al.,* 2009). While one of these pathways, represented by the Wsp system, apparently affects the rotation speed of the flagellum, two other pathways, represented by the GacAS system and the SadB protein, regulate the amount of flagellin and the lenght of the flagella. We have previouly shown that both the GacAS system and the SadB protein repress the expression of the *fleQ* gene (Navazo *et al.,* 2009), which encodes the master regulatory protein directing synthesis and assembly of flagella components. The aim of this work was to elucidate the way in which these two pathways exert repression of the expression of *fleQ*.

Materials and Methods

Pseudomonas fluorescens mutants were generated by homologous recombination and checked by Southern blot and PCR. Gene overexpression was performed by cloning the whole gene in an expression vector under the control of an inducible promoter. Swimming motility assays were performed by inoculating bacteria with an sterile toothpick in the center of SA plates containing 0.3% purified agar. Swimming was measured as the halo diameter after 18 h incubation at 28C. Exoprotease and pyoverdine production were determined as previously described (Martinez-Granero *et al.,* 2005).

Results and Discussion

The GacAS system is a post-transcriptional regulatory system that functions through activating the expression of small RNAs such as *rsmXY* and *Z*, which titrate small RNA binding proteins such as RsmA and E, avoiding the binding of these proteins to the leader sequences of messenger RNAs. Binding of RsmAE to the messenger RNAs precludes their translation. Therefore, activation of the GacAS system results in activation of the translation of target mRNAs. However, the final result of activation of the GacAS system results in repression of motility. Therefore, in order to know whether repression through the GacAS system was also through the same pathway, we overexpressed the *rsmA* gene in the wild-type

strain. Overexpression of *rsmA* resulted in a swimming phenotype identical to a *gacS* mutant, indicating that repression of motility follows the same pathway that activation of other genes through this system.

These results suggested that the GacAS system should act through activation of the translation of a *fleQ* repressor. Three repressors of *fleQ* have been described in different pseudomonads: Vfr, MorA and AmrZ and we made mutants in each of the genes. A *vfr* mutant did not present a swimming phenotype and a *morA* mutant showed reduced motility with respect to the wt strain, so were discarded as candidates for Gac and SadB signalling. Conversely, an *amrZ* mutant was hypermotile, showing a motility higher than *gac* or *sadB* mutants. These results made AmrZ a good candidate for co-regulation by both system. This was confirmed by the analysis of double mutants *gacSamrZ* and *sadBamrZ*. Swimming assays showed that both *gacS* and *sadB* were epistatic over *amrZ*.

Expression of *amrZ* requires the alternative sigma factor AlgT. An *algT* mutant showed the same phenotype than the *amrZ* mutant and wt swimming phenotype was restored by ectopic expression of *amrZ* in this background, confirming that the phenotype of the *algT* mutant was due to the lack of production of AmrZ. Furthermore, analysis of double mutants showed that both *gacS* and *sadB* were epistatic over *algT*. Expression analysis also showed that *algT* expression was lower in *gacS* and *sadB* mutants than in the wild-type strain.

We propose a model in which the GacAS and SadB pathways converge in the expression of *algT*, being the GacAS branch mediated by the Rsm pathway. AlgT in turn is required for AmrZ production. Repression of the *fleQ* gene by AmrZ results in lower amounts of flagellin produced and reduced motility.

Acknowledgments

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Screening of rhizobacteria in rice plants under different stress situations

García Cristóbal J*, Bonilla Martínez A, García-Villaraco A, Ramos Solano B, Gutiérrez Mañero FJ, Lucas García JA

*Departamento de Biología, Universidad San Pablo-CEU, 28668, Spain. *jgcristobal@ceu.es*

Summary

A screeening of rhizobacteria in rice plants in two different growth stages was conducted in the province of Badajoz. The use of rhizobacteria in order to elicit the secondary metabolism of plants and increase their resistance in situations of biotic and/or abiotic, is a biotechnological alternative very interesting, since the EU legislation on the use of pesticides is becoming more restrictive. Rhizobacteria isolated from the samples were characterized by their functional diversity based on a number of biochemical tests to verify certain skills related to elicitation phenomena. We showed that the phenological stage of the plant plays a key role, finding a greater functional diversity in the first sampling than in the second sampling. We also observed a clear difference in the biochemical capabilities of these rhizobacteria depending on the stress situation in which the plant was. Currently, these bacteria are undergoing a thorough study to be used in future in this crop.

Introducción

El cultivo de arroz (*Oryza sativa*) es de gran importancia en España, siendo Extremadura una de las zonas de mayor producción (>186.000 Tm en 2007). Los mayores problemas para la producción en esta zona provienen de la presencia del hongo patógeno *Pyricularia oryzae* y un problema asociado a la presencia de sulfuros que deriva en anaerobiosis radical produciendo su podredumbre y su menor desarrollo conocido como Akiochi (Armstrong y Armstrong, 2005). Las plantas han desarrollo un complejo metabolismo secundario que les ayuda a afrontar cualquier situación desfavorable. Se sabe que ese metabolismo secundario puede ser elicitado por ciertas bacterias no patógenas denominadas PGPR (Plant Growth Promoting Rhizobacteria (Kloepper *et al.,* 1980)). Actualmente existe un especial interés en la investigación dirigida al desarrollo de inoculantes con este tipo de rizobacterias beneficiosas para hacer un manejo agrícola no perjudicial para el medioambiente. El objetivo principal de este trabajo es la búsqueda y caracterización de nuevas rizobacterias provenientes de la rizosfera de arroz capaces de poner en marcha rutas metabólicas secundarias que permitan el cultivo de arroz en situaciones desfavorables por algún estrés biótico o abiótico.

Materiales y Métodos

Se escogieron tres zonas de muestreo afectadas por los problemas más comunes en la zona arrocera de Badajoz: una zona aquejada por Akiochi, otra que por sus condiciones de humedad y temperatura era susceptible al ataque del hongo *Pyricularia*, y una zona control con ausencia de los dos problemas antes mencionados. Los muestreos se realizaron en dos momentos fenológicos del cultivo de arroz: (i) en máximo ahijamiento (Julio) y (ii) en grano pastoso-lechoso (Septiembre). Se cogieron 9 raíces de arroz con sus correspondientes suelos rizosféricos, éstos fueron homogenizados y de este homogenado se resuspendió un 1 g por zona en 10 ml de agua destilada estéril y se llevaron a cabo una serie de diluciones seriadas con una posterior siembra en placa en tres medios: (i) agar para métodos estándar (PCA), (ii) medio de selección de *Pseudomonas* (King B), y (iii) medio mínimo (M9). Tras una incubación de 48-96 h a 28ºC, se seleccionaron al azar 50 cepas por medio y por tiempo de muestreo dando un total de 900, de las que se seleccionaron la mitad al azar, siendo sometidas a pruebas bioquímicas relacionadas con su potencial capacidad como PGPR: (i) capacidad para solubilizar fosfatos insolubles; (ii) capacidad para producir sideróforos; (iii) capacidad para producir moleculas de comunicación de tipo AHLs; (iv) capacidad para utilizar el ácido 1-aminociclopropanocarboxílico (ACC) como única fuente de nitrógeno; y (v) capacidad para producir quitinasas. Con las cepas que dieron positivo en una o más de estas pruebas se llevó a cabo una secuenciación parcial de la región rDNA del gen 16s. Dichas secuncias fueron introducidas en las bases de datos del GeneBank estableciendo el grado de homología con cepas allí depositadas.

Resultados y Discusión

Los muestreos se realizaron en dos estados fenologicos diferentes. Se puede observar como durante la primera etapa, maximo ahijamiento (fuerte crecimiento vegetativo de las planta), la diversidad funcional de las rizobacterias aisladas fue mucho mayor que en el estado de grano pastoso lechoso (minimo desarrollo vegetativo de las plantas). Es bien cocnocido que la planta es capaz de seleccionar las bacterias que se desarrollan en sus rizosfera, y que este hecho está controlado por la cantidad y calidad de los exudados liberados en cada momento por la planta (Lucas García *et al*., 2001a,b). Uno de los factores mas determinantes en este hecho es el estado fenológico de la palnta (Grayston *et al.,* 1996).

Es destacable que en las plantas aquejadas por Akiochi en septiembre (condiciones de anaerobiosis y niveles de sulfuros muy acentuados), la gran mayoria de bacterias seleccionadas por estas plantas son capaces de degradar ACC (precursor del Etileno). Plantas con altos grados de estrés producen gran cantidad de etileno, y por ende de su precursor (ACC). Probablemente la planta esté seleccionando este tipo de bacterias con la intención de disminuir la concentración de etileno en el interior de la planta minimizando así el efecto sobre el crecimiento radical (Glick *et al.,* 1998).

Tabla. Porcentaje de bacterias por zona y por tiempo de muestreo capaces de realizar las actividades bioquimicas indicadas en Materiales y Métodos.

Actividades \ Zonas	JULIO			SEPTIEMBRE		
	Control	Akiochi	Pyricularia	Control	Akiochi	Pyricularia
ACC	6.25	16.7	7.7	0	75	23.53
AHL	37.5	27.7	15.38	16.67	0	0
CAS	75	44.4	30.77	83.3	0	4.17
PDYA	12.5	27.7	30.77	50	25	35.3
AHL+CAS	18.75	5.5	0	16.67	0	0
ACC+PDYA	3.125	0	0	0	0	0
CAS+PDYA	12.5	0	0	33.3	0	5.9
ACC+CAS	0	0	0	0	0	5.9
ACC+AHL	0	5.5	0	0	0	0

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Phytoremediation of aqueous solutions of lubricants from industrial activities using maize plants grown in hydroponic conditions inoculated with different bacterial strains

Lucas García JA, Bonilla A, Gutiérrez Mañero FJ, Grijalbo Fernández L

Departamento de Biología. Universidad San Pablo – CEU, 28668 Boadilla del Monte, Madrid, Spain.

Summary

Many companies produce industrial waste rich in oils with DQO values above those permitted by law. In most cases this value is reduced by diluting with water. In this study we have developed a bioremediation system using maize plants growing in hydroponic culture, which have been inoculated with different bacterial strains. In all experiments the system was able to reduce the DQO below the values allowed by law in a very short period of time. In all cases bacterial inoculation reduced the value of DQO less than in the non-inoculated treatments, although differences were not significant. The study was complemented by measuring the pH after the remediation as well as biometric measurements of plants.

Introduction

La biorremediación se define como la acción de los microorganismos u otros sistemas biológicos para degradar contaminantes ambientales (Caplan, 1993; Watanabe, 2001; Dua *et al.,* 2002). Es una técnica muy utilizada para limpiar lugares contaminados tanto con metales pesados como con distintos tipos de hidrocarburos. Existen distintas estrategias de biorremediación (atenuación natural, bioaumento, fitorremediación, etc). En nuestros experimentos se ha utilizado la fitorizorremediación (un tipo de fitorremediación) que consiste en utilizar las raíces de las plantas para extraer los contaminantes (Marín *et al.,* 2005), y la rizorremediación que es la contribución de poblaciones bacterianas de la rizosfera al proceso de biorremediación (Schwab y Banks, 1994; Anderson *et al.,* 2003).

Materiales y Métodos

Se colocaron 20 semillas pregerminadas de *Zea mays* en un sistema hidropónico con un material inerte que permitía el enraizamiento de las plantas, éstas se regaron con una solución nutritiva formada por agua, Hoagland (1 g/l) y caldo nutritivo (8 g/l). A los 12 días se realizó un primer inóculo (10^9 ufc/ml) y tres días después, cuando las plantas tenían un tamaño de unos 28 cm, se cambió el agua por agua de taladrina (DQO entre 1500 y 1800 ppm) con Hoagland (1 g/l) y caldo nutritivo (8 g/l) y se realizó un nuevo inóculo (10^9 ufc/ml). Cinco días después de añadir agua de taladrina se realizaron las medidas de: altura, nº hojas, DQO y pH. En el primer experimento se utilizó el maíz con material inerte como soporte y también el maíz sin material inerte pero no se inoculó, mientras que en el resto se inoculó con: una levadura (Esp21), una cepa bacteriana del género *Enterobacter* (Esp1), una cepa de *Pseudomonas fluorescens* (Aur 6) y una cepa *Bacillus licheniformis* (TP9) aislada de las propias taladrinas.

La taladrina es una emulsión de agua y aceite (2%) que se utiliza para refrigerar la zona de contacto entre las herramientas de corte y los materiales a mecanizar. Una vez que ha perdido sus propiedades se somete a un proceso físico que consta de varias etapas en las que se separa parcialmente el aceite del agua y que tiene como resultado el agua de taladrina utilizada en este trabajo.

Resultados y Discusión

El primer objetivo de este trabajo fue determinar si la reducción de la DQO del agua de taladrina (A.T.) era mejor con el material inerte o sin dicho soporte. Se obtuvo que la combinación material inerte y planta era la que garantizaba una mayor reducción de la DQO, de donde se dedujo la posible presencia de cepas con capacidad biorremediadora en el material inerte.

En segundo lugar, se quiso determinar si la inoculación de la cepa elegida mejoraba el proceso de biorremediación (Tabla). En todos los casos se observó que la reducción era mayor cuando no se inoculaba la cepa, aunque no presentaban diferencias estadísticamente significativas entre los tratamientos con y sin inóculo. Sin embargo, sí existían diferencias significativas entre la DQO inicial y la DQO obtenida después del tratamiento de biorremediación (49 y 50% de reducción con y sin inóculo). Las plantas podrían disminuir la DQO absorbiendo algunos compuestos acumulándolos en el interior de las células de la raíz, hecho ya demostrado por otros autores (Gao y Zhu, 2004). Además, los exudados radicales son fuente de nutrientes para los microorganismos que viven cerca de sus raíces, acelerando los procesos degradativos bacterianos hasta 100 veces con respecto a bacterias no asociadas a raíces (Parrish *et al.,* 2005).También se observó una reducción del pH (Tabla) en un punto en los tratamientos con inóculo frente a los que no lo llevan, siendo la reducción significativa cuando se añadía la levadura.

En tercer lugar se quiso conocer el efecto del A.T. en las plantas. Para ello se estudió la altura y el número de hojas. En la altura se encontró una disminución significativa en los tratamientos con A.T. frente a los controles. En cuanto al nº de hojas, se encontró que este parámetro disminuía en los tratamientos con A.T. frente a los controles, aunque esta disminución no es significativa cuando se inoculó con la levadura. La reducción en estos parámetros puede deberse a que la habilidad de las plantas para extraer PAHs (como el fluoreno y el pireno presentes en el A.T.), translocarlos, transformarlos y acumularlos es un factor limitante por la fitotoxidad de estos compuestos y puede afectar cuantitativa y cualitativamente a varios procesos bioquímicos y fisiológicos que conllevan a la producción de biomasa (Kummerová *et al.,* 2006)

Tabla. Reducción de la DQO y pH en los experimentos con maíz y cepa inoculada.

Cepa	DQO inicial	pH inicial	DQO final sin bacteria	DQO final con bacteria	pH final sin bacteria	pH final con bacteria
TP9	1678 ppm	-	490,96 ppm	533,3 ppm	-	-
Aur 6	1726 ppm	9,41	784 ppm	819 ppm	8,66	8,52
Levadura	1800 ppm	9,43	902 ppm	921,7 ppm	8,62	8,54
Esp1	1779 ppm	9,71	632,9 ppm	683, 8 ppm	8,65	8,56

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Analysis of proteins involved in biofilm formation through the regulation of c-di-GMP levels in different species of *Sinorhizobium*

Baena I[1], Crespo-Rivas JC[2], Margaret I[2], Buendía-Clavería AM[2], Bonilla I[1], Vinardell JM[2], Lloret J[1]

[1]Departamento de Biología, Universidad Autónoma de Madrid, 28049 Madrid, Spain; [2]Departamento de Microbiología, Universidad de Sevilla, Avda. Reina Mercedes 6, 41012 Sevilla, Spain.

Summary

Bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP) is a widespread second messenger in bacteria that regulates the transition between the sessile and the motile "lifestyle" of the cell through its concentration levels. Hence, high levels stimulate exopolysaccharide production and biofilm formation, whether low levels promote motility. Proteins with characteristic GGDEF and EAL domains are responsible for synthesis and degradation of c-di-GMP, respectively. Our aim in this work is to study these types of proteins in two species of rhizobia, specially in relation to biofilm formation, to understand the role of c-di-GMP levels in either plant associated or free living bacteria.

Introducción

Durante los últimos años se ha puesto de manifiesto la gran importancia que tiene la formación de biopelículas bacterianas en las relaciones, tanto simbióticas como patógenas, que establecen las bacterias con las plantas (Danhorn y Fuqua, 2007). Sin embargo, en el caso particular de las rizobiáceas son pocos los estudios que analicen el papel que juega la formación de biopelículas tanto en vida libre como en el establecimiento de la simbiosis. Hay que destacar que trabajos recientes han relacionado la formación de biopelículas en distintas especies de rizobio con polisacáridos de la superficie celular, que juegan un papel importante en el desarrollo del nódulo (Rinaudi y Gonzalez, 2009). Además, en *S. meliloti* se ha visto que los genes *nod* están implicados en la formación y arquitectura de las biopelículas formadas en vida libre por esta bacteria (Fujishige *et al.*, 2008), sugiriendo un nexo entre el proceso de nodulación y el de formación de biopelículas.

El dinucleótido bis-(3',5')-monofosfato de guanosina cíclico (c-di-GMP) es una molécula pequeña pero de gran importancia en la formación de biopelículas. Esta molécula actúa como segundo mensajero en las bacterias, modulando fenotipos relacionados con la adaptacion a diversos estilos de vida y con la interacción con organismos eucariotas (Camilli and Bassler, 2006). Los niveles internos de c-di-GMP son cruciales para que las bacterias pasen de vida móvil a sésil y formen biopelículas. Así, cuando aumentan los niveles de c-di-GMP, las bacterias adoptan estilos de vida sésiles e incrementan la expresión de genes relacionados con la adhesión como los exopolisacáridos (Lee *et al.*, 2007); por el contrario, cuando disminuyen los niveles de c-di-GMP, las bacterias aumentan su movilidad (swiming, swarming y twitching) e incrementan la expresión de genes relacionados con la virulencia (Lee *et al.*, 2007; Tamayo *et al.*, 2008).

Los niveles de c-di-GMP están a su vez regulados por proteínas que lo sintetizan a partir de dos moléculas de GTP, y proteínas que lo hidrolizan. Las primeras presentan actividad diguanilato ciclasa, y se caracterizan por presentar un dominio conservado GGDEF; las segundas tienen actividad fosfodiesterasa, y poseen un dominio EAL (Christen *et al.*, 2005). Las bacterias Gram negativas poseen un número considerable de estas proteínas, muchas asociadas a la membrana, que presentan al menos uno de estos dos dominios. Frecuentemente asociados a éstos, se han descrito otros dominios N-terminales, generalmente de tipo sensor como los PAS, lo que sugiere que numerosas señales medioambientales y celulares son

integradas en las vías de señalización del c-di-GMP. Hasta ahora, un número muy pequeño de estas señales han sido identificadas: oxígeno, luz visible, falta de nutrientes, antibióticos, sales biliares, pequeñas moléculas de señalización intercelular, etc (Jenal y Malone, 2006).

En la actualidad, nuestro grupo de investigación está centrado en estudiar la función de las proteínas que sintetizan y degradan el c-di-GMP en dos especies distintas de rizobio: *Sinorhizobium meliloti* y *Sinorhizobium fredii*. La obtención de mutantes afectados en dichas proteínas nos permitirá analizar posteriormente su posible efecto sobre aspectos fisiológicos importantes de la bacteria como la movilidad, la producción de exopolisacáridos, la formación de biopelículas y, además, su papel en la interacción con sus huéspedes.

Agradecimientos

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Preliminary analysis of the *Sinorhizobium fredii* HH103 type III-secreted effectors NopL and NopC

Jiménez-Guerrero I[1*], Cubo MT[1], Dahl E[1], Parada M[2], Pérez-Montaño F[1], Espinar-Marchena F[1], Toimil A[1], Lavado-Roldán A[1], Gutiérrez-Patricio S[1], Ollero FJ[1], López-Baena FJ[1]

[1]*Departamento de Microbiología, Facultad de Biología, Universidad de Sevilla, Avda. Reina Mercedes 6, 41012 Sevilla, Spain. *irejimgue@alum.us.es*

Summary

In this work we show our preliminary studies about the *Sinorhizobium fredii* HH103 effector proteins NopL and NopC. We previously confirmed that secretion of both proteins depended on flavonoids and on a functional type III secretion system (T3SS). The set of Nops secreted by HH103 are beneficial in the symbiosis with soybean but detrimental with the tropical legume *Erythrina variegata*. Two mutants in the HH103 *nopL* and *nopC* genes were constructed. Inactivation of *nopL* blocked secretion of NopL but did not prevent secretion of the rest of Nops. However, the absence of NopL did not significantly affect symbiosis with American soybeans. Mutation of *nopC* completely blocked secretion of Nops, suggesting that this protein could be a component of the type III-secretion machinery and not a secreted effector protein.

Introduction

Some Gram-negative bacteria possess a specialized apparatus for protein secretion called type III secretion system (T3SS). Symbiotic and pathogenic bacteria use the T3SS to translocate effectors, directly into the host cytoplasm, that are involved in the modulation of host defense responses. *S. fredii* HH103 secrete at least eight proteins, called Nops, through the T3SS: NopA, NopB, NopC, NopD, NopL, NopM, NopP, and NopX. HH103 Nops are necessary for an effective nodulation of soybean. However, the absence of only the effector protein NopP is beneficial for nodulation in this plant. In this work, we report a preliminary analysis of the HH103 putative effector genes *nopL* and *nopC*. NopL of *Rhizobium* sp. NGR234 is phosphorylated by plant kinases and seems to modulate host defense responses. The function of NopC has not been identified yet but it has been proposed to be a putative effector protein and not a component of the T3SS-pilus, the filament that connects the bacteria with the host cell.

Materials and Methods

Recombinant DNA techniques were performed according to general protocols. Primer pairs used for amplification of the HH103 *nopL* and *nopC* genes were, respectively: nopLF (5'- GTTGTCTCTATCATCATGGA) and nopLR (5'- AACGCCTACTCTGCAGGAAA); fy1secF (5'- CCAGGGAGTCCAGATCGTGCA) and fy1secR (5'- GAGGCGTGGTTTACCGATCGA). The NCBI BLAST program was used for homology searches. Plasmids pMUS943 and pMUS793 were obtained, respectively, by cloning a 1.2 kb PCR fragment containing the *nopL* gene and its *tts* box and a 1.3 kb PCR fragment containing the *nopC* gene and its *tts* box into vector pGEM-T Easy. Both genes were mutated by insertion of the Ω interposon into the coding region of the genes. Plasmid pK18mob, which is suicide in rhizobia, was used for the homogenotization of the mutated versions of the *nopL* and *nopC* genes in HH103. Plasmids were transferred from *E. coli* to *Sinorhizobium* strains by conjugation using plasmid pRK2013 as helper. Extracellular proteins extraction and separation and nodulation tests were carried out as previously described.

Results and Discussion

The HH103 *nopL* and *nopC* genes were isolated and sequenced. HH103 NopL was 100%, 95% and 25% identical to the NopL proteins of *S. fredii* USDA257, *Rhizobium* sp. NGR234 and *Bradyrhizobium japonicum* USDA110, respectively. The deduced HH103 NopC protein (98 amino acids) showed 100% and 55% identity to NopC of NGR234 and *Bradyrhizobium elkanii*, respectively.

Inactivation of the HH103 *nopL* gene blocked secretion of one of the proteins that were previously identified by mass spectrometry as NopL but did not affect secretion of the rest of Nops (Figure). By contrast, mutation of *nopC* completely blocked secretion of Nops suggesting that NopC could be a component of the T3SS machinery and not an effector (data not shown).

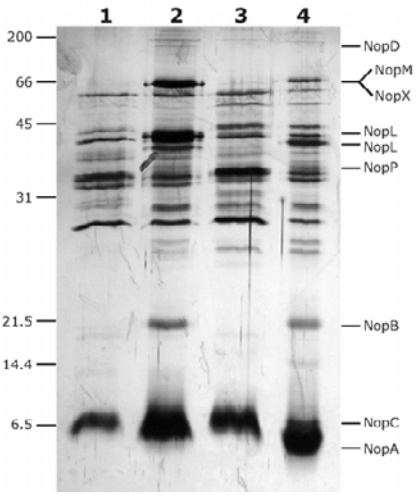

Figure. Silver-stained gel of secreted proteins of noninduced cultures of *S. fredii* HH103 (lane 1) and HH103 *nopL*::Ω (lane 3), and secreted proteins of induced cultures (genistein 3.7 μM) of HH103 (lane 2) and HH103 *nopL*::Ω (lane 4). Proteins whose secretion depends on genistein and on a functional T3SS are shown on the right of the figure. Molecular masses (kDa) of the marker are shown on the left.

The symbiotic phenotype of the HH103 *nopL*::Ω mutant strain in Williams soybean showed no significant differences when compared to the parental strain (Table). Therefore, NopL does not seem to play a key role in the symbiosis with soybeans.

Table. Plant responses to inoculation of *Glycine max* cv. Williams with *S. fredii* HH103 Rif^R and HH103 Rif^R *nopL*::Ω.

Inoculants	Number of nodules	Nodules fresh mass (g)	Plant-top dry mass (g)
None	0	0	1.175 ± 0.46
S. fredii HH103 Rif^R	229.3 ± 87.2	2.45 ± 0.83	4.545 ± 1.369
S. fredii HH103 Rif^R *nopL*::Ω	166 ± 28.8	1.65 ± 0.43	3.4 ± 0.862

Data represent the mean ± SD of five jars containing two plants.

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Sinorhizobium fredii HH103 type III-secreted proteins are involved in the regulation of soybean defense responses

López-Baena FJ[1*], Monreal JA[2], Pérez-Montaño F[1], Guasch-Vidal B[1], Espuny MR[1], Cubo MT[1], Bellogín RA[1], Vinardell JM[1], Ollero FJ[1]

*[1]Departamento de Microbiología and [2]Departamento de Fisiología Vegetal, Facultad de Biología, Universidad de Sevilla, Avda. Reina Mercedes 6, 41012 Sevilla, Spain. *jlopez@us.es*

Summary

S. fredii HH103 secretes at least 8 nodulation outer proteins (Nops), necessary for an effective nodulation of soybean, through the type III secretion system (T3SS). In this work we studied whether Nops affect the expression of the pathogenesis-related genes *PR1*, *PR2*, and *PR3* in soybean roots and shoots. In the presence of HH103, expression of the *PR1* gene was induced in soybean roots 4 days post inoculation (d.p.i) and increased 8 d.p.i. The absence of Nops provoked a higher induction of *PR1* in both soybean roots and shoots and a decrease on the symbiotic performance, suggesting that Nops diminish plant defense responses during the early stages of infection. However, the inactivation of *nopP*, which codes for an effector, led to a decrease in *PR1* expression and to an increase in nodule number. Therefore, the absence of NopP or that of the complete set of Nops seems to have opposite effects on the symbiotic performance and on the elicitation of soybean defense responses.

Introduction

Plants have evolved various defense mechanisms to resist colonization by microbial pathogens, including the synthesis of pathogenesis-related proteins. Some phytopathogens have the capacity to deliver effectors directly into the host cell via the T3SS to overcome plant defense responses. These effectors suppress host defenses to promote disease but can also be recognized by specific receptors, triggering a hypersensitive response that serves to eliminate the pathogen. It has been proposed that during the initial steps of nodulation rhizobia are recognized as pathogenic agents, triggering defense responses that are usually weak, local, and transitory. The T3SS has also been found in some rhizobial strains, such as HH103, and is involved in host-range determination and symbiotic efficiency. In HH103, Nops are necessary for an effective nodulation with soybean, but the absence of the effector NopP is highly beneficial.

Materials and Methods

For soybean RNA extraction, 5 pregerminated *Glycine max* cv. Williams seeds were aseptically transferred to a stainless-steel lattice placed in a glass cylinder containing 150 mL of a 1M Fåhraeus solution and grown in a controlled environment chamber. The hydroponics system was inoculated with *c.* 10^6 bacteria. 8 days after inoculation, shoots and the upper third of the roots were excised and fast-frozen in liquid nitrogen. For the time-course experiments, root samples were obtained 4 h and 2, 4, and 8 days post-inoculation. RNA extraction and cDNA synthesis were performed using the RNAeasy plant minikit and the Quantitect kit (Qiagen), respectively. To quantify soybean gene expression, the primer pairs employed for the detection of transcripts of the soybean *PR1*, *PR2*, *PR3*, and *UBI3* genes have been previously described. Expression was calculated relative to noninoculated plants. The fold change in the target gene, normalized to *UBI3* and relative to the gene expression in the control sample, was calculated.

Results and Discussion

Plant-pathogenic and rhizobial T3SSs are well conserved and have been proposed to play similar roles in pathogenicity and symbiosis, respectively. We have investigated whether HH103 Nops modulate the expression of three soybean *PR* genes in roots and shoots. Our results show that only the expression of *PR1* was clearly affected because no significant differences in *PR2* and *PR3* transcript levels in roots and shoots were scored between noninoculated control plants and those inoculated with either HH103 and a *ttsI* or a *nopP*

mutant derivative (Figure, **A**). Time-course experiments showed that the expression of *PR1* started to increase in roots 4 days after inoculation (Figure, **B**). Consideration the time lapse between the recognition of the possible elicitors and the synthesis of the PR proteins, these results suggest that HH103 Nops are recognized in the early infection stages of symbiosis.

Whereas Nops are necessary for an effective nodulation of soybean, the absence of NopP increased nodule number. Thus, in the symbiotic relationship established between HH103 and Williams soybean, Nops could have a double function during the early stages of infection: **i**) they could be involved in the elicitation of a localized and controlled defense response in roots that would not compromise nodulation; and **ii**) they could block the induction of a stronger defense response in roots and the systemic expression of the *PR1* gene. This stronger defense response would clearly affect nodulation. The fact that Nops modulate plant defenses suggests that rhizobia may manipulate host metabolic responses using mechanisms common to pathogens.

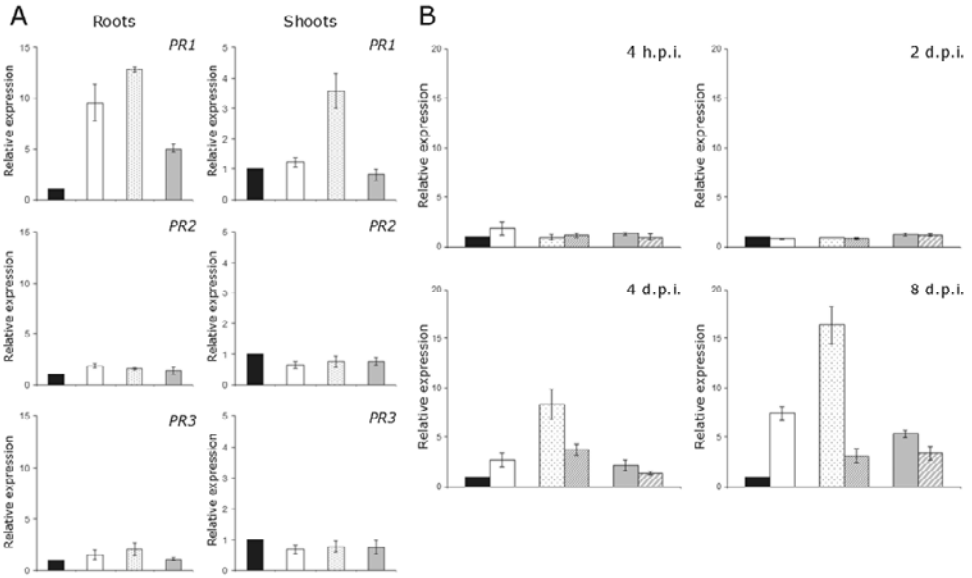

Figure. A. Analysis of the expression of the *PR1*, *PR2*, and *PR3* genes in soybean roots and shoots 8 d.p.i. using *q*RT-PCR. Plants were inoculated with HH103 (white bars), HH103 *ttsI*::Ω (dotted bars), and HH103 *nopP*::*lacZ* (grey bars). **B.** Time-course analysis of the expression of the *PR1* gene in soybean roots. Plants were inoculated with HH103 (white bars), HH103 *ttsI*::Ω (dotted bars), HH103 *ttsI*::Ω complemented (black-stripped bars), HH103 *nopP*::*lacZ* (grey bars), and HH103 *nopP*::*lacZ* complemented (grey-stripped bars). Data shown are the means ± SD for two biological triplicates. Expression was calculated relative to noninoculated control plants (black bars).

Acknowledgments

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Characterization of the quorum sensing systems of *Sinorhizobium fredii* SMH12 and *Rhizobium etli* ISP42

Pérez-Montaño F[1*], Espinar-Marchena F[1], Guasch-Vidal B[1], López-Baena FJ[1], Ollero FJ[1], Jiménez-Guerrero I[1], Cámara M[2], Bellogín RA[1], Espuny MR[1]

*[1]Departamento de Microbiología, Facultad de Biología, Universidad de Sevilla, 41012 Sevilla, Spain; [2]School of Molecular Medical Sciences, Centre for Biomolecular Sciences, University of Nottingham, Nottingham, NG7 2RD, UK. *fperezm@us.es.*

Summary

Legume-nodulating rhizobia use *N*-acyl homoserine lactones (AHLs) to regulate several physiological traits related to the symbiotic plant-microbe interaction. When compared to other genera of bacteria, rhizobia produce the largest diversity of AHLs. In this work we show that *Sinorhizobium fredii* SMH12 and *Rhizobium etli* ISP42, two rhizobial strains with different nodulation ranges, produced a similar pattern of AHL molecules, sharing in both cases the production of *N*-octanoyl homoserine lactone and its 3-oxo and/or 3-hydroxy derivatives. To gain a better understanding of the QS systems in these bacteria, mutants in the genes coding for the AHL synthases and transconjugants expressing a lactonase, an enzyme that opens the AHL lactone ring, were constructed and assayed in nodulation, swimming and biofilm formation assays.

Introduction

Many bacteria regulate gene expression in response to changes in population density by a process known as quorum sensing (QS), using small diffusible signal molecules called autoinducers (AI). Quorum sensing-regulated genes are involved in adaptive changes in the physiology of bacterial populations in a coordinated manner.. Furthermore, quorum sensing-mediated gene expression can regulate complex interactions between bacteria of different species and even between bacteria and some eukaryotic organisms they closely interact with.

In Gram-negative bacteria, the best described AIs are derivates of *N*-acyl homoserine lactones (AHLs). Different bacterial species can produce the same AHL or AHLs with similar structures and properties, suggesting the frequent occurrence of crosstalk between them using these signal molecules. Phenotypes regulated by QS in rhizobia include plasmid transfer, surface polysaccharide production, growth inhibition, stationary-phase adaptation, nodulation efficiency, symbiosome development and nitrogen fixation, all of which are important for the establishment of a successful symbiosis.

Materials and Methods

Identification of the AHLs produced by *Sinorhizobium fredii* SMH12 and *Rhizobium etli* ISP42 was carried out by thin layer chromatography (TLC) analysis using *Agrobacterium tumefaciens* NT1 (pZLR4) as biosensor and by mass spectrometry. Mutants in the AHL synthase genes (*traI* in SMH12 and *raiI* in ISP42) and transconjugant strains expressing the *aiiA* lactonase were obtained. Nodulation tests were assayed in soybean cv. Osumi (SMH12 and its QS-defective derivatives) and in common bean cv. BBL (ISP42 and its QS-defective derivatives). Swimming assays were carried out using YM 0.2% and 0.3% agar (m/v) as media. Biofilm formation was tested using TY and YM media.

Results and Discussion

TLC and mass spectrometry analysis showed that SMH12 produced at least C8-HSL and 3-oxo-C8-HSL. In the case of ISP42, the analysis showed that this bacterium produced at least C8-HSL, 3-OH-C8-HSL and 3-oxo-C14-HSL. Thus, both strains produced AHLs with C8 chains, a common feature shared with other rhizobia.

To gain a better understanding of the role of the QS systems in *R. etli* ISP42 and *S. fredii* SMH12, we constructed mutants the AHL synthase genes (*traI* in SMH12 and *raiI* in ISP42) and expressed the *aiiA* lactonase in these organisms. The SMH12 *traI*::*lacZ* strain showed a reduced production of the AHL molecules mentioned above, suggesting that there is another AHL-synthase gene involved in the production of C8-HSL and 3-oxo-C8-HSL. The *raiI*::Ω strain was defective in the production of C8-HSL and 3-OH-C8-HSL. Thus, there must be another gene involved in the biosynthesis of 3-oxo-C14-HSL. Strains expressing lactonase did not produce any AHLs.

The SMH12 strain carrying the *aiiA* lactonase gene showed a significant decrease in the induction of nodule numbers and nodule fresh mass (40% compared to the wild type) when tested in soybean cv. Osumi (Table). Hence, the SMH12 QS system seems to be necessary for an effective nodulation. Only slight decreases were obtained in the rest of the derivatives when compared to the wild-type strains in all the nodulation parameters analyzed (Table).

The SMH12 and ISP42 derivatives were impaired in their swimming ability compared to the wild-type strains, especially using YM 0.2% agar. Finally, reduced biofilm formation was observed in QS defective derivatives for both bacteria. In the case of the SMH12 derivatives, differences appeared using TY as medium. In contrast, in ISP42 derivatives, differences in biofilm formation appeared only using YM medium. Both swimming and biofilm formation play an important role in the root colonization process.

Table. Plant responses to inoculation of *Phaseolus vulgaris* cv BBL with *Rhizobium etli* ISP42 and derivatives and *Glycine max* cv. Osumi with *Sinorhizobium fredii* SMH12 and derivatives.

	***Rhizobium etli* ISP42**				***Sinorhizobium fredii* SMH12**			
	NI	**WT**	***raiI*::Ω**	**Lactonase+**	**NI**	**WT**	***traI*::*lacZ***	**Lactonase+**
Nodule number	0	299.6 ± 78.8	239 ± 92.7	271.2 ± 104.6	0	124.3 ± 5.4	91.1 ± 18.2	76.2 ± 16*
Nodule fresh mass (g)	0	1.3 ± 0.4	1.12 ± 0.4	1.04 ± 0.2	0	1.56 ± 0.2	1.24 ± 0.3	1.04 ± 0.2*
Plant top dry mass (g)	0.48 ± 0.1	0.99 ± 0.3	0.92 ± 0.4	0.68 ± 0.1	0.48 ± 0.1	1.34 ± 0.2	1.11 ± 0.3	1.1 ± 0.3

Data represent the mean ± SD of five jars containing two plants. The derivatives were individually compared with its wild-type strain by using the Mann–Whitney non-parametric test. * Numbers in the same lane significantly different at the level α = 5%.

Acknowledgments

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A mutation on *Rhizobium tropici* strain CIAT899 *glnD* gene is unstable in B$^-$ minimal medium containing nitrate as the sole nitrogen source

Guasch-Vidal B[1*], Díaz-Santín LM[1], Manyani H[2], Pérez-Montaño F[1], Toimil A[1], Cubo MT[1], Camacho M[3], Ollero FJ[1], Bellogín RA[1], Espuny MR[1], Megías M[2]

*[1]Departamento de Microbiología, Facultad de Biología, Universidad de Sevilla, 41012, Sevilla; [2]Departamento de Microbiología y Parasitología, Facultad de Farmacia, Universidad de Sevilla, 41012, Sevilla; [3]I.F.A.P.A. Centro "Las Torres-Tomejil", Alcalá del Río, Sevilla, Spain. *beagv@us.es*

Summary

The global nitrogen regulation (*ntr*) system modulates nitrogen metabolism in response to the prevailing nitrogen source and the needs of the cell (Merrick & Edwards, 1995). One of the key enzymes in this complex regulation system, the uridylyltransferase/uridylyl-removing enzyme (UTase/UR protein), is encoded by the *glnD* gene. We selected a *Rhizobium tropici* CIAT899 *glnD* insertion mutant, C8, because, unlike the wild type, it was not able to produce Nod factors (LCOs) in B$^-$ minimal medium with salt stress in the absence of apigenin. Mutants with defects in nitrogen stress response regulation are often unable to grow on nitrogen-containing compounds because they fail to induce catabolic enzymes needed to mobilize nitrogen. Here we analyze the ability of C8 to grow and produce LCOs in B$^-$ minimal medium containing different nitrogen sources. Results show that the Tn*5* insertion in *glnD* is unstable when C8 grows in B$^-$ minimal medium with nitrate as the sole nitrogen source, and that both the Tn*5* instability and the C8 production of LCOs are restored either with the addition of glutamine to the growth media or by *glnD* complementation.

Introduction

Most legume-nodulating rhizobia produce Nod factors (LCOs) in response to flavonoids exuded from legume roots. *Rhizobium tropici* strain CIAT899 can synthesize and secrete LCOs under salt stress conditions in the absence of flavonoid apigenin. In order to understand this particular LCO synthesis mechanism, we obtained the C8 mutant which shows a lower β-galactosidase activity and does not produce LCOs under salt stress condition in the absence of apigenin compared to the wild type. Cloning and sequencing of the DNA flanquing the transposon in C8 revealed that the Tn*5* insertion was located in *glnD* gene. In many bacteria, the uridylyltransferase/uridylyl-removing enzyme, encoded by the *glnD* gene, has been found to be a key regulator in nitrogen control. C8 mutant does not grow on B$^-$ minimal medium with nitrate as the sole nitrogen source but displays a requeriment for glutamine to grow in B$^-$ medium. We observed the C8 *glnD* mutation appears to be unstable in B$^-$ medium containing nitrate as the unique nitrogen source.

Materials and Methods

To evaluate bacterial growth on various nitrogen sources, *R. tropici* strains were first grown at 28ºC to late log phase in TY medium (Behringer, 1974) and then diluted 1/100 in B$^-$ minimal medium (Spaink *et al*., 1992) containing KNO_3 or glutamine as unique nitrogen sources added at 1 $g \cdot L^{-1}$. Growth rates were monitored by measuring the absorbance at 600 nm every 2 hours for 120 hours. Initial and final recounts of Colony Form Units (CFU) were done in each condition. All the experiments were done in triplicate. A statistical significant number of big and small colonies were taken from the final recounts TY plates in order to confirm the presence of the *glnD* and *Km*^{R} (Tn*5*-Mob) genes by PCR. To complement the *glnD* mutant, the complete *glnD* gene of *R. tropici* CIAT899 was amplified by PCR and cloned into pBBR1MCS to give plasmid pMUS1068 which was conjugated into *R. tropici* strain C8.

Results and Discussion

Sequencing of the cloned DNA flanking the Tn*5*-Mob insertion in C8 mutant revealed that the mutation was located in the *glnD* gene, the sensory component of the nitrogen regulation system. C8 mutant does not produce LCOs under salt stress condition in the absence of apigenin. This inability is due to C8 has growth defects with nitrate as the sole nitrogen source and develops two types of colonies. We estimated that 7% of the C8 mutant viable cells grown in B^- liquid medium will yield big colonies when spread in a solid medium plate. Analyzing the growth of the bacteria from the different C8 colonies, we found that the bacteria from the big colonies have a wild-type growth rate in B^- minimal medium with nitrate while the bacteria from the small colonies do not grow in these conditions. We used PCR to analyze the Tn*5*-Mob insertion site from the different colonies. We could amplify the Km^R gene but could not amplify the *glnD* gene on small colonies, so we concluded all the small colonies we analyzed carried the Tn*5*-Mob transposon in the *glnD* gene. Selected one of these colonies, it was demonstrated that it does not produce LCOs under salt stress conditions. Anyway we could amplify *glnD* gene on big colonies and only a few of them were positive for Km^R amplification, so we concluded that all the big colonies we analyzed had the *glnD* gene intact and, selected one of them, it was able to produce LCOs under salt stress condition. Besides, C8 mutant growth was restored in B^- minimal medium in the presence of glutamine. All the colonies from cultures growing in the presence of glutamine that we analyzed were positive for the *Km* gene-fragment amplification and negative for the *glnD* gene amplification due to the insertion of Tn*5*-Mob on *glnD* gene. In *Corynebacterium glutamicum* and *Sinorhizobium meliloti*, *glnD* insertion mutants also appear to be unstable (Jakoby *et al*. 1999; Colnaghi *et al*. 2001).

Complementation of the C8 mutant with plasmid pMUS1068, which carries the complete *glnD* gene in plasmid pBBR1MSC, demonstrates that *glnD* gene is essential for *R.tropici* CIAT899 to grow on B^- minimal medium with nitrate. As O´Connell *et al*. (1998) reported, our data do suggest that GlnD is required for nitrogen utilization by CIAT899.

Acknowledgments

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Study on endophytic bacteria belonging to the genus *Pantoea* in rice plants

Megías E[1*], Ollero FJ[2], Megías M[1]

[1]*Departamento de Microbiología y Parasitología, Facultad de Farmacia and* [2]*Departamento de Microbiología, Facultad de Biología, Universidad de Sevilla, Sevilla, Spain. *mesau@us.es*

Summary

Rice (*Oryza sativa*) is a primary source of food for more than half the world population. Therefore, due to the excessive population growth, it won't be possible to cover the demand for rice in the near future. In the present work we have confirmed the presence of bacteria inside rice plants cultivated under sterile conditions which were previously inoculated with the appropriate endophytic bacterium. However, a confocal microscopy approach is needed in order to confirm the endophytic character of a given bacterium. Our preliminary studies in confocal microscopy suggest that bacteria are inside the plants. Furthermore, the bacteria used in this assay have some features that make them capable of improving plant output. In other words, they act as PGPR (Plant Growth Promoting Rhizobacteria) bacteria. Our primary objective is to find any bacteria within our collection which has the capacity to improve rice crops and to confirm our hypothesis about the location of the bacteria inside the rice plant.

Introducción

El arroz se utiliza en su inmensa mayoría para consumo humano directo y representa más del 21% del consumo mundial de cereales. La producción mundial es superior a 625 millones de toneladas y dependen de este cultivo la alimentación de más de 2.500 millones de personas (40% de la población mundial), sobre todo en los países en desarrollo.

La producción de arroz en la Unión Europea se centra en los países del Mediterráneo, destacando Italia y España con un 55,91% y un 26,72% respectivamente del total comunitario. La producción media del arrozal sevillano se estima en unas 321.422 Tm de arroz-cáscara con unos rendimientos de aproximadamente 8.500 Kg/ha (FAS, 2007) lo que representa más del 40% del total nacional. En un año de pluviometría suficiente, Andalucía se erige como la primera Comunidad Autónoma productora de arroz en España. Concentra unas 36.071 ha de cultivo en el estuario del río Guadalquivir, en Sevilla, en zonas ubicadas dentro de los limites del Parque Nacional de Doñana.

Las bacterias endófitas pueden ser definidas como aquellas bacterias que colonizan los tejidos internos de la planta sin que haya síntomas externos de infección o efectos nocivos en el hospedador. De forma indirecta evitan el desarrollo de enfermedades mediante la competencia por nutrientes o hierro, la síntesis de compuestos antibióticos o la inducción de una respuesta sistémica en la planta. El efecto directo viene determinado por sus capacidades como bacterias PGPR (bacterias promotoras del crecimiento vegetal): producción de fitohormonas, fijación de nitrógeno (en aquellas diazotrofas) y solubilización de hierro y fosfatos, entre otras actividades.

Asociados al arroz se han descrito numerosos endófitos entre los que encontramos especies de géneros tan diversos como *Azorhizobium*, *Bradyrhizobium*, *Rhizobium*, *Agrobacterium* y *Sphingomonas* (α Proteobacteria); *Azoarcus*, *Burkholderia*, *Chromobacterium* y *Herbaspirillum* (β Proteobacteria); *Klebsiella*, *Pantoea* y *Serratia* (γ Proteobacteria). De estas bacterias queremos destacar el género *Pantoea*, antiguamente ubicado entre los géneros *Erwinia* y *Enterobacter,* descrito para incluir diversas especies anteriormente ubicadas en el complejo "*Erwinia herbicola-Enterobacter agglomerans*". Actualmente el género lo comprenden 7 especies.

Materiales y Métodos

Aislamiento de DNA. El aislamiento del DNA total genómico de las 8 bacterias del genero *Pantoea* se llevó a cabo mediante el kit *High Pure PCR Template* de Roche. La pureza y cantidad de DNA extraído se midió mediante el aparato NanoDrop 1000 de Thermo Scientific.

Detección por PCR del gen nifH. Para la detección del gen *nifH* en las diferentes cepas testadas se usaron parejas de cebadores: nifH, polE, nifHKle, nifHPseu y nifHent. Se realizaron las PCR adecuadas y se secuenciaron tras la selección del producto con el kit *Illustra^{tm} GFX^{tm} PCR DNA Gel Band Purification* de GE Healthcare.

Amplificación por PCR del gen del ARN ribosómico 16s. Para la amplificación del gen del ADNr 16s se usó la pareja de primers hD1 y rD1. Se realizaron las PCR adecuadas y se secuenciaron tras la selección del producto con el kit *Illustra^{tm} GFX^{tm} PCR DNA Gel Band Purification* de GE Healthcare.

Esterilización de semillas y preparación de plantas. Las semillas se descascarillaron y se esterilizaron superficialmente mediante agua oxigenada al 10% e hipoclorito sódico al 1%. Las semillas se pregerminaron en placas de agar agua al 1,5% durante 48 h en oscuridad y temperatura controlada (28ºC). Las semillas se depositaron en tubos de cristal estériles de 20 cm. Como soporte se usaron rejillas de acero inoxidable. Como solución nutritiva usamos Hoagland al 0.25X,. Las plantas se incubaron en fitotrón durante 7-10 días .

Marcaje fluorescente de la cepa AMG 501. Con el fin de confirmar el carácter endófito de la cepa AMG 501 (*Pantoea* sp.) marcamos la bacteria con dos proteínas fluorescentes de diferente color de emisión. Por un lado usamos la proteína verde fluorescente (GFP) y por otro la proteína "Red Cherry", que confiere fluorescencia roja a la bacteria. No hemos llevado a cabo el doble marcaje.

Preparación y observación de las muestras para microscopía confocal. Se hicieron cortes transversales de raíz y tallo, intentando hacerlos lo suficientemente finos como para que alguno quedara dispuesto en posición coronal. Las muestras se observaron inmediatamente tras su preparación, usando un microscopio confocal fluorescente espectral de barrido láser Leica TCS-SP2. Las imágenes se tomaron con objetivos de inmersión de 40X y 63X.

Resultados y Discusión

A las bacterias inicialmente seleccionadas, pertenecientes al género *Pantoea*, se les practicaron diversos estudios con el fin de confirmar su "carácter" endofito. Las aproximaciones experimentales se han llevado a cabo mediante la reinoculación bacteriana en plantas de arroz y determinación de su presencia en el interior de la misma, mediante aislamiento en medios adecuados y mediante el estudio de microscopia confocal.

Se han realizados estudios por PCR que permitan demostrar la presencia de genes los genes *nif* en estas cepas, utilizando varias parejas de primers específicos, no se ha podido determinar la presencia de estos genes en las cepas estudiadas. Actualmente se están redefiniendo las cepas por estudios finos de secuenciación del gen completo de 16S y estudios por MLSA utilizando 4 parejas de primers (gyr, rho, atpD, inf) que permiten, en la mayoría de los casos, la clasificación en diferentes especies del género *Pantoea.*

Se están llevando a cabo estudios que permitan establecer parámetros fisiológicos fácilmente seleccionables (producción de EPS o movilidad) y poder realizar mutaciones al azar con transposones que nos permitan estudiar su implicación en el "carácter endófito" de las cepas.

Agradecimientos

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Sinorhizobium fredii HH103 mutants in the *rkp-3* region are affected in biosynthesis of capsular polysaccharides and lipopolysaccharides and are impaired in their symbiotic interaction with host legumes

Crespo-Rivas JC[1*], Margaret I[1], Acosta-Jurado S[1], Hidalgo A[2], Murdoch P del S[2], Buendía AM[1], Rodríguez-Carvajal MA[3], Cubo T[1], Gil-Serrano A[3], Bonilla I[4], Rodríguez DN[5], Soto MJ[6], Sanjuán J[6], Ruiz-Sainz JE[1], Vinardell JM[1]

*[1]Departamento de Microbiología and [2]Departamento de Bioquímica Vegetal y Biología Molecular, Facultad de Biología, Universidad de Sevilla, Avda. Reina Mercedes 6, 41012 Sevilla, Spain; [3]Departamento de Biología, Universidad Autónoma de Madrid, 28049 Madrid, Spain; [4]Departamento de Química Orgánica, Facultad de Química, Universidad de Sevilla, c/ Profesor García González 1, 41012 Sevilla, Spain; [5]Centro Las Torres-Tomejil (IFAPA), Alcalá del Río, Sevilla, Spain; [6]Departamento de Microbiología del Suelo y Sistemas Simbióticos, Estación Experimental del Zaidín, CSIC, Apartado 419, 18080 Granada, Spain. *jcrespo@us.es*

Summary

The outcome of a successful symbiotic interaction between rhizobia and legumes requires the participation of diverse bacterial surface polysaccharides such as exopolysaccharides (EPS), lipopolysaccharides (LPS), capsular polysaccharides (KPS or K-antigens), and cyclic β-glucans. In this work we have isolated and sequenced six genes of the *rkp-3* region of the broad host range *Sinorhizobium fredii* HH103 strain. This cluster is composed by the *rkpLMNOPQ* genes, which encode enzymes involved in the synthesis of the specific repetition subunit of the KPS, a derivative from pseudoaminic acid. At this moment we have obtained independent mutants in *rkpM*, *rkpP*, and *rkpQ*. These mutants not only lack KPS but also display an altered LPS as determined by SDS-PAGE and immunoblotting experiments. Their mobility in semi-solid medium is reduced as compared to that of the parental strain HH103. In addition, they are clearly impaired in their symbiotic properties as they only induce pseudonodules or empty nodules in host plants forming determinate nodules such as soybean (*Glycine max*) or *Vigna unguiculata*, and the number of effective nodules formed by *Glycyrrhiza uralensis*, an indeterminate-nodule forming legume, is clearly reduced.

Introducción

Los polisacáridos superficiales bacterianos juegan un papel esencial en la simbiosis entre los rizobios y las leguminosas. El KPS (o polisacárido capsular tipo antígeno K) es un polisacárido ácido anclado en la bacteria y que presenta una estructura específica de estirpe. *Sinorhizobium fredii* HH103 sintetiza un KPS que consiste en un homopolisacárido formado por un derivado del pseudoamínico. Actualmente se conocen tres agrupaciones génicas que son necesarias para la producción del KPS y que han sido identificadas en *S. meliloti* Rm41: *rkp-1*, *rkp-2*, *rkp-3*. La región *rkp-3* contiene genes implicados en el transporte del KPS y genes implicados en la subunidad de repetición específica de estirpe. En este trabajo informamos sobre el aislamiento de la región *rkp-3* de *S. fredii* HH103 así como de la caracterización fisiológica y simbiótica y diversos mutantes afectados en algunos de los genes que componen esta región génica. Nuestros resultados indican que estos genes están implicados en la síntesis del KPS y de los LPS que son esenciales para la capacidad simbiótica de *S. fredii* HH103 con las leguminosas hospedadoras probadas.

Materiales y Métodos

Las técnicas microbiológicas y de biología molecular se realizaron según lo descrito por Crespo-Rivas *et al.* (2009). Las extracciones de polisacáridos superficiales y su visualización mediante técnicas electroforéticas así

como la realización de ensayos de nodulación se realizaron como se describe por Buendía-Clavería *et al*. (2003) y Parada *et al.* (2006).

Resultados y Discusión

Se han secuenciado seis genes de la región *rkp-3* (*rkpLMNOPQ*) de HH103 que mantienen la misma organización génica que en *S. meliloti* Rm41. Se han obtenido mutantes independientes en los genes *rkpM*, *rkpP* y *rkpQ*. Nuestros estudios hasta la fecha indican que los mutantes carecen del KPS silvestre (Fig. 1A) y que presentan un perfil electroforético del LPS alterado (Fig. 1B) que no es reconocido por un anticuerpo monoclonal anti-LPS específico del *S. fredii* HH103. Su movilidad en medio semi-sólido fue claramente inferior a la de la estirpe parental (Fig. 2). Estos mutantes sólo inducen la formación de pseudonódulos y algunos pocos nódulos vacíos al interaccionar con *Glycine max* (soja) y *Vigna unguiculata* (nódulos determinados). En *Glycyrrhiza uralensis* (que forma nódulos indeterminados) se forman menos nódulos que con la estirpe parental pero éstos sí fijan nitrógeno. Estudios de expresión de los genes *rkpM* y *rkpQ* muestran que no se inducen por flavonoides ni por el pH del medio de cultivo. La complementación de los mutante *rkpM* y *rkpQ* con un cósmico, pMUS664, que porta la región *rkp-3* completa restituye las características de la estirpe silvestre HH103 en cuanto a movilidad, producción de KPS y LPS, así como la capacidad de formar nódulos fijadores de nitrógeno en la soja.

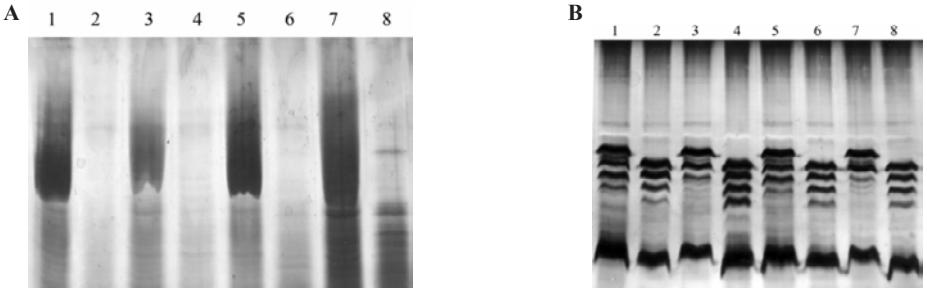

Figura 1. Electroforesis en geles de poliacrilamida de polisacáridos de extractos crudos de *Sinorhizobium fredii* HH103-Rif^R sin SDS (**A;** KPS**)** y con SDS **(B;** LPS). Carriles 1 y 5 : HH103Rif^R (SVQ269), carril 2: SVQ581 (HH103Rif^R *rkpM*::Ω), carril 3: SVQ581pMUS664, carril 4: SVQ582 (HH103Rif^R *rkpM*::*lacZ*-Gm^R), carril 6: SVQ592 (HH103Rif^R *rkpQ*::Ω), carril 7: SVQ592pMUS664, carril 8: SVQ594 (HH103Rif^R *rkpQ*::*lacZ*-Gm^R).

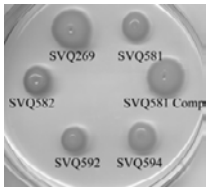

Figura 2. Placa de TY semisólido inoculado con la estirpe parental, los mutantes en la región *rkp-3* y la estirpe complementada de *rkpM*.

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Mutants in the *lpsB* region of *Sinorhizobium fredii* HH103 are impaired for nodulation with soybean

Margaret I[1*], Buendía-Clavería A[1], Crespo-Rivas JC[1], Hidalgo A[1], Alías C [1], López-Remijo A[1], Bolaños L[3], Rodríguez Carvajal MA [2], Vinardell JM[1], Ruiz-Sainz JE[1]

*[1]Departamento de Microbiología and [2]Departamento de Química Orgánica, Universidad de Sevilla, Spain; [3]Departamento de Biología, Universidad Autónoma de Madrid, Spain. *isa_margaret@us.es*

Summary

We have sequenced a 5.6 kb region of the *Sinorhizobium fredii* HH103 genome that contains four putative genes implicated in LPS biosynthesis: *lpsB, lpsE, lpsD* and *lpsC*. The *greA* gene, which codes for a transcriptional factor, is located upstream *lpsB*. The organization of all these five genes is equal to that of *S. meliloti* 1021 and *Rhizobium* sp. NGR234. We have constructed mutants in the *lpsB*, *lpsE* and *greA* genes. All of them show changes in their LPS electrophoretic profiles and their symbiotic capacity with soybean (*Glycine max*) is severely reduced. *lpsB* mutants also showed symbiotic impairment with *Vigna unguiculata* and *Glycyrrhiza uralensis* (an indeterminate nodule-forming legume). *S. fredii* HH103 *greA* mutants produced more exopolysaccharide (EPS) and its "swimming" mobility was reduced. Changes in the capsular polysaccharides (KPS), cyclic glucans (CG) and exopolysaccharides (EPS) produced by *lpsB* mutants have not been detected. LPS profiles and symbiotic deficiencies of the *lpsE* and *greA* mutants were complemented by introducing their respective wild-type gene. The *lpsB* and *greA* genes are transcribed in the same orientation but it is not clear yet whether they form a single transcriptional unit. Preliminary experiments indicated that *greA* might regulate the *lpsB* gene expression. These results indicate that LPS is of crucial importance for the symbiotic capacity of *S. fredii* HH103.

Introducción

En la simbiosis rizobio-leguminosa la formación de los nódulos requiere un complejo intercambio de señales entre ambos simbiontes. Además de los factores de nodulación, diversos polisacáridos superficiales rizobianos son necesarios para el establecimiento de una simbiosis funcional (Kannenberg *et al.,* 1998). Una clase de ellos, los lipopolisacáridos, nunca han sido estudiados en *S. fredii* HH103, una estirpe capaz de nodular tanto leguminosas formadoras de nódulos determinados como indeterminados. Las principales funciones atribuidas a los LPS durante la nodulación serían actuar como barrera estructural frente a los compuestos antimicrobianos secretados por las plantas, intervenir en el reconocimiento y adhesión a la planta, modular la respuesta defensiva de la planta y participar en la infección e invasión del nódulo. En este trabajo se presenta la región *lpsB* de *S. fredii* HH103, involucrada en la biosíntesis del LPS en esta bacteria, así como la caracterización de diversos mutantes en genes de dicha región.

Materiales y Métodos

Los mutantes en los genes *lpsB, lpsE* y *greA* de S. *fredii* HH103 se contruyeron usando el plásmido pK18mob mediante la inserción del casete *lacZ*-Gm^R o del interposón Omega. Los ensayos de nodulación en *Glycine max* (L.) Merr. cv. Williams, *Vigna unguiculata* y *Glycyrriza uralensis* se llevaron a cabo como se describe en Crespo-Rivas *et al.* (2009). Las electroforesis en geles de poliacrilamida con SDS y su posterior tinción con plata así como los ensayos de inmunodetección se realizaron como se describe en Buendía-Clavería *et al.* (2003).

Resultados y Discusión

El primer objetivo de este trabajo fue localizar genes involucrados en la biosíntesis del LPS y determinar su importancia en el establecimiento de una simbiosis funcional con plantas formadoras de nódulos determinados e indeterminados. Se secuenciaron 5,6 kb del genoma de *S. fredii* HH103 que contenían 4 genes posiblemente implicados en la biosíntesis del LPS de

esta bacteria, *lpsB*, *lpsE*, *lpsD* y *lpsC*. Aguas arriba de *lpsB* y en el mismo sentido de transcripción se localiza el gen *greA*, un factor de transcripción común a muchos organismos. Mediante la inserción del casete *lacZ*-Gm^R o del interposón Omega se obtuvieron los siguientes mutantes: en el gen *lpsB*, SVQ613 (*lpsB*::Ω) y SVQ615 (*lpsB*::*lacZ*-Gm^R); en el gen *lpsE*, SVQ642 (*lpsE*::*lacZ*-Gm^{R}en el mismo sentido de transcripción) y SVQ647 (*lpsB*::*lacZ*-Gm^R en sentido opuesto); y en el gen *greA* SVQ655 (*greA*::*lacZ*-Gm^R en el mismo sentido de transcripción), y SVQ656 (*greA*::*lacZ*-Gm^R en sentido opuesto). En experimentos de SDS-PAGE, el LPS de los mutantes *lpsB* y *lpsE* presentaron una mayor movilidad que el de la estirpe silvestre y no fueron reconocidos por un anticuerpo monoclonal anti-LPS específico de HH103 disponible en nuestro laboratorio. De los mutantes en el gen *greA* tan sólo el mutante SVQ656 mostró un perfil de LPS alterado comparado con el de la estirpe parental (Figura).

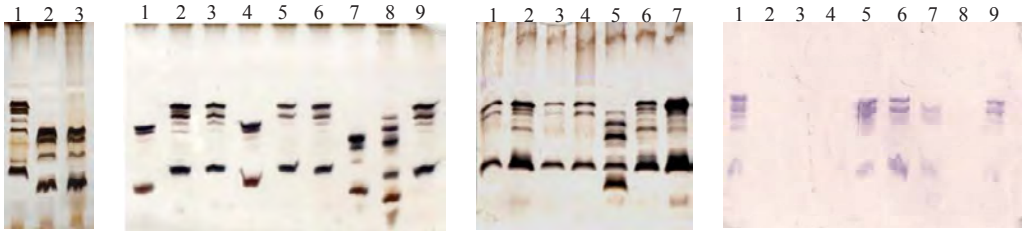

Figura. Paneles A-C: SDS-PAGE teñidos con plata de LPS de extractos crudos de *S. fredii* HH103 rifR y de los mutantes *lpsB*, *lpsE* y *greA*. **A**: carril 1, HH103 Rif^R; 2, SVQ613 (*lpsB*::Ω); 3, SVQ615 (*lpsB*::*lacZ*). **B**: carril 1, SVQ642 (*lpsE*::*lacZ*→→); 2, estirpe SVQ642 complementada; 3, HH103 Rif^R; 4: SVQ647 (*lpsE*::*lacZ*→←); 5, estirpe SVQ647 complementada; 6, HH103 Rif^R; 7, SVQ613; 8, SVQ656; 9, HH103 Rif^R. **C:** carril 1, HH103 Rif^R; 2, SVQ655 (*greA*::*lacZ*→→); 3, strain SVQ655 complementada; 4, HH103 Rif^R; 5, SVQ656 (*greA*::*lacZ*→←); 6, estirpe SVQ656 complementada; 7, HH103 Rif^R. **Panel D**: inmunodetección con el anticuerpo monoclonal NB622822 anti-LPS. Carril 1: HH103 Rif^R; 2, SVQ613; 3, SVQ642; 4, SVQ647; 5, HH103 Rif^R; 6, SVQ655; 7, SVQ656; 8, SVQ613; 9, HH103 Rif^R.

Todos los mutantes de la región *lpsB* inducen un menor número de nódulos fijadores en los ensayos con soja. Los mutantes *lpsB* se han ensayado además en plantas de *V. unguiculata* (formadora de nódulos determinados) y *G. uralensis* (formadora de nódulos indeterminados), que también tienen gravemente afectada su capacidad simbiótica. Transfiriendo por conjugación un plásmido que portaba una versión silvestre del gen afectado en cada caso, se obtuvieron estirpes complementadas de los mutantes *lpsE* y *greA*, que recuperaban tanto el perfil de LPS silvestre como la capacidad simbiótica con soja. Los mutantes en el gen *greA* presentaron además otros fenotipos como una menor capacidad de movimiento tipo "swimming" y un aumento en la producción de EPS.

Agradecimientos

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Regulation of *Sinorhizobium fredii* HH103 exopolysaccharide production by *nodD* and *nolR*

Vinardell JM[1*], Murdoch P del S[2], Crespo-Rivas JC[1], Acosta-Jurado S[1], Hidalgo A[2], Margaret I[1], Baena I[3], Lloret J[3], Ollero, FJ[1], López-Baena FJ[1], Rodríguez-Carvajal MA[4], Buendía AM[1], Rodríguez-Navarro DN[5], Gil-Serrano A[4], Ruiz-Sainz JE[1]

*[1]Departamento de Microbiología and [2]Departamento de Bioquímica Vegetal y Biología Molecular, Facultad de Biología, Universidad de Sevilla. Avda. Reina Mercedes 6, 41012 Sevilla, Spain; [3]Departamento de Biología, Universidad Autónoma de Madrid, 28049 Madrid, Spain; [4]Departamento de Química Orgánica, Facultad de Química, Universidad de Sevilla. c/ Profesor García González 1, 41012 Sevilla, Spain; [5]Centro Las Torres-Tomejil (IFAPA), Apartado Oficial, Alcalá del Río, Sevilla, Spain. *jvinar@us.es*

Summary

Bacterial surface polysaccharides play a crucial role in the rhizobia-legume symbiotic interaction. Here we report that the production of *Sinorhizobium fredii* HH103 exopolysaccharide (EPS) is influenced by the regulatory proteins NodD1 and NolR. The presence of flavonoids inducing *nod* genes diminished the amount of mucus produced in solid medium in a *nodD1*-dependent manner. This effect is not observed in the close related bacterium *S. meliloti*. Previously we have determined that NolR enhanced amount of mucus produced by HH103 in solid medium. Thus, in *S. fredii* HH103 the production of EPS is regulated in an opposite manner to that of Nod factors and Nops (Nodulation Outer Proteins): it is repressed by NodD1 and flavonoids, which are both required for the expression of genes involved in Nod factors and Nops production, but enhanced by the regulatory protein NolR, which in turns represses the expression of all the *nod* and *nop* genes tested so far including *nodD1*.

Introduction

In the rhizobia-legume symbiotic interaction, bacterial surface polysaccharides appear to play a crucial role either acting as signals required for the progression of the interaction and/or preventing host defence mechanisms. At present, EPS, lipopolysaccharides (LPS), capsular polysaccharides (KPS or K-antigens), and cyclic β-glucans (CG) are the rhizobial polysaccharides most commonly investigated for their roles in the nodulation process (Fraysse *et al.,* 2003). The symbiotic relevance of the EPS has been reported to be higher in indeterminate-nodule than in determinate-nodule forming symbiosis. According to this, the absence of EPS in *S. fredii* HH103 did not decrease the number of nodules formed by determinate-nodule forming legumes, such as soybean, but diminished the bacterial symbiotic ability with *Glycyrrhiza uralensis*, an indeterminate-nodule forming legume (Parada *et al.,* 2006; our unpublished results). Previous results of our research group have shown that in *S. fredii* HH103 the mucus produced in solid medium is negatively affected by flavonoids, but enhanced by the regulatory protein NolR (Vinardell *et al.,* 2004).

Materials and Methods

The microbiological techniques employed in this work were done according to Vinardell *et al*. (2004). Briefly, to observe EPS production in solid medium, rhizobial strains were grown in solid YMA plates 96 h at 28ºC. After this, plates were incubated at 20ºC for 72 h and scanned. For chemical analysis of bacterial EPS, rhizobial strains were cultured in 400 ml of YM medium at 28ºC for 168 h and cells were collected by centrifugation. EPS was recovered from the culture supernatants and subjected to SEC-HPLC (Size Exclusion Chromatography-High Performance Liquid Chromatography) as described by Crespo-Rivas *et al.* (2009).

Results and Discussion

The aim of this work was to further investigate the regulation of the production of *S. fredii* HH103 EPS by flavonoids and NolR. First, we analysed whether this phenomenon is observed in the close-related species *S. meliloti*. As shown in Figure (**A**), the mucus amount

of *S. meliloti* strains Rm41 and Sm1021 is apparently not affected, in contrast to that observed in *S. fredii* HH103, by the presence of the flavonoid luteolin, an inducer of *nod* gene expression in both sinorhizobial especies. This result points out that a flavonoid negative effect on EPS production is not a universal phenomenon among sinorhizobia.

Second, we assessed the effect of flavonoids on EPS production in *S. fredii* HH103 mutant derivatives lacking NodD1, NodD2, or NolR. Figure (**B**) shows that in a *nodD1* background the presence of genistein did not affect EPS production in HH103. Neither the presence of flavone, a flavonoid which is not able to induce *nod* gene expression in *S. fredii*, nor the absence of a functional copy of *nodD2* influenced HH103 EPS production. As expected, the lack of NolR diminished EPS production by *S. fredii* HH103 regardless the presence or not of flavonoids. These results suggested that in HH103 the production of EPS is regulated in an opposite manner to that of Nod factors and Nops (Nodulation Outer Proteins): it is repressed by NodD1 and flavonoids, which are required for the expression of genes involved in Nod factors and Nops production, but enhanced by the regulatory protein NolR, which in turns represses the expression of *nod* and *nop* genes (Vinardell *et al.,* 2004).

Third, we investigated whether the molecular weight of the EPS produced by HH103 was affected by the presence of genistein and by the lack of functional copies of either NodD1 or NolR, by using SEC-HPLC (data not shown). Surprisingly, preliminary results indicate that those conditions which diminished bacterial amount of mucus on solid plates (presence of genistein, absence of a functional copy of NolR) provoked an increase in the molecular weight of the EPS produced. This increase was not observed in the presence of genistein if a functional copy of *nodD1* was not present.

In conclusion, our results indicate that the EPS of *S. fredii* HH103 is modulated quantitative and qualitatively by the symbiotic regulatory proteins NodD1 and NolR.

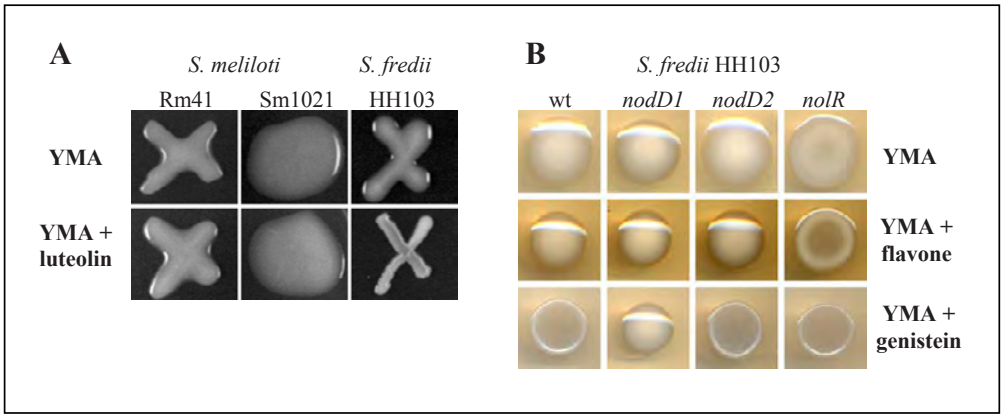

Figure. EPS production by sinorhizobial strains in the presence or absence of flavonoids.

Acknowledgments

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Identification and functional characterization of *Rhizobium leguminosarum* bv. *viciae* genetic systems involved in nickel homeostasis

Rubio L[1*], Prieto RI[2], Imperial J[3], Palacios JM[2], Brito B[2]

[1]Departamento de Química y Análisis Agrícola, E.U. Ingeniería Técnica Agrícola, Ciudad Universitaria s/n, 28040 Madrid and Centro de Biotecnología y Genómica de Plantas (CBGP), Campus de Montegancedo, Pozuelo de Alarcón, 28223 Madrid; [2]Departamento de Biotecnología, E.T.S. Ingenieros Agrónomos, Ciudad Universitaria s/n, 28040 Madrid and CBGP, Campus de Montegancedo, Pozuelo de Alarcón, 28223 Madrid; [3]CSIC and CBGP, Campus de Montegancedo, Pozuelo de Alarcón, 28223 Madrid, Spain. [*]laura.rubio@upm.es

Summary

A collection of *Rhizobium leguminosarum* bv. *viciae* strains isolated from ultramafic and contaminated soils in Italy and Germany, respectively, was analyzed for resistance to nickel (Ni) and cobalt (Co) ions. These assays led to the identification of strain UPM1137, which is able to grow at high concentrations of Ni and Co. In order to identify genetic systems involved in the homeostasis to these metals, a random mutagenesis was carried out in UPM1137 by inserting a Tn5-derivative minitransposon. As a result 4313 transconjugants were obtained, being 39 of them (0.90%) unable to grow at 1.5 mM $NiCl_2$. The identification of the transposon insertion sites in these mutants showed that the disrupted genes encode proteins belonging to different functional categories, where the secreted and membrane proteins were the most numerous. The analysis of heavy metal resistance and phenotypes in symbiotic and free-living cells will define the contribution of these genes to metal homeostasis.

Introduction

Soils with high concentrations of heavy metals, either naturally or by contamination, are becoming more abundant. High metal concentration in soils affects fertility as well as microbial activity. Bacteria are able to develop several mechanisms to detoxify the cell and resist those stress conditions (Bruins *et al.,* 2000). Analysis of bacteria isolated from ultramafic soils, or soils contaminated with heavy metals, might allow the identification of mechanisms required for survival in such inhospitable conditions.

Materials and Methods

The nitrogen fixation and nodulation phenotypes of strains UPM1131, UPM1132, UPM1133, UPM1134, UPM1135, UPM1136, UPM1137, UPM1138, UPM1139, UPM1140, UPM1141 and UPM1142 of *Rhizobium leguminosarum* bv. *viciae* (Fernández *et al.,* 2005) were analyzed by inoculation of pre-germinated seeds of *Pisum sativum*. The analysis of Ni and Co resistance in strains of *R. leguminosarum* was performed in plates of TY medium supplemented with increasing concentrations of $NiCl_2$ and $CoCl_2$. For disk diffusion tests of metal resistance (Bauer *et al.,* 1966), exponential cultures were inoculated in TY medium and disks soaked in 100 mM, 200 mM and 500 mM $NiCl_2$ or 20, 50 and 100 mM $CoCl_2$, were placed on the plate. The diameter of the inhibition zone (mm) was measured after 48 h of incubation.

Plasmid pSS240, containing the minitransposon Tn*5*SSoriR*gusA*, was introduced in strain UPM1137 by conjugation. Transconjugants affected on their growth on 1.5 mM $NiCl_2$ were selected, and the disrupted gene was identified by cloning in *Escherichia coli* and sequencing of the regions adjacent to the minitransposon with the aad1846 primer, complementary to vector pSS240. The sequences obtained were compared using BLAST program at the NCBI database. Additional information about genes and classification were obtained from the database RhizoBase (http://genome.kazusa.or.jp/rhizobase/).

Results and Discussion

A collection of *Rhizobium leguminosarum* bv. *viciae* strains isolated from soils of Italy and Germany containing high concentrations of heavy metals were analyzed for resistance to Ni and Co. Growth in TY medium supplemented with increasing concentrations of $NiCl_2$ or

$CoCl_2$ and disk diffusion tests of these metals revealed a large diversity of resistance to Ni and Co (Table). Strain UPM1137 showing the minimum diameter of the inhibition zone in the disk diffusion tests, and strain UPM1136 showing the maximum value, were selected as the most and less resistant strains, respectively.

Table. Susceptibility of *R. leguminosarum* bv. *viciae* strains to transition metal ions as determined by disk diffusion tests.

Strains	Inhibition zone diameter (mm)	
	$NiCl_2^*$	$CoCl_2^+$
UPM1131[2]	20	20
UPM1132[2]	18	26
UPM1133[2]	16	18
UPM1134[2]	18	16
UPM1135[1]	16	22
UPM1136[2]	26	26
UPM1137[2]	12	15
UPM1138[2]	22	26
UPM1139[2]	14	24
UPM1140[1]	17	18
UPM1141[1]	22	20
UPM1142[1]	22	15
3841	20	20

[1] Isolated from contaminated soil (Stuttgart, Germany).
[2] Isolated from ultramafic soil (Gorro, Italy).
*200 mM $NiCl_2$. +100 mM $CoCl_2$

In order to identify genetic systems involved in Ni and Co resistance, a random mutagenesis was carried out in strain UPM1137 by the insertion of a Tn5-derivative minitransposon. The screening for Ni resistance revealed that 39 out of 4313 transconjugants were unable to grow at the Ni concentration established (1.5 mM $NiCl_2$). So far, the disrupted genes have been identified in 26 mutants, and the comparison of the translated sequences enabled protein classification in functional categories. The most frequent category corresponded to secreted and membrane proteins (26%), followed by proteins involved in the metabolism of small molecules (23%). Proteins identified in this analysis included a homolog to the *E. coli* Ni/Co efflux system RcnA, and a putative transmembrane protein found in other *Rhizobiaceae* species and encoded in a *locus* adjacent to the copper homeostasis *cop* operon. The characterization of Ni and Co resistance levels of the mutants established two different groups, one of them consisting of mutants only affected in Ni resistance and the other being sensitive to both metals. Further phenotypic characterization of these mutants will determine the role of the genetic systems identified in metal homeostasis.

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Diversity of nickel ligands in nodule cytosol, nickel transport, and expression of a nickel-dependent enzyme in endosymbiotic bacteria as affected by the legume host

Brito B[1], del Cacho C[2], Pérez-Novillo C[2], Cámara C[2], Ruiz-Argüeso T[1], Palacios JM[1*]

*[1]Centro de Biotecnología y Genómica de Plantas and Departamento de Biotecnología Universidad Politécnica de Madrid, Campus de Montegancedo, M-40, km 37.7, 28223 Pozuelo de Alarcón, Madrid; [2]Departamento de Química Analítica, Facultad de CC. Químicas, Universidad Complutense de Madrid, 28040 Madrid, Spain. *jose.palacios@upm.es*

Summary

Provision of metals to endosymbiotic bacteria represents a potential limitation for metalloenzyme synthesis inside legume nodules. Metal ions are usually bound to organic ligands in the cell cytoplasm, and the nature of such metal-ligand complexes might affect metal availability. We have observed a strong effect of the legume host on hydrogenase synthesis when the same *Rhizobium leguminosarum* bv. *viciae* strain establishes a symbiotic interaction with pea (*Pisum sativum*) or lentil (*Lens sculenta*) plants. These data, along with the different phenotypes of mutants altered in nickel (Ni) transport in these hosts, suggest a role for the chemical form of Ni on metal provision to the bacteroid. The biochemical analysis of cytosolic fractions of pea and lentil nodules has revealed the different nature and concentration of organic ligands chelating Ni in these hosts.

Introduction

In natural environments metals are often chelated by organic ligands due to their high binding affinity (Waldron & Robinson, 2009). In the case of endosymbiotic bacteria, bacteroids are surrounded by plant cytoplasm in which metal ions are also complexed by different ligands. Some of these ligands are organic acids, which, at the same time, are the main carbon substrate transferred to bacteroids in legume nodules (den Herder & Parniske, 2009). We are interested in the effect of such complexes on the supply of metals to the bacteroids. Previous reports indicated that the synthesis of [NiFe] hydrogenase in the nodules is limited by the availability of Ni (Brito *et al.,* 1994; Ureta *et al.,* 2005). Also, expression of this metalloenzyme is strongly affected by the legume host (López *et al.,* 1983; Brito *et al.,* 2008). In this work we have studied the nature of organic acids complexing Ni in the cytosol fraction of nodules induced in different legume hosts, correlating it with Ni transport and hydrogenase activity in bacteroids.

Materials and Methods

The *R. leguminosarum* wild type SPF25(pALPF1) and *hupE*-deficient mutant SPF22A(pALPFE) strains were previously described (Brito *et al.,* 2010). Organic ligands present in nodule cytoplasm were resolved by HPLC-UV/MS using a C18 reversed phase column. For separation and identification of Ni complexes, a silica column coupled to UV/MS was used. Identification of Ni compounds in the chromatography profile was performed by ICP-MS (Cacho *et al.,* 2010). Hydrogenase activities from bacteroids were determined by an amperometric method using oxygen as electron acceptor.

Results and Discussion

Two lines of evidence indicate that host-dependent differences in the chemical form of Ni in the nodule cytoplasm affects the expression of NiFe hydrogenase. First, we found that the lack of expression of hydrogenase in a non-permissive legume host can be partially complemented by the addition of Ni to the plant nutrient solution. Second, we observed differences in hydrogenase phenotype from a *R. leguminosarum* mutant deficient in the Ni transporter HupE when in symbiosis with pea vs. lentils, suggesting a different pathway of Ni uptake potentially due to the presence of different Ni ligands in both plants (Figure).

We have determined the nature of Ni complexes present in the cytosol of pea and lentil nodules induced by the same *R. leguminosarum* bv. *viciae* strain. By HPLC-UV/MS using a C18 reversed phase column we have observed that cytosol fractions of pea and lentil nodules differs in organic acid composition; however, Ni complexes could not be resolved by this approach. A new methodology using a normal phase chromatography with a silica column and a hexane:ethanol mobile phase coupled to UV/MS has been developed. By this method we have determined that Ni-malate and Ni-citrate are the main Ni complexes in pea nodules. In contrast, in nodules induced by the same strain in lentil plants, Ni is present mostly as Ni-citrate, although low amounts of Ni-malate and Ni-tartrate are also present. These results are consistent with the concentrations of each free ligand in the nodule cytosol of these hosts as corrected by the affinity constant of each ligand for Ni.

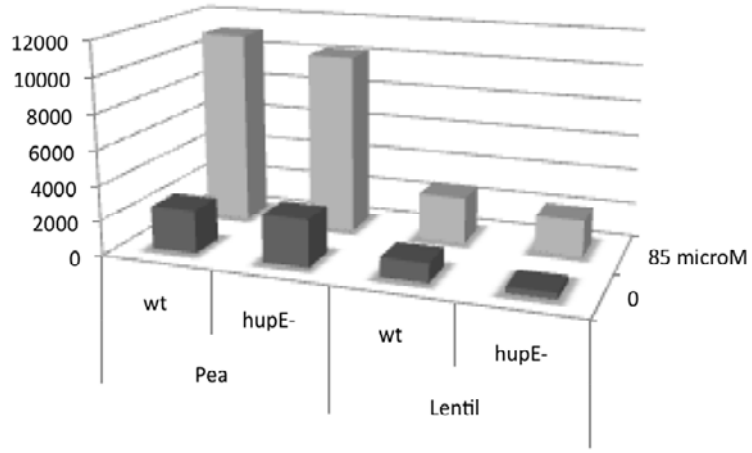

Figure. Effect of host and Ni transport on symbiotic hydrogenase activity. The graph shows the values of hydrogenase activity of pea and lentil bacteroids induced by *R. leguminosarum* SPF25(pALPF1) wild-type strain or by its *hupE*-deficient derivative SPF22A(pALPFE). Pea (*Pisum sativum* cv Frisson) and lentil (*Lens sculenta* cv Magda) plants were grown in nutrient solutions without Ni supplementation or supplemented with 85 μM $NiCl_2$. Values are expressed in nmoles of H_2 oxidized per hour per mg protein, and represent the average of at least two experiments (adapted from Brito *et al.,* 2010).

Acknowledgments

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Structure of the O-specific polysaccharide chain of the lipopolysaccharide isolated from *Pantoea sp.* AEP17

Contreras R[1*], González Barroso S[1], Rodríguez Carvajal MA[1], Espuny MR[2], Espinar-Marchena F[2], Gil Serrano AM[1]

[1]Departamento de Química Orgánica, Facultad de Química, Universidad de Sevilla; [2]Departamento de Microbiología, Facultad de Biología, Universidad de Sevilla, Spain.

Summary

The lipopolysaccharide (LPS) of *Pantoea sp.* AEP17 was isolated by the hot phenol/water method. The O-specific polysaccharide (O-PS) was released by mild acid hydrolysis and isolated by gel filtration chromatography, and its structure was elucidated using NMR spectroscopy and GC-MS. The polysaccharide is composed of an hexasaccharidic repeating unit with the structure: 2)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→2)-α-L-Rha*p*-(1→3)-β-D-Glc*p*NAc-(1→3)-[α-D-GalAN-(1→2)]-α-L-Rha*p*-(1→, where GalAN is galacturonamide. The structure of the O-antigen of *Pantoea sp.* AEP17 is similar to the O-specific polysaccharide from *Escherichia coli* O35 and *Salmonella arizonae* O62.

Introduction

Pantoea sp. AEP17 is a Gram-negative bacterium that has been isolated from agricultural soil and studied for their plant growth-promoting activities such as production of indole acetic acid (IAA) and siderophores. Bacterial lipopolysaccharide is a component of the outer membrane in the cell wall of Gram-negative bacteria and it is composed of three distinct regions: the O-antigen (the so-called O-chain) polysaccharide, a short core oligosaccharide, and lipid A. This O-chain is responsible for the antigenic properties of the LPS. The aim of this work is to isolate and elucidate the structure of the O-specific polysaccharide chain of *Pantoea sp*. AEP17 lipopolysaccharide.

Materials and Methods

Bacterial culture conditions and growth. *Pantoea sp*. AEP17 was grown at 28 ºC in TY medium (Beringer, 1974) and the LPS was isolated from dried bacterial cells by hot phenol-water extraction (Westphal *et al.,* 1965).

Isolation of the O-specific polysaccharide. Delipidation of the LPS (60 mg) was performed with 1% AcOH at 100ºC until lipid precipitation. The precipitate was removed by centrifugation (4000 rpm, 20 min), and the supernatant was fractionated on a column (70 x 1.5 cm) of Bio-gel P-2 (Bio-Rad) in water to separate the O-antigen from core oligosaccharide.

Monosaccharide analysis. Monosaccharides were identified on GLC-MS separation of their trimethylsilylated methyl glycosides obtained as described (Gil-Serrano *et al.,* 1998). The absolute configuration of monosaccharides in the O-antigen was studied by the formation of trimethylsilylated (*S*)- and (*R*,*S*)-2-butyl glycosides (Gerwig *et al.,* 1978).

Methylation analysis. Polysaccharide was methylated by a modificated method of Ciucanu and Costello (Ciucanu *et al.,* 2003). Methylated mononosaccharides were reduced with $NaBD_4$ (Abe *et al.,*1984), hydrolyzed and acetylated (Kim *et al.,* 2006) to obtain partially methylated and acetylated alditols, which were analysed by GLC-MS.

Determination of molecular size. The molecular size of the O-antigen was determined by size exclusion chromatography on an HPLC system (Waters) with a Zorbax PSM1000 column (6.2 x 250 mm, 5 μm) and monitored with a differential refractometer (Waters).

NMR analyses. Spectra were recorded at 303K on a Bruker AV500 spectrometer operating at 500.13 MHz (^{1}H) and 125.75 MHz (^{13}C). The ^{1}H and ^{13}C NMR spectra of the polysaccharide were assigned using 2D DQF-COSY, TOCSY, NOESY, HSQC, HSQC-TOCSY and HMBC experiments. Chemical shifts are reported related to internal DHO signal (δ_H 4.71 ppm) and external dimethylsulfoxide (δ_C 39.5 ppm) as references.

Results and Discussion

The O-specific polysaccharide was obtained by mild acid degradation of the LPS isolated hydrolysis of the polysaccharide revealed L-Rha, D-GlcNAc and D-GalA in the ratio 4:1:1. Methylation analysis revealed 2- and 3-substituted Rha*p*, 2,3-disubstituted Rha*p* and 3-substituted Glc*p*NAc.

The assignment of ^{1}H and ^{13}C signals (Table) was carried out using two-dimensional NMR experiments as described above. The sequence of the residues in the repeating unit was deduced from the NOESY and HMBC experiments.

Table. ^{1}H- and ^{13}C NMR chemical shifts of O-antigen isolated from *Pantoea sp.* AEP17. [a]GalAN: galacturonamide.

Residue		Position							
		1	2	3	4	5	6	Ac	NH
→2)-α-L-Rha*p*	^{1}H	5.17	4.10	3.96	3.46	3.82	1.31		
	^{13}C	101.0	77.9	69.9	72.2	69.3	16.7		
α-D-Gal*p*AN[a]	^{1}H	5.12	3.83	3.94	4.35	4.89			~8
	^{13}C	96.7	67.9	69.2	69.9	71.5	173.9		
→2,3)-α-L-Rha*p*	^{1}H	5.06	4.40	3.93	3.55	3.73	1.27		
	^{13}C	99.3	74.7	78.4	71.6	69.7	16.7		
→2)-α-L-Rha*p*	^{1}H	5.01	3.78	3.88	3.46	3.99	1.24		
	^{13}C	99.8	80.1	69.9	72.3	69.2	16.7		
→3)-α-L-Rha*p*.	^{1}H	4.86	4.12	3.82	3.54	3.77	1.28		
	^{13}C	102.4	70.0	77.7	71.7	69.3	16.8		
→3)-β-D-Glc*p*NAc	^{1}H	4.69	3.73	3.58	3.32	3.41	3.67 ; 3.92	2.06	
	^{13}C	102.6	56.2	81.6	69.4	75.9	61.5	22.7 ; 174.4	

On the basis of these data, it was concluded that the repeating unit of the O-specific polysaccharide of *Pantoea sp.* AEP17 has the following structure:
2)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→2)-α-L-Rha*p*-(1→3)-β-D-Glc*p*NAc-(1→3)-[α-D-GalAN-(1→2)]-α-L-Rha*p*-(1→.

A backbone made of rhamnose units agrees with the O-antigen found in other species of *Pantoea* (Cimmino *et al.,* 2008). The structure of the hexasaccharide repeating unit is very similar to the O-PS present in *E. coli* O35 (Rundlöf *et al.,* 1998) and *S. arizonae* O62 (Vinogradov *et al.,* 1994). Remarkable is also the presence of a rarely occurring component of bacterial polysaccharide, GalAN and has been identified only twice as a component of lipopolysaccharides.

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Effect of cellulase *celC2* heterologous expression in the *Sinorhizobium meliloti-Medicago sativa* symbiosis

Robledo M[1], Menéndez E[1*], Rivera LP[1], Jiménez-Zurdo JI[2], Bober M[1], Velázquez E[1], Martínez-Molina E[1], Mateos PF[1]

*[1]Departamento de Microbiología y Genética, CIALE, Universidad de Salamanca, Spain; [2]Estación Experimental del Zaidín, CSIC, Apartado 419, 18080 Granada, Spain. *esthermenendez@usal.es*

Summary

Nitrogen is required for plant development. Despite its abundance, it is unavailable for plants. Rhizobia-legume symbiosis is the most efficient source of biological nitrogen fixation in agricultural crops. The establishment of this well-known partnership begins with bacterial infection of plant root hairs. We have previously shown that *Rhizobium* cellulase CelC2 plays a crucial role in primary symbiotic infection process in legume roots (Robledo *et al.* 2008). This cellulase can specifically hydrolyze the non-crystalline apex of clover (*Trifolium repens*) root hairs, leading to rhizobia penetration. CelC2 knockout mutants are unable to complete the infection process. On the opposite, CelC2 over-producing mutants produce an excessive degradation of the root hair tip. In order to analyze the effect of a heterologous expression, we have introduced the *celC2* gene into *Sinorhizobium meliloti* 1021, the natural alfalfa (*Medicago sativa*) symbiont. This strain can not infect or nodulate clover and does not contain *celC2* homologs in its genome. A *S. meliloti* 1021 mutant that constitutively expresses CelC2 displayed several relevant differences in root hair deformation and invasion both in alfalfa and clover. This was a result of extensive degradation of non-crystalline cellulose located at the root hair tips. We also observed that some alfalfa plants, when inoculated with the 1021C2^{+} mutant, develop brown aberrant nodules with disorganized internal structures; these nodules do not fix nitrogen, as there was a delay in plant development under nitrogen-free conditions. These results suggest that endoglucanase expression may mediate invasion of non-host legumes and that this feature may not be restricted to clover plants.

Introduction

Plant development mainly depends on the availability of mineral nutrients, such as nitrogen. Although nitrogen is one of the most abundant elements on earth, it is not readily available to plants. Therefore, rhizobial microorganisms play a crucial role in nitrogen availability because they may convert atmospheric nitrogen into ammonia. These nitrogen-fixing endophytic bacteria can invade their plant hosts through colonization of intercellular epidermal spaces, crack entry at emerging lateral roots, or infection of root hairs. The later is the best characterized infection pathway, elicited by a complex molecular dialogue between the symbionts.

A key event of the infection process required for development of the *Rhizobium*-legume root-nodule symbiosis is the highly localized, complete erosion of the host root hair cell wall through which the bacterial symbiont penetrates to establish a nitrogen-fixing, intracellular endosymbiotic state. Once an infection thread has penetrated to the base of a root hair cell, the bacteria must grow through the developing meristematic zone to the underlying cell. Each bacterial cell penetrates into a target nodule cell in an individual, unwalled membrane compartment that originates from the infection thread and forms a unit which is known as the symbiosome.

Here we report on a cell-bound bacterial cellulase from *R. leguminosarum* bv. *trifolii* ANU843 that fulfils a significant role in both of these symbiotic infectivity events. CelC2 cellulase is a 1,4-ß-D-endoglucanase with high substrate specificity for noncrystalline cellulose. Previous studies demonstrated that CelC2 knockout mutants were unable to breach the host wall at the root-hair tip and form infection threads that are necessary to invade nodules allowing them to fix nitrogen. They also showed that the transfer of the cloned wild-

type gene into the CelC2 knockout mutant restored these symbiotic phenotypes (Robledo *et al.,* 2008).

To further analyze the symbiotic function of this bacterial endoglucanase, we engineered *S. meliloti* cellulase CelC2- expressing mutants and monitored their symbiotic phenothype through the different steps of the infection process in alfalfa and clover. Our results show that CelC2 highly localized site of cell wall degradation in alfalfa plants may match the restricted distribution of the isotropic noncrystalline wall architecture at root hair tips and infection threads found in clover.

Materials and Methods

Construction of *Sinorhizobium meliloti* 1021*celC2*$^{+}$ strain was performed introducing vector pJZC2 (Robledo *et al.,* 2008) in *S. meliloti* 1021 wild-type strain by triparental mating. Detection of CM-cellulase isozymes present in sonicated cells extracts from 1021C2^{+} strain was performed as described (Mateos *et al.,* 1992). The GFP encoding plasmid pHC60 (Cheng *et al.,* 1998) was introduced into 1021 wild-type and its derivative strain as described above to yield fluorescent label bacteria. To test the symbiotic phenotypes, sterile plants of *Medicago sativa* cv. Aragón and *Trifolium repens* cv. HUIA were inoculated with *S. meliloti* strain 1021, 1021C2^{+} or gfp-tagged derivatives, for infection and nodulation assays as previously described (Robledo *et al.,* 2008).

Results and Discussion

The activity stain overlay of *S. meliloti* strain 1021 CelC2^{+} assay confirms that cellulase CelC2 is expressed, processed and localized in this strain in the same way as for *R. leguminosarum* bv. *trifolii* ANU843. We can also confirm that the *S. meliloti* 1021 wild-type strain does not produce this enzyme.

Differential interference contrast (DIC) and confocal micrograpy of alfalfa and clover root hairs infected with *S. meliloti* 1021*celC2*$^{+}$ showed alterations on root hair invasion. Alfalfa nodules after 40 days of the beginning of the assay show an aberrant morphology especially in nodular development zone. The percentage of abnormal nodules increase with the inoculum size. Histological sections of aberrant nodules show disorganized cellular structures.

Plant phenotypes at the end of the nodulation test inoculated with *S. meliloti* 1021C2^{+} display a significant lower development than the inoculated ones with the wild-type strain. All this data encourage and spread *celC2* enzyme role in root hair invasion of legumes.

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Rhizobium leguminosarum bv. *trifolii* E11 as multifunctional bioinoculant in legumes and non-legumes

Rivera LP[1*], Robledo M[1], Velázquez E[1], Yanni RY[2], Dazzo F[3], Martínez-Molina E[1], Mateos PF[1]

*[1]Departamento de Microbiología y Genética, CIALE, Universidad de Salamanca, Spain; [2]Sakha Agricultural Research Station, Kafr El-sheikh 33717, Egypt; [3]Department of Microbiology and Molecular Genetics, Michigan State University, USA. *lprr@usal.es*

Summary

The mobilization of nutrients has been studied for decades, focusing mainly on biological nitrogen fixation and, in particular, on the symbiosis between microorganisms and higher plants. It has been generically designated rhizobia to those microorganisms capable of establishing nodule endosymbiosis with legumes; this interaction has been widely studied because of its importance in human nutrition. However, rice (*Oryza sativa*) is the most important crop in human nutrition according to FAO. The surface dedicated to rice cultivation in the world is *c.* 150 millions of hectares, with a production of 600 millions of tons per year. It has been reported that rhizobia have the capacity to establish endophytic associations with several crops (*i.e.* rice, wheat, barley, corn, sorghum), increasing vegetative growth, grain yield, and the efficient use of nitrogen fertilizer. The aims of this work are to apply this knowledge to *Rhizobium leguminosarum* bv. *trifolii* E11, a selected bioinoculant, and to analyze the role of cellulases in infection and colonization of rice and legume roots.

Introducción

La asociación endofítica natural entre *Rhizobium leguminosarum* bv. *trifolii* y las raíces de arroz fue descrita por primera vez por nuestro grupo de investigación, en colaboración con otros grupos internacionales (Yanni *et al.,* 1997, 2001). En estos trabajos se describe el aislamiento y selección de varias cepas de *R. leguminosarum* bv. *trifolii* con capacidad de nodular y fijar nitrógeno en trébol (*Trifolium repens*) y que además tenían potencial para promover el crecimiento del arroz y mejorar productividad tanto en condiciones de laboratorio como en ensayos de campo Los beneficios de esta asociación *Rhizobium*-arroz no se deben a la fijación biológica de nitrógeno sino a otros factores que promueven el crecimiento vegetal. Por ejemplo, la cepa seleccionada para este estudio, *R. leguminosarum* bv. *trifolii* E11, produce auxinas (ácido indolacético) giberelinas (GA_7), celulasas, poligalacturonasas, bacteriocinas y sideróforos, y es capaz de solubilizar fosfato tanto orgánico (fitato) como inorgánico (cálcico). Esta cepa incrementa significativamente el rendimiento de grano de arroz con una disminución en la necesidad de aporte externo de fertilizantes químicos. Esta capacidad es de gran importancia para la agricultura sostenible, aumentando la producción sin dañar irreparablemente la base de recursos naturales donde se puedan llevar acabo estos cultivos.

Nuestro grupo de investigación ha comprobado que una de las celulasas (C2) producidas por el microsimbionte *R. leguminosarum* bv. *trifolii* ANU843 puede hidrolizar de una forma muy localizada y específica la pared celular del trébol, evitando la destrucción de la misma y facilitando la entrada de la bacteria (Robledo *et al.,* 2008) a través de los pelos radicales. Con este trabajo pretendemos determinar la influencia de esta celulasa en la habilidad que tiene la cepa E11 de infectar tanto leguminosas como cereales y comprobar si esa universalidad también se puede aplicar a la capacidad de promover el crecimiento vegetal en un amplio rango de hospedadores.

Materiales y Metodos

La cepa seleccionada para este trabajo, *Rhizobium leguminosarum* bv. *trifolii* E11, fue aislada del interior de raíces de arroz cultivado en el Delta del Nilo en rotación con trébol (Yanni *et al.,* 1997). Tanto la preparación de ADN de plásmidos como las técnicas de ADN recombinante se realizaron siguiendo los protocolos estándar (Sambrook *et al.,* 1989). Para obtener el mutante E11C2+, un fragmento de ADN que contenía la celulasa CelC2, así como 78 nucleótidos aguas arriba del codón de inicio de la traducción, fueron insertados entre los sitios de reconocimiento de las enzimas *Xho*I y *Sal*I del vector pBBRMS-2. Esto dio lugar al plásmido pJZC2, que fue movilizado al interior de la estirpe *R. leguminosarum* bv. *trifolii* E11 mediante una unión triparental, usando el plásmido pRK2013 como ayuda. Las bacterias fotoluminiscentes por expresión de GFP fueron obtenidas de igual forma mediante la conjugación del plásmido pHC60 en las respectivas estirpes (E11 o E11C2+).

Resultados y Discusion

La obtención de mutantes superproductores de la actividad celulásica CelC2 nos ha permitido comprobar que estas cepas tienen incrementada su capacidad infectiva. Mediante el uso de distintas técnicas de microscopía (óptica, confocal y electrónica) hemos comprobado que esta cepa superproductora tiene incrementado su mecanismo de invasión no canónica a través de los espacios intercelulares, manteniendo su mecanismo de invasión canónico intracelular en trébol y arroz.

Como consecuencia de esta mayor infectividad realizamos ensayos de inoculación en diferentes cultivos y variedades de leguminosas y cereales. Los datos obtenidos revelan que la cepa E11C2^{+} mejora la capacidad biofertilizadora de la cepa parental actuando como un buen bioinoculante multifuncional en leguminosas (tanto como PGPR como FBN) y además en cereales (PGPR).

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Role of *Rhizobium* endoglucanase CelC in cellulose biosynthesis and attachment to plant roots and abiotic surfaces

Robledo M[1*], Rivera L[1], Jiménez-Zurdo JI[2], Dazzo F[3], Velázquez E[1], Martínez-Molina E[1], Hirsch AM[4], Mateos PF[1]

*[1]Centro Hispano Luso de Investigaciones Agrarias, Universidad de Salamanca, Spain; [2]Estación Experimental del Zaidín, CSIC, Apartado 419, 18080 Granada, Spain; [3]Department of Microbiology and Molecular Genetics, Michigan State University, USA; [4]Department of Molecular, Cell and Developmental Biology, University of California-Los Angeles, USA. *martarg@usal.es*

Summary

The *celC* gene encodes an endoglucanase required for canonical infection in *Rhizobium leguminosarum* bv. *trifolii*. This protein is a product of the *celABC* operon, predicted to be involved in bacterial cellulose biosynthesis. CelC has a high substrate specificity for carboxymethyl cellulose (CMC) and does not degrade other polysaccharides. *The celC*$^-$ and *celC*$^+$ mutants, lacking and overproducing cellulase CelC of *R. leguminosarum* bv. *trifolii* strain ANU843, produced greatly increased and reduced amounts of cellulose, respectively, as revealed by Congo red staining. They also developed a different colony morphology and thickness compared to the wild-type strain. For example, cellulose microfibrils were considerably longer as visualized by Calcofluor-staining *celC*$^-$ bacteria. The *celC*$^-$ phenotype correlated with a significant increase in culture flocculation, whereas fibrils and flocculation were undetectable in *celC*$^+$ cells. Because these strains constitute an excellent model system to clarify the role of cellulose synthesis in aggregation and attachment in *Rhizobium*, we analyzed their ability to produce biofilms on plant roots and abiotic surfaces. Our results indicate that CelC plays a role in cellulose biosynthesis by modulating the length of the cellulose fibrils that mediates attachment among *Rhizobium* bacteria and that cellulose-deficient mutants as well as cellulose overproducers exhibit reduced biofilm phenotypes *in vivo* and *in vitro*.

Introduction

Among the eubacteria that synthesize cellulose (*Agrobacterium, Rhizobium, Gluconacetobacter, E. coli, Salmonella*, and others), we can find orthologs to the *celC* endoglucanase-coding gene located near putative cellulose synthase genes, *celABC*, in a region of the chromosome, which is involved in bacterial cellulose biosynthesis (Wong *et al.*, 1990; Matthysse *et al.,* 1995b; Ausmees *et al.*, 1999). The postulated involvement of the *Agrobacterium* CelC cellulase enzyme in bacterial cellulose biosynthesis is to transfer new oligomer primers for chain elongation (Matthysse *et al.*, 1995a), but this biochemical function has not been definitively established.

Our research group detected *Rhizobium*-produced cellulose microfibrils that are anchored to clover (*Trifolium repens*) roots (Mateos *et al.,* 1995), but these structures are not essential for nodulation (Ausmees *et al.*, 1999). However, we found that *Rhizobium leguminosarum* bv. *trifolii* strain ANU843 CelC endoglucanase activity eroded the noncrystalline tip of the clover root hair wall, and furthermore, that it was involved in both primary and secondary infection of clover (Robledo *et al.*, 2008). During the course of our research on the CelC protein, we obtained a knockout mutant (*celC*$^-$) and a *celC* overproducing strain (*celC*$^+$). Curiously, we observed that cellulose microfibrils produced by the *celC*$^+$ overexpression mutant were undetectable by Calcofluor staining, and that the microfibrils of the *celC*$^-$ deletion mutant were significantly longer than those seen in wild type, suggesting that *R. leguminosarum* bv. *trifolii* cellulose is cleaved by CelC.

Cellulose is likely to play an important role in both rhizosphere colonization and attachment to surfaces in bulk soil. However, the role of cellulose in *Rhizobium* biofilm formation has not been fully characterized. We gfp-tagged our *celC*$^-$ and *celC*$^+$ mutant strains to analyze attachment to clover roots and abiotic surfaces such as plastic microtiter plates,

tabs and sand. We found that both deletion and overexpression of the *celC* gene reduced the ability of *R. leguminosarum* bv. *trifolii* to develop a mature biofilm.

Materials and Methods

Cellulose production was qualitatively assayed by Congo Red and Calcofluor staining and quantified by acid-alkali extraction followed by X-ray diffraction analysis using commercial cellulose (Avicel) as a template to confirm the presence of cellulose.

In vitro biofilms were established on microtiter plates, tabs and sand as described earlier (Fujishige *et al.,* 2006a). Root biofilms were prepared as in (Fujishige *et al.,* 2006b) and analyzed by confocal laser scanning microscopy using green-fluorescent-protein-labeled bacteria. Root and sand-attached cells were quantified by counting cfus.

Results and Discussion

Once we observed that Congo red staining of *celC*$^-$ and *celC*$^+$ mutants detected greatly increased or reduced amounts of cellulose, respectively, compared to the wild type, we performed Calcofluor staining to observe the bacterial cellulose microfibrils. Staining showed that the microfibrils in the *celC*$^-$ mutant were longer than those of the wild-type and that they were absent in the *celC*$^+$ mutant. Although the theoretical possibility existed that the altered characteristics of the *celC*$^-$ mutant were due to the formation of a new polysacharide, X-ray diffraction analysis of polysaccharides present in bacterial cultures from the *celC*$^-$ mutant demonstrated that the pattern of the curve obtained using avicel as control followed the one observed in the wild-type strain. Therefore, the increase in flocculation, Congo red staining, and estimated microfibril length observed in the *celC*$^-$ mutant is most likely due to the overproduction of cellulose microfibrils.

In this work, we analyzed biofilm formation by these cellulose mutants because such mutants have often been shown to be impaired in development of this structures in other bacterial species. In an examination of the *R. leguminosarum celC*$^+$ strain by the microtiter plate assay method, we found that biofilm formation was reduced compared to the wild-type strain in a number of different media. The most striking change was found between the *celC*$^-$ and wild-type because prior to washing, the biofilms of the cellulose-overproducing mutant appeared to be robust and compact. However, *celC*$^-$ cells were easily removed with each successive washing step, whereas almost all of the wild type cells remained attached. This suggests that the biofilm does not develop normally if cellulose microfibrils are not cleaved into shorter lengths by *celC.* Sand and plant attachment assays of gfp-tagged bacterial cultures confirmed the role of cellulase in modulation of the biofilm structure by cellulose. We propose that cellulase CelC is associated with cellulose cleavage and processing and that cellulose is essential for mature biofilm formation in *R. leguminosarum* bv. *trifolii.*

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Protein N-glycosylation patterns are altered during development of legume symbiotic nodules under boron deficiency

Reguera M, Villarejo A, Abreu A, Bonilla I, Bolaños L*

*Departamento de Biología, Facultad de Ciencias, Universidad Autónoma de Madrid, Darwin 2, 28049 Madrid, Spain. *luis.bolarios@uam.es*

Summary

The symbiotic interaction between legume and rhizobia is largely dependent on plant glycoproteins able to interact with borate ions. We have used biochemical and immunological techniques to investigate the effects of boron (B)-deficiency on N-linked glycoproteins during pea (*Pisum sativum*) nodule development. Binding to concanavalin A lectin revealed that proteins with a high mannose type N-glycan, typical of the endoplasmic reticulum, accumulated in nodules developed under low B. Moreover, immunoblot analysis with antibodies against xylose and fucose sugar residues, which are added in the Golgi apparatus, also revealed a high accumulation of proteins with complex type N-glycans as well as a very different glycosylation pattern in B-deficient nodules. Specific antibodies allowed further identification of particular glycoproteins involved in nodule organogenesis and symbiosome development which were either undetectable or identified as abnormally glycosylated forms in low B nodules, leading to development of tumour-like nodules containing undifferentiated bacteroids. The overall results suggest that B is involved in the protein N-glycosylation pathways that take place in the endomembrane system and/or the secretory sytem itself during nodule organogenesis.

Introduction

The legume root symbiotic nodule encloses an interesting process of organogenesis highly regulated by molecular plant–bacteria interactions and B deficiency has a strong effect on legume–rhizobia symbioses affecting rhizobia-legume cell-surface interactions and cell-to-cell signalling during symbiotic events (Bonilla & Bolaños, 2004) and nodule organogenesis (Reguera *et al.,* 2009). The capacity of borate to interact with *cis*-diols in sugars and other biomolecules is considered the basis of any function of B (Bolaños *et al.,* 2004a). Many of such plant-microbe interactions are driven by several plant and bacterial glycoconjugates (Brewin, 2004) that are potential candidates to react with B (Reguera *et al.,* 2010). The glycosylation mechanisms in the host plant cell must function very accurately for a correct symbiosis, and mutations affecting protein glycosylation leads to non-functional, abnormally developed nodules (Sherrier *et al.,* 1997). Therefore, investigating plant-glycoproteins developmentally regulated during nodule development is important for understanding the symbiotic interaction and should assist in elucidating specific roles of B in plant and nodule development.

Materials and Methods

Pea (*Pisum sativum* cv. Lincoln) inoculated with *Rhizobium leguminosarum* bv. *viciae* strain 3841 were grown in B-sufficient or B-deficient conditions (Reguera *et al.,* 2010). Nodules were harvested, homogenized, and fractionated, or sectionated for immunogold staining and microscopy (Rae *et al.,* 1991). Nodule fractions were processed and proteins resolved by SDS-PAGE, electroblotted, and subjected to concanavalin A (ConA) lectin specific affiniblot for detection of proteins with high mannose type N-glycans. Blots were also probed with specific antibodies to detect complex type N-glycans (anti-fucose or anti-xylose antigens) (AgriSera, Sweden), nodule membrane glycoproteins sharing antigenicity with rhamnogalacturonan II, or nodule lectin-like PsNLEC-1 isoforms secreted to the symbiosome compartment.

Results and Discussion

Pea nodule organogenesis proceeds abnormally in the absence of B (Figure): nodule tissue, host cell or bacteroid development failed under B deficiency. But, why is cell differentiation highly sensitive to B deficiency? Several studies on B in plant and animal development point to a role of B in the stability of secreted and/or in membrane glycoproteins involved in cell signalling (Bonilla *et al.,* 2009). The analysis of nodule extracts by ConA specific affinoblots to detect proteins harbouring high mannose type N-glycans, typical for the endoplasmic reticulum or immunodetection of complex type N-glycans with anti-fucose or anti-xylose demonstrated a very different glycosylation pattern and accumulation of several proteins decorated with those glycans in B-deficient nodules. Particular glycoproteins expressed during differentiation of invaded cells and during symbiosome maturation (Redondo-Nieto *et al.,* 2007) were never detected in B-deficient nodules. Furthermore, two isoforms of the lectin-like PsNLEC1 that mediates peribacteroid membrane-lipopolysaccharide interactions important for bacteroid differentiation (Bolaños *et al.,* 2004a), were abnormally glycosylated in B-deficient nodules and not secreted to the symbiosome compartment.

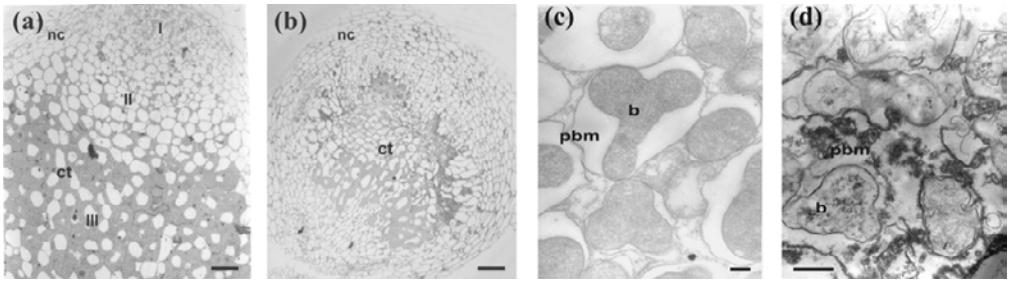

Figure. Toluidine blue-stained semithin sections (**a**, **b**) and transmission electron micrographs of infected cells (**c**, **d**) of pea +B (**a, c**) or –B (**b**, **d**) nodules harvested three weeks post-inoculation, and showing abnormal development of nodule tissues and symbiosomes. I, nodule meristem; II, infection zone; III, maturation nitrogen-fixing zone. ct, Central tissues; nc, nodule cortex; pbm, peribacteroid membrane; b, bacteroid. Bars: 0.1 mm (**a**, **b**); 0.5 μm (**c**, **d**).

Borate deprivation is a major constraint of nodule, plant and animal organogenesis. The lack of such a small molecule affects every step of the rhizobia-legume interaction suggesting either a multiplicity of B functions or a role in crucial processes during development. Since protein N-glycosylation is one of the most important post-translational modifications in eukaryotes and is involved in processes such as cell signalling, our overall results provide evidence that plant or/and bacteria cell-to-cell communication mechanisms depend on B.

Acknowledgments

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Rhizobia-bean interaction under different environments in the northwest of Spain

Rodiño AP*, Fuente M, Santalla M, Castro A, De Ron AM

*Plant Genetic Resources Department, Misión Biológica de Galicia (MBG-CSIC), Apartado 28, 36080 Pontevedra, Spain. *aprodino@mbg.cesga.es*

Summary

The low soil fertility can have a negative impact on the legume-rhizobia symbiotic relationship reducing the ability of rhizobia to form nodules with optimal N_2-fixing capacity. The SNF provides an ecologically acceptable alternative to high applications of nitrogenous fertilizers. It seems that there is an important genotypic variability associated with SNF potential and amount of N_2 fixing that emphasizes the need to explore the potential of indigenous rhizobial strains for improving the symbiotic performance of common bean (*Phaseolus vulgaris*). The objective of this work was to study the effect of the soil environment on the ability of rhizobia to form nodules and the need for rhizobial inoculation when introducing common bean into different soil environments. There are differences in the nodulation-bean interaction in different environments that could be exploited in breeding programs for enhanced nodulation and N_2 fixation in different bean market class.

Introduction

Common bean is a relevant source of protein in human diet for centuries, where animal protein is very expensive and sometimes unsafe or unhealthy. In many parts of the world, satisfying the food needs of growing population has resulted in the adoption of inadequate agricultural practices, forcing agriculture onto more marginal land where nutrient availability may be jeopardised by soil chemical and physical conditions. Reduced soil fertility occurs as a consequence of poor soil management, and nitrogen is often the nutrient most limiting to crop growth, where the situation is frequently aggravated by the chemical fertilizers, which have indirect cost, related by the consumption of non-renewable energy necessary for ammonia synthesis, as well as environmental implications, including the contamination of surface and ground water by excess nitrate and increased production of nitrogen-oxide greenhouse gases. Symbiotic nitrogen fixation (SNF) is important for the sustainability of agriculture and still provides more nitrogen to the agricultural ecosystems worldwide than the total amount of N fertiliser applied and constitutes an ecological alternative to the high application of N fertilisers, particularly in Europe, and an economic alternative to the limited access of developing countries to N fertilisers (Egamberdiyeva, 2007). The objective of this work was to study the effect of the soil environment on the ability of rhizobia to form nodules and the need for rhizobial inoculation into different soil environments.

Material and Methods

Soils samples were collected in the three field trials in 2004-2005 that were chosen to represent prevalent agrosystems in the bean growing areas in North of Spain (ENV1, ENV2 and ENV4). Chemical properties of air-dried soil samples were determined using standard procedures. The experience was conducted in acid soils derived from granites or schist with loamy sand texture. Thirty bean accessions from the Iberian Peninsula were chosen for the field trials and the experimental design was a randomized complete block with two replications. Symbiotic parameters, nodule dry weight (NDW), shoot dry weight (SDW) and yield were measured on each plant individually. The analyses of variance and regressions were performed using the general linear model procedure of the Statistical Analysis System package.

Results and Discussion

Results from this study (Table) show that soils from ENV1 and ENV4 present high levels of exchangeable aluminium as well as of available phosphorus, probably due to repeated yearly

applications of pig manures, whereas soil from ENV2, had been limed in the past with magnesium limestone. The availability of P was found to be significantly correlated with population size of rhizobia. Considerable efforts over years have been directed toward selecting the optimal combination of rhizobial inoculum and the legume genotype for acidic soils were high H^+, Al or Mn may limit the effectiveness of some rhizobia-host combinations (Wakelin *et al.,* 2007). Significant differences among the different environments were noted for all agronomic and symbiotic characters evaluated. Figure shows the correlation between yield and nodulation for the different environments where a large variation was observed. In the ENV1 there were more efficiency in the interaction bacterial strain-common bean accession, and PHA-0019, PHA-0118, PHA-0418, PHA-0519, and PMB-0222 could be distinguished for their nodulation and yield. In the other environments there were also accessions with as high yield and nodulation such as PHA-0019, PHA-0418, and PMB-0222. Many common-bean accessions showed deficient SNF in agreement with the conclusion that common bean was originally domesticated as a home-garden crop, thus receiving organic residues resulting in deficient selection pressure on the symbiosis with rhizobia. Obtaining sufficient knowledge on the characteristics of indigenous rhizobia populations in Spanish soils will be valuable for developing strategies to improve SNF and thus increase bean yields at low cost (Abaidoo *et al.,* 2007). The results from the present study reinforce the importance of conducting trials in multiple environments, to provide strong support for recommending the use of inoculants.

Table. Average values of selected soil chemical properties from the different environments.

	pH (H_2O)	pH KCl	O.M. (%) [a]	Ca^{2+}	Mg^{2+}	Na^+	K^+	Al^{3+}	ECEC [c]	P [d] mgkg^{-1}
				(cmol(+) kg^{-1}) [b]						
Env1	5.08 (0.271)	4.33 (0.294)	5.58 (1.021)	5.57 (1.454)	0.43 (0.121)	0.11 (0.008)	0.83 (0.090)	1.18 (0.513)	8.12 (1.208)	141.0 (27.71)
Env2	5.96 (0.182)	5.28 (0.130)	7.7 (0.354)	15.08 (1.973)	1.52 (0.291)	0.20 (0.037)	0.44 (0.154)	0.18 (0.053)	17.42 (2.112)	29.4 (10.04)
Env4	5.00 (0.632)	4.12 (0.223)	4.42 (1.272)	2.82 (1.191)	0.35 (0.152)	0.10 (0.020)	0.74 (0.238)	1.62 (0.477)	5.63 (1.505)	158.8 (21.02)

[a]Organic matter by weight loss-on ignition. [b]Extracted with 1 M NH_4Cl. [c]Effective Cation Exchange Capacity. [d] Phosphorus availability by Olsen's method.

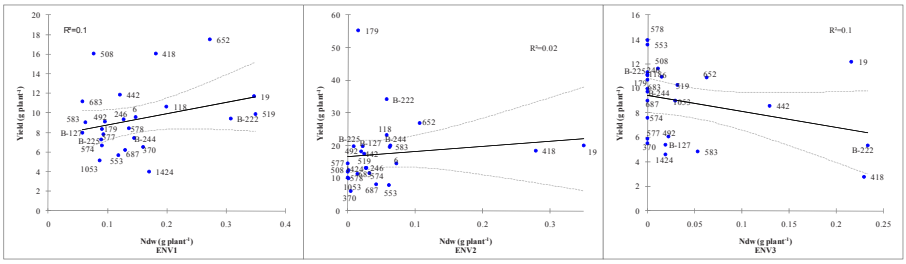

Figure. Relation between yield (g plant^{-1}) and NDW (mg dry weight of nodules plant^{-1}) of the accessions in the field trials.

Acknowledgments

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Session 4

Physiology and metabolism

Nitric oxide and lipopolysaccharides in *Rhizobium*-legume symbiosis

Uchiumi T[1*], Murakami E[1], Nagata M[1], Takayama H[1], Shigeoka M[2], Sahara M[2], Uchida M[2], Nagano Y[3], Muto S[3, 4], Sato S[5], Shimoda Y[5], Kucho K[1], Higashi S[2], Abe M[1]

*[1]Graduate School of Science and Engineering, Kagoshima University, Kagoshima 890-0065, Japan; [2]Department of Chemistry and Bioscience, Kagoshima University, Kagoshima 890-0065, Japan; [3]Analytical Research Centre of Experimental Science, Saga University, Saga 840-8502, Japan; [4]Graduate School of Agriculture, Saga University, Saga 840-8502, Japan; [5]Kazusa DNA Research Institute, Chiba 292-0818, Japan. *uttan@sci.kagoshima-u.ac.jp*

Summary

Crude lipopolysaccharides (LPS) of *Mesorhizobium loti* induced nitric oxide (NO) production in the roots of its host plant *Lotus japonicus*. LPS of *M. loti* was purified and divided chemically or enzymatically to identify the component which is responsible for NO production. Lipid A and lipo-oligosaccharides showed higher activity of NO production compared with that of other fractions including polysaccharides and oligosaccharides. Four candidates of Lipopolysaccharide Binding Protein (LBP), *LjLBP1*, *LjLBP2*, *LjLBP3* and *LjLBP4*, were identified on the genome of *L. japonicus*. RNA interference (RNAi) transgenic hairy roots were generated. Although the inoculation of *M. loti* induced nodule formation on these transgenic roots, *M. loti* was not able to establish symbiosis on the RNAi roots of *LjLBP1* and *LjLBP 2*. After penetration into the host plant roots, LBP might be essential for establishing the symbiosis. Further investigation is required to understand whether LBP is responsible for NO production by LPS.

Introduction

Nitric oxide (NO), an inducer of plant defense system, is produced transiently in the roots of leguminous plants when their symbiotic rhizobia are inoculated (Nagata *et al.,* 2008). However, the plant defense system is not induced or is suppressed by an unknown mechanism. Even after symbiosis is established, NO is produced inside the nodules by an unknown mechanism. Plants respond to LPS in the same way as to flagellin, elongation factor and other molecules of bacterial PAMPs (pathogen associated molecular patterns). The recognition of LPS by host plants must function in *Rhizobium*-legume symbiosis, considering that many of the LPS mutants are defective in symbiosis (Ferguson *et al.,* 2005). However, the plant molecules involved in LPS recognition have not been identified yet. To understand LPS-recognition system of plants, we tried to identify the component responsible for NO production and the plant protein associated with bacterial LPS. We identified four candidate genes for LBP of *L. japonicus* and showed that these LBP genes will be essential for symbiotic nitrogen fixation.

Materials and Methods

Lotus japonics MG20 Miyakojima was used as a host plant throughout experiments. *Mesorhizobiium loti* MAFF303099 was used as a microsymbiont of *L. japonicus*. *Escherichia coli* DH5α was used for the cloning host and *Agrobacterium rhizogenes* LBA1334 was used for generation of transgenic hairy roots of *L. japonicus*. Plasmids pUB-GW-GFP and pUB-GWS-GFP (http://resourcedb.nbrp.jp/resource/list.jsp) were used for overexpress- and RNAi-constructs of *LjLBP*s, respectively. NO was observed by fluorescent microscope using NO specific fluorescent dyes DAF-FM DA (4-amino-5-methylamino-2',7'-difluorofluorescein diacetate) and DAR-4M AM (Sekisui Medical, http://www.sekisuimedical.jp/english) (Nagata *et al.,* 2008., Shimoda *et al.,* 2009.). LPS of *M. loti* was isolated by hot water-phenol method and purified by column chromatography. Gene expression levels were estimated by quantitative reverse transcription-PCR with appropriate gene-specific primers. Nitrogen fixation activity was measured as acetylene reduction activity.

Results and Discussion

LPS covering the cell surface of gram-negative bacteria have been shown to activate innate immune responses in both plants and animals. For instance, LPS from various bacterial strains induce NO production in cultured *Arabidopsis thaliana* cells. We focused on the LPS of *M. loti* as a potential inducer of NO production and expression of *LjHb1*, a class 1 hemoglobin gene, in *L. japonicus*. When *L. japonicus* plants (14 days after germination) were treated with crude LPS of *M. loti* MAFF303099, we observed a transient expression of *LjHb1* and a distinct DAF-fluorescence in the roots, and this response pattern was similar to that elicited after inoculation with rhizobia (Nagata *et al.,* 2009). These results suggest that the LPS of *M. loti* is a genuine candidate as inducer of NO production in *L. japonicus*.

In mammals, LPS signaling is well understood and LBP has been identified as a molecule that directly binds to bacterial LPS (Chaby, 2002). However, the LPS signaling system is unknown in plants. Our first aim was to identify a gene encoding LBP in the *L. japonicus* genome. Four genes were found as LBP candidates of *L. japonicus* and were designated as *LjLBP1*, *2*, *3*, and *4*, respectively. The expression of *LjLBP3/4* was up-regulated in response to *M. loti*, whereas expression of *LjLBP1* and *LjLBP2* was stable in all tissues examined. The phylogenetic tree, based on the predicted amino acid sequences of known LBPs, shows that *LjLBP1*, *3*, and *4*, are located on the same branch and separately from *LjLBP2* (Figure). Recombinant proteins of the N-terminal barrel of LjLBP1 and LjLBP2, which would be responsible for LPS binding, were produced in *E. coli*. Both recombinant proteins were purified in the same fraction of LPS of *E. coli*, suggesting that all LjLBPs are able to bind to LPS. To understand the function of LjLBP, transgenic hairy roots bearing RNAi or overexpression constructs of *LjLBP*s were generated and inoculated with *M. loti*. Although *M. loti* induced nodules on the RNAi hairy roots, the nodules were pale and ineffective with biolysis. Overexpression had no significant effect, except for the increased number of bacteroids inside the symbiosomes. These observations suggest that LPS signaling of the host plant will be essential for establishing symbiosis, especially after microsymbiont invasion.

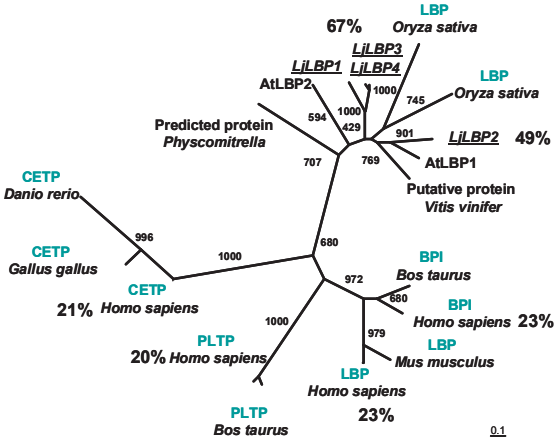

Figure. Phylogenetic tree of the LBP/BPI/CEPT family based on the predicted amino acid sequence. LBP, lipopolysaccharide binding protein; BPI, bactericidal permeability increasing protein; CETP, cholesterol ester transfer protein; PLTP, phospholipid transfer protein. %, amino acid sequence identity *vs* LjLBP1.

Acknowledgments

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Host genes involved in nodulation preference in the common bean-*Rhizobium etli* symbiosis

Aguilar OM[1*], Beker MP[1], Battaglia ME[1], Zanetti ME[1], Blanco FA[1], Nicoletti K[1], Soria-Diaz ME[2], Gil-Serrano A[2], Megías M[3]

*[1]Instituto de Biotecnología y Biología Molecular, CONICET-La Plata, Facultad Ciencias Exactas, Universidad Nacional de La Plata, 1900-La Plata, Argentina; [2]Departamento de Química Orgánica, Facultad de Química, Universidad de Sevilla, 41071 Sevilla, Spain; [3]Departamento de Microbiología y Parasitología, Facultad de Farmacia, Universidad de Sevilla, 41071 Sevilla, Spain. *aguilar@biol.unlp.edu.ar*

Summary

Common beans (*Phaseolus vulgaris*) and rhizobia coevolved in the two main centres of host domestication. In order to characterize the symbiosis, the physiology of nodulation was examined in combinations of host x lineages of *Rhizobium etli*. Analysis of nodulation factors produced by strains of *R. etli* from both centres of diversification and competitiveness by *nol*L mutant, suggested that factors other than Nod factors (NFs) might be involved in strain preference. A host gene coding for a subunit of the NF-Y transcriptional factor was identified in the Mesomerican beans and found to be activated in response to inoculation with its cognate rhizobia

The common bean-rhizobia symbiosis in the Americas

Common beans are believed to have been domesticated in the Americas with two centres of diversification, one of them in Meso-America and the other in the Southern Andes dispersed in Ecuador, Perú, Bolivia and Argentina. Beans are used for direct human nutrition all over the world particularly, in Latin American and African countries. We have demonstrated nodulation preference between beans and lineages of *R. etli* from the same host region. Our results invited us to speculate that the major genetic pools of beans and rhizobia coevolved independently after geographic separation (Aguilar *et al*., 2004).

By applying a suppressive subtractive hybridization approach in which cDNA from a Mesoamerican cultivar inoculated either with the more or the less efficient strain was used as driver and tester, a set of genes were identified as being involved in the early preferential symbiotic interaction (Peltzer-Meschini *et al*., 2008). The role in nodulation, of one of these genes -coding for a monomeric GTPase with similarities to RabA2a- has been studied in bean composite plants by using a reverse genetic approach. Examination of root morphology in PvRabA2a RNAi plants revealed that the number and length of the root hairs were severely reduced. Upon inoculation with *R. etli*, nodulation was completely impaired and induction of early nodulation genes (ENODs) was undetectable in silenced hairy roots (Blanco *et al*., 2009).

Early response to different lineages of *R. etli*

We have further characterized the interaction between beans x lineage of *R. etli*. Inoculated roots showed earlier response to infection with cognate strains as compared to alopatric strains. These responses include root hair curling, formation of infection threads and number of nodule per plant. In order to gain insight on the differential response, the NF produced by strains from the Mesoamerican center of diversification and from the Andean center of diversification was analyzed. The molecule signal of one out of three Andean *R. etli* strains we examined showed a chitolipooligosaccharidic molecule that is devoid of the acetyl residue on the fucosyl moiety at the reducing end of the molecule. The structure of the other Andean strains as well as the Mesoamerican strains were found to be similar each other. We assessed the competitiveness for nodule occupancy of Mesoamerican beans, by an *R. etli nol*L mutant derived of the Mesoamerican strain CE3, which fails to acetylate the fucosyl residue. Results

indicated that, although the *nol*L mutation affects negatively the competitiveness in co-inoculations with any of the two Mesoamerican *R. etli* wild-type strains that we assayed (CE3 and SC15), it does not affect the large nodule occupancy in co-inoculation experiments with the alopatric strain *R. etli* 55N1 from the Southern Andean region. These results suggest that factors other than NFs could play a role in determining affinity for nodulation by certain lineages of *R. etli*. Within this frame, we have performed competition experiments in beans from both centers of host diversification by using a combination of strains that included *Rhizobium tropici* CIAT899, a genotype other than *R. etli*. Nodules of the Mesoamerican cultivar occupied by strains CIAT899 and *R. etli* 55N1 were found to be comparable to each other, whereas in the Andean bean cultivar Alubia, occupancy by the sympatric strain 55N1 clearly overcomes strain CIAT899. This result indicated that competitiveness is expressed in the host x strain combination of the same centre of diversification.

Identification of host genes involved in the efficient nodulation by specific *R. etli* strains

A host gene was identified that is differently expressed in the Mesoamerican cultivar inoculated with its cognate strains. PvNF-YC, identified in a survey of genes that are differential expressed in the Mesoamerican cultivar with its cognate strains, encodes the C subunit of the nuclear factor Y (NF-Y) heterotrimeric transcription factor (Peltzer Meschini *et al*., 2008). RNA interference (RNAi)-mediated gene silencing of this gene in bean hairy roots led to a reduced nodulation phenotype and defects in initiation/progression of infection threads. Interestingly, expression of early nodulin genes such as RIP, ERN and ENOD40 was not affected in these roots, indicating that PvNF-YC might act independently or downstream of these early nodulins. By contrast, induction of G2/S transition cell cycle genes in response to rizobia was impaired in PvNF-YC RNAi roots, suggesting that this gene might be required for first cortical cell divisions that lead to the formation of initial primordia in determined nodules. In order to identify proteins that can physically interact with this transcription factor, a yeast two hybrid (Y2H) screening was performed using PvNF-YC as bait and a cDNA library from root tissue inoculated with *R. etli*. A total of eight clones that potentially interacts with NF-YC were isolated and sequenced. Among them, two encode receptor-like kinases and another clone encodes a putative transcriptional regulator. Interaction of PvNF-YC with these gene products was verified by retransformation of yeast and is being validated by co-immunoprecipitation assays and bimolecular fluorescent complementation in *Agrobacterium*-infiltrated *Nicotiana benthamiana* leaves. The function of these PvNF-YC interacting proteins in nodulation efficiency and bacterial infection is being evaluated by both RNAi and overexpression. This will help to elucidate the signal transduction pathway specifically activated in Mesoamerican beans in response to its cognate *R. etli* strain.

Acknowledgments

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Differential roles of HypC and HupF proteins for hydrogenase synthesis in *Rhizobium leguminosarum*

Albareda M[1ψ], Manyani H[2ψ], Brito B[1], Böck A[3], Imperial J[1,4], Ruiz-Argüeso T[1], Palacios JM[1*]

*[1]Departamento de Biotecnología, Escuela Técnica Superior de Ingenieros Agrónomos, and Centro de Biotecnología y Genómica de Plantas (CBGP), Universidad Politécnica de Madrid, Campus de Montegancedo - 28223 Pozuelo de Alarcón, Madrid, Spain; [2]ResBioAgro, S.L., Centro de Investigación, Tecnología e Innovación, Universidad de Sevilla, 41012 Sevilla, Spain; [3]University of Munich, Munich, Germany; [4]CSIC, Centro de Biotecnología and Genómica de Plantas (CBGP), Universidad Politécnica de Madrid (UPM), Campus de Montegancedo - 28223 Pozuelo de Alarcón, Madrid, Spain. *jose.palacios@upm.es; [ψ]Both authors contributed equally to this work.*

Summary

Some diazotrophic bacteria induce [NiFe] hydrogenases to recycle the hydrogen evolved by nitrogenase during the nitrogen fixation process. Biosynthesis of *Rhizobium leguminosarum* [FeNi] hydrogenase requires a number of accessory proteins (products of *hup* and *hyp* genes) that mediate the incorporation of Ni and Fe into the active site. Among them, HypC-paralog HupF and HupK are present in bacteria that synthesize hydrogenase in the presence of oxygen. Hydrogenase activity in mutant strains lacking either *hupF* or *hypC* genes was severely reduced, indicating that both proteins are essentials for biosynthesis of hydrogenase. Co-purification of StrepTag labelled variants of HupF and HypC by an affinity chromatography-based approach demonstrated interactions between HupL-HupF and HypC-HupK. Experiments carried out with strains induced for hydrogenase under 3% oxygen tensions indicated that HupF might provide additional stability to HupL under these conditions.

Introduction

The generation of hydrogen (H_2) as a consequence of the nitrogenase activity is a source of inefficiency for the nitrogen fixation process. Some rhizobia synthesize a hydrogen uptake (Hup) system with a [NiFe] hydrogenase that catalyses the oxidation of H_2 evolved during N_2 fixation.

[NiFe] hydrogenase is a membrane-bound enzyme that contains a large subunit (HupL) and a small subunit (HupS) in a αβ configuration. HupL contains the catalytic site ($NiFe(CN)_2CO$ cofactor). HupS contains three Fe-S clusters (two 4Fe-4S and one 3Fe-4S) through which electrons from H_2 are conducted to their primary acceptor. In *R. leguminosarum* bv. *viciae* 18 genes (*hupSLCDEFGHIJKhypABFCDEX)* clustered in the symbiotic plasmid are required for hydrogenase synthesis (Ruiz-Argüeso *et al*., 2001). Analysis of *Escherichia coli* hydrogenase-3 has shown that a HypC-HypD complex carrying the $Fe(CN^-)_2CO$ cofactor intermediate is formed through the concerted action of these two proteins with HypF and HypE. Then HypC, after HypD dissociation, transfers the precursor cofactor to HupL (Blockesh & Böck, 2006).

Two of *R. leguminosarum* genes cited above, *hupF* and *hupK*, are absent in bacteria that synthesize hydrogenase under anaerobic conditions, such as in *E. coli*. Since these two genes are present in other hydrogenase systems expressed in the presence of oxygen, our working hypothesis is that both proteins might participate in a modified pathway adapted to the presence of oxygen. In this work, we have studied the functional roles of both paralog metallochaperones (HupF and HypC) and their interaction with other components of the biosynthetic system.

Materials and Methods

In-frame delections in each of the *hupL*, *hupD*, *hupF*, *hupK* and *hypC* genes were generated in plasmid pALPF1 as described by Manyani *et al*. (2005). This plasmid contains the whole *hup* cluster under the control of the promoter of *fixN* gene allowing microaerobic expression of hydrogenase activity in free-living cells. The resulting plasmids were transferred by conjugation to Hup$^-$ *R. leguminosarum* UPM1155 strain. Hydrogenase activity was induced in cultures grown under continuous bubbling with a gas mixture containing O_2 concentrations of 1 or 3%. Hydrogenase activity was measured using an amperometric method with oxygen as electron acceptor as previously described (Ruiz-Argüeso *et al.,* 1978).

Results and Discussion

Hydrogenase activity of *hypC* and *hupF*-deleted mutant strains was drastically reduced (<10%) compared to the wild type, indicating that both proteins are essential for biosynthesis of hydrogenase. When expressed from a pBBR1MCS-based plasmid, HypC-StrepTag and HupF-StrepTag proteins complemented the Δ*hypC* and Δ*hupF* mutations, respectively, exhibiting levels of hydrogenase activity similar to those associated with wild type pALPF1. A cross complementation was not observed between HypC and HupF proteins. These data suggest that both proteins have differential effect on hydrogenase synthesis.

Co-purification experiments using StrepTag-labelled variants of HypC and HupF and antisera specific for HupL and HupK revealed the existence of direct interactions between HupL-HupF and HypC-HupK. This data are consistent with evidence reported for the hydrogenase system of *Ralstonia eutropha* (Ludwig *et al*., 2009). Furthermore, in our case the latter complex associates to the former one in a HupK-dependent manner, suggesting that the four proteins form a single complex that mediates the transfer of cofactor precursor into HupL.

Since HupF participates in hydrogenase systems induced in the presence of oxygen, we hypothesized that this protein might be involved in the adaptation of the system to higher oxygen tensions. To test this hypothesis, bacterial cultures from wild type strain (pALPF1) and *hypC* and *hupF*-deleted mutants were induced for hydrogenase activity in atmospheres containing either 1 or 3% O_2. Immunoblot analysis with antibodies against *R. leguminosarum* HupL showed that crude extracts from bacterial cultures of both mutant strains grown at 1% O_2 accumulated the unprocessed form of HupL. Analysis of crude extracts of Δ*hupF* mutant strains grown at 3% O_2 showed that unprocessed form of HupL was absent whereas it was present in the Δ*hypC* mutant, that contained normal levels of HupF. These data suggest that HupF might have a protective role of hydrogenase structural subunit HupL against higher oxygen levels.

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Role of polyols in the response of alfalfa to salt stress

Palma FJ*, Iribarne C, Tejera NA, Lluch C

*Departamento de Fisiología Vegetal, Facultad de Ciencias, Universidad de Granada, 18071 Granada, Spain. *fpalma@ugr.es*

Summary

Accumulation of polyols (*myo*-inositol, ononitol and pinitol) in response to salinity stress and its contribution to osmotic adjustment or protection mechanisms were investigated in alfalfa (*Medicago sativa*) root nodules. Changes on plant growth, symbiotic nitrogen fixation and polyols content in nodules were determinated at the vegetative and flowering stages in alfalfa plants under salinity stress (100 and 150 mM NaCl). Plant dry weight and nitrogenase activity were lower in plants subjected to salinity stress; nevertheless, maximum values of this activity were detected for all treatments at the beginning of the flowering stage, declining thereafter. This suggests that plant development could be limited by nitrogen fixation. *Myo*-inositol, ononitol and pinitol increased with salinity. However, polyols showed an opposite tendency with plant age: *myo*-inositol and ononitol decreased and pinitol increased. Our results show that polyols could be involved in the salt tolerance mechanism of alfalfa by means of osmotic adjustment and/or their protection function.

Introducción

Las leguminosas están clasificadas como especies relativamente sensibles a la sal (Lauchli, 1984), y su producción está particularmente afectada por el estrés salino al depender de la fijación simbiótica de nitrógeno (Elsheikh y Wood, 1995). La limitación de la productividad está asociada con un menor crecimiento de la planta y una disminución del desarrollo simbiótico, con la consecuente reducción de la capacidad de fijación de nitrógeno (Delgado *et al.,* 1993). En condiciones de estrés, se desencadenan mecanismos fisiológicos y bioquímicos, como la acumulación de solutos compatibles (Sairam *et al.,* 2006). La principal función de estos compuestos es el ajuste osmótico, aunque también intervienen en mecanismos de protección, manteniendo la integridad de las membranas, estabilizando proteínas y reduciéndo el daño oxidativo (Sairam *et al*., 2006). Los azúcares solubles y sus derivados polialcoholes o polioles son los osmolitos más comunes que se acumulan en plantas en respuesta al bajos potenciales hídricos. El D-pinitol es el principal poliol en las familias *Pinaceae*, *Leguminosae* y *Caryophyllaceae,* asociado con el estrés hídrico (Streeter *et al.,* 2001) y la nodulación de leguminosas (Streeter, 1980). La biosíntesis del pinitol ocurre en dos etapas: a partir de su precursor, el mioinositol, se forma ononitol que finalmente formará pinitol (Chiera *et al.,* 2006). En general, los polioles alcanzan concentraciones elevadas en especies de plantas tolerantes a la sal y a la sequía, y sus propiedades químicas y su naturaleza inerte, los convierte en moléculas ideales para actuar como solutos compatibles o moléculas protectoras de tejidos (McManus *et al.,* 2000).

Materiales y Métodos

Material biológico y condiciones de crecimiento. Las semillas de alfalfa (*M. sativa* cv. Aragón) desinfectadas y germinadas se transfirieron a bandejas de cultivo con solución nutritiva, y se inocularon con *S. meliloti* GR4. En estas condiciones crecieron hasta el día 28 (simbiosis establecida), en ese momento se aplicó el tratamiento salino de NaCl (0, 100 y 150 mM) y se mantuvo hasta el final del tiempo de experimentación. Las plantas fueron cosechadas coincidiendo con cuatro estados fisiológicos del desarrollo de la planta: crecimiento vegetativo (42 días), inicio de floración (56 días), floración (70 días) y fructificación (84 días).

Fijación de nitrógeno. La actividad nitrogenasa se determinó cuantificando el desprendimiento de H_2 en un sistema de flujo abierto (Witty y Minchin, 1998). La actividad nitrogenasa aparente (ANA) fue determinada en una atmósfera de N_2:O_2 (80%:20%) y la actividad nitrogenasa total (ANT) en Ar:O_2, ambas con un flujo total de 0.4 l/min. La tasa de fijación de nitrógeno (TFN) fue calculada como (ANT-ANA)/3.

Mio-inositol, ononitol y pinitol. El material vegetal (0.2 g) se maceró con nitrógeno líquido y 1.5 ml de etanol al 80%. El homogeneizado se centrifugó a 10000*g* durante 15 min a 4ºC, y el sobrenadante se secó al vacío a 40ºC en SpeedVac. Las muestras se resuspendieron en agua mili-Q a 80ºC y los polioles se cuantificaron por cromatografía iónica (Dionex Sunnydale, CA, USA).

Resultados y Discusión

La salinidad provoca una reducción significativa del crecimiento y desarrollo de las plantas de alfafa (Tabla), tal como se ha descrito en otras leguminosas como *Medicago truncatula* (Lopez *et al*., 2008) entre otras. La relación raíz/parte aérea reflejó los cambios inducidos por la salinidad, evidenciándose una mayor sensibilidad de la parte aérea al NaCl, ya que en estas plantas incrementó esta relación. En general, el estrés salino provoca la inhibición de la actividad nitrogenasa y de la tasa de fijación de nitrógeno en nódulos a lo largo de la ontogenia del cultivo (Tabla) coincidiendo con lo descrito en nódulos de *M. truncatula* (Lopez *et al*., 2008). Los datos de la tabla revelan que en nódulos de plantas expuestas al estrés salino se acumula mayor contenido de polioles (mio-inositol, ononitol y pinitol), intensificandose este acumulo con el crecimiento, además el comportamiento de cada uno de ellos es diferente, el mio-inositol y ononitol descienden, el pinitol incrementa y alcanza el máximo en fructificación. Streeter *et al*. (2001) describen la acumulación de pinitol en soja como consecuencia del estrés hídrico, y en transgénicas de tabaco que acumulan ononitol se incrementó la tolerancia a la sequía y a salinidad (Sheveleva *et al*., 1997).

Tabla. Efecto del tratamiento con NaCl en el peso seco de planta (PSP) [expresado en g planta^{-1}], relación raíz/parte aérea (RPA), actividad nitrogenasa aparente (ANA) y actividad nitrogenasa total (ANT) [expresadas en µmol H_2 g^{-1} PSN h^{-1}], tasa de fijación de nitrógeno (TFN) [expresada en µmol N_2 g^{-1} PSN h^{-1}] y contenidos de mio-inositol, ononitol y pinitol [expresados en mg g^{-1} PF nódulo] en nódulos de alfalfa.

Tiempo (días)	NaCl	PSP	RPA	ANA	ANT	TFN	Mio-inositol	Ononitol	Pinitol
	0	0.156^{g}	0.315^{ef}	86^{de}	709^{b}	251^{a}	0.463^{de}	4.61^{c}	5.76^{h}
42	100	0.136^{g}	0.387^{def}	200^{a}	427^{c}	65^{e}	2.925^{a}	6.50^{ab}	8.89^{f}
	150	0.113^{g}	0.534^{de}	104^{cd}	333^{cd}	52^{e}	2.981^{a}	7.22^{a}	8.51^{fg}
	0	0.472^{e}	0.271^{f}	201^{a}	1051^{a}	284^{a}	0.442^{e}	3.92^{cd}	7.28^{g}
56	100	0.317^{f}	0.619^{e}	182^{a}	777^{b}	201^{b}	1.464^{b}	6.50^{ab}	13.75^{d}
	150	0.283^{f}	1.003^{c}	129^{bc}	636^{b}	170^{b}	1.210^{c}	4.52^{c}	20.07
	0	0.983^{c}	0.876^{c}	111^{cd}	475^{c}	121^{c}	0.407^{e}	5.88^{b}	10.62^{e}
70	100	0.761^{d}	0.896^{c}	90^{de}	439^{c}	116^{c}	0.551^{d}	4.56^{c}	16.10^{c}
	150	0.775^{d}	1.256^{b}	65^{ef}	393^{c}	109^{cd}	0.425^{e}	2.74^{e}	21.05^{b}
	0	2.026^{a}	1.078^{bc}	146^{b}	371^{cd}	75^{de}	0.240^{f}	4.06^{cd}	13.80^{d}
84	100	1.562^{b}	1.809^{a}	47^{f}	190^{e}	47^{e}	0.497^{de}	3.63^{d}	21.07^{b}
	150	1.032^{c}	1.260^{b}	52^{f}	235^{de}	60^{e}	0.435^{e}	3.65^{d}	27.21^{a}
MDS ($P \leq 0.05$)		0.103	0.237	30	142	34	0.090	0.73	1.37

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The role of *Bradyrhizobium japonicum nirB* gene in nitric oxide detoxification in soybean nodules

Sánchez C[1*], Cabrera JJ[1], Meakin GE[2], Gates AJ[2], Richardson DJ[2], Bedmar EJ[1], Delgado MJ[1]

*[1]Estación Experimental del Zaidín, CSIC, Apartado 419, 18080 Granada, Spain; [2]Centre of Molecular Strcuture and Biochemistry (CMSB), School of Biological Sciences, University of East Anglia, Norwich, NR4 7TJ, UK. *cristina.sago@gmail.com*

Summary

The identification of nitric oxide (NO) bound to leghemoglobin (LbNO) and free NO within soybean nodules has led to the question of how *Bradyrhizobium japonicum* bacteroids overcome the toxicity of this NO. It has previously been reported in some bacteria that ammonia-generating nitrite reductases that catalyze 6-electron reduction of nitrite to ammonia are also capable to reduce NO to ammonia or nitrous oxide. A gene, *nirB*, encoding a putative ammonia-generating sirohaem nitrite reductase has been identified in the *B. japonicum* USDA110 genome. The involvement of NirB in NO detoxification in soybean nodules was assessed by inoculating plants with a *nirB* deficient mutant. LbNO complexes were analyzed in nodules by using electron paramagnetic resonance and UV-visible spectroscopies. Levels of LbNO complexes and NO in *nirB* nodules were significantly higher than those observed in wild-type nodules. After incubation with a NO scavenger, the increase of NO accumulation in *nirB* nodules compared to wild-type nodules was suppressed. These results suggest that NirB has a role in NO detoxification in soybean nodules.

Introduction

Bradyrhizobium japonicum forms root nodules when is symbiotically associated with soybean. Several authors have demonstrated the presence of NO bound to leghemoglobin (nitrosylleghemoglobin, LbNO) and free NO within soybean nodules (Meakin *et al.,* 2006; Sánchez *et al.,* 2010). NO is a signal molecule involved in diverse physiological processes in plants, but it can become very toxic under certain conditions determined, for example, by its rate of production and diffusion, and the redox state of the cell. In fact, NO has been reported as a potent inhibitor of nitrogenase activity by several authors, and even of nitrogenase genes (Sánchez *et al.,* 2010). Meakin and coworkers (2006) demonstrated that knocking-out nitric oxide reductase (Nor), which catalyses the reduction of NO to nitrous oxide (N_2O) through denitrification, does not affect the level of NO within nodules, leading to the suggestion that other systems may be involved in NO detoxification. In several bacteria, ammonia-generating nitrite reductases, are capable to detoxify NO to NH_4^+ or N_2O, anaerobically (Poock *et al.,* 2002, Mills *et al.,* 2008). The presence of a gene, *nirB*, putatively encoding a sirohaem ammonia-generating nitrite reductase has been identified in the USDA110 genome (http://genome.kazusa.or.jp/rhizobase). In this work, a *nirB* deletion mutant has been obtained, and its capacity to detoxify NO in soybean nodules has been investigated.

Materials and Methods

Plants were inoculated with the wild-type (WT) *B. japonicum* USDA110 strain, and *nirB* 1901 or *norC* GRC131 mutants, and grown in a controlled environmental chamber under controlled conditions. After growth for 15 days, plants were supplemented with 4 mM KNO_3. Nodules were harvested from 34-day-old plants. As previously described Sánchez *et al.* (2010), whole nodule EPR spectroscopy was used to detect LbNO, UV-visible spectroscopy was used to quantify the proportion of Lb bound to NO in nodule extracts, and free NO was analyzed by using the specific fluorescent probe DAF-FM.

Results and Discussion

The EPR LbNO signal from *nirB* nodules was significantly higher than that observed in WT or *norC* nodules (Figure, **A**). Similarly, interpretation of the absorption spectra found that levels of LbNO were about 2 times higher in *nirB* nodules than in WT or *norC* nodules (data

not shown). Fluorometric NO detection showed that NO production by *nirB* nodules was *c.* 2 times higher than that produced by WT or *norC* nodules (Figure, **B**). To make clear that the induction of fluorescence observed in *nirB* nodules was due to NO, nodules were treated with a NO scavenger (c-PTIO) and such induction was clearly suppressed (Figure, **B**), indicating that the fluorescence was representative of NO production. According to Meakin and associates (2006), the EPR spectrum of the *norC* nodules showed a similar spectrum to that of the WT nodules (Figure, **A**), supporting the previous suggestion that Nor is not responsible for NO detoxification within soybean nodules (Meakin *et al*., 2006; Sánchez *et al*., 2010). Since *nirB* nodules accumulated more NO and LbNO complexes than WT nodules, a role for NirB in NO detoxification in soybean nodules might be suggested. Recently, it was proposed that in soybean nodules is Lb and not NirK or Nor bacteroidal enzymes the main system involved in detoxifying nitrite and NO produced by bacteroidal denitrification in response to flooding conditions (Sánchez *et al*., 2010). Whether NirB contributes to the protection of simbiotic nitrogen fixation against NO need to be addressed.

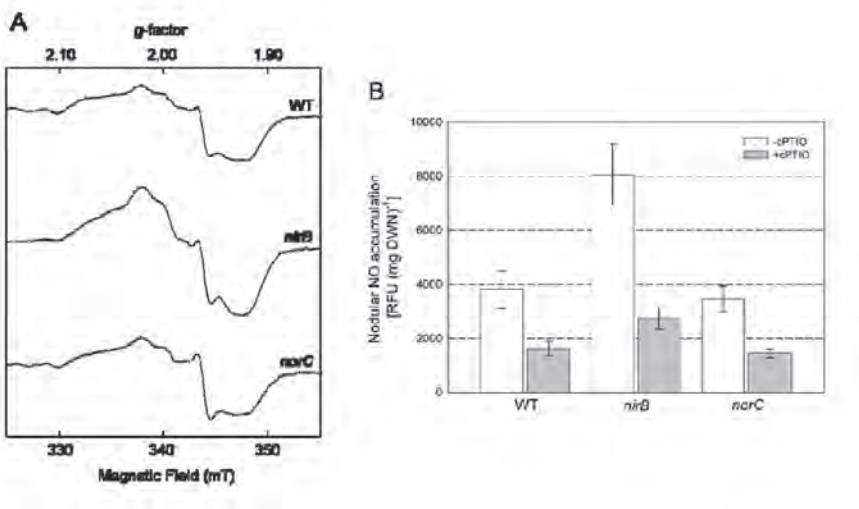

Figure. A. EPR spectra from intact nodules. The y-axis is the same scale on all panels. Spectra are representative of EPR spectra recorded from triplicate nodule samples from two or three different nodule harvests. **B.** NO detection in nodules using DAF-FM and expressed as relative fluorescence units (RFU). Data are means ± SE from three independent experiments assayed by using six replicates. DWN, dry weight nodule.

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Evidence for a differential nitrogen nutrition regulated drought stress response mechanism observed in *Medicago truncatula*

Staudinger CE*, Wienkoop S

Department of Molecular Systems Biology, University of Vienna, Althanstrasse 14, 1090 Vienna.
**christiana.staudinger@univie.ac.at*

Summary

Physiological characterisation studies in the model legume *Medicago truncatula* revealed a differentially regulated stress response to drought strongly related to nitrogen supply. Results will be presented here, indicating a significant stress alleviation in symbiotically N_2-fixing plants compared to N-fertilized plants. Anatomical dissection of symbiotic and non-symbiotic plants revealed higher stomatal density for nodulated plants. Withholding water resulted in a decline of stomatal conductance, which was less pronounced in plants inoculated with *Sinorhizobium meliloti*. In addition, these plants showed a significantly higher photosystem II operating efficiency under severe drought stress than non-symbiotic individuals. After two weeks N fertilized plants exhibited distinct senescence features while symbiotic plants remained vital. To investigate the molecular mechanisms underlying this divergence, temporal and spatial level metabolomic and proteomic studies are under progress.

Introduction

Symbiotic nitrogen fixation in legumes provides considerable advantages for agricultural productivity from an ecological and economical point of view. Water scarcity has important negative effects on this symbiosis, however little is known about the role of nitrogen nutrituion regime in response to abiotic stresses. In *Medicago truncatula*, nitrogen fixation is decreasing fast in response to drought. After 6 days a 70% drop in apparent nitrogenase activity was observed (Larrainzar *et al.,* 2007). However little is known about the regulatory influence of different nitrogen nutrition systems in response to abiotic stresses.

Materials and Methods

Medicago truncatula cv. Jemalong plants were cultivated in 0.8 l pots filled with a 5:2 (v/v) vermiculite:perlite, under controled environmental conditions (14 h photoperiod, photosynthetic photon flux density of 500-600 µmol m^{-2} s^{-1}, 22°C/16°C day/night temperature, 50% relative humidity). Plants were daily watered with Evans nutrient solution containing 0.5mM (first 2 weeks) and 2.5mM ammonium nitrate (subsequent weeks). After 4 weeks of growth, one randomly chosen set of plants was inoculated with *Sinorhizobium meliloti* and provided with nutrient solution free from synthetic N fertilizer. After 7 weeks, one subset of inoculated and N fertilized plants, respectively, was exposed to drought stress, while a second subset served as controls. One day before withheld irrigation and on days 3 and 6 of drought stress, primary chlorophyll fluorescence parameters of light adapted plants (Imaging-PAM chlorophyll fluorometer; Walz GmbH; Effeltrich, Germany) and stomatal conductance were measured (PMR-4 steady state porometer; PP Systems, Inc.; Amesbury, Massachusetts, USA).

Roots and shoots of stressed and control plants were additionally analysed using mass spectrometry (MS). An integrative liquid chromatograohy coupled MS/MS Orbitrap based proteomics approach (MAPA, Höhenwarter *et al.,* 2008) along with metabolite profiling using GC-MS was carried out as described previously (Wienkoop *et al.,* 2010).

Results and Discussion

When exposed to long-term drought stress, N fertilized plants died while symbiotic individuals remained vital. On day six of drought stress, chlorophyll fluorescence light induction curves of symbiotic plants showed significantly higher photosystem II operating efficiencies at photon flux densities higher than 76 µmol m^{-2} s^{-1}. This indicates that light energy absorbed by photosystem II is more efficiently used for acceptor reduction of the

photosynthetic electron transport chain preventing detirmental effects of excitation energy. Comprehensive MS analyses and computational data mining revealed differential proteomic and metabolomic pattern in response to drought stress. These results describe and distinguish specific regulatory mechanisms towards improved stress tolerance caused by different nitrogen nutrition states**.**

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Nitrogen fixation response in soybean to changes in vapor pressure deficit

Arrese-Igor C[1,2*], Sinclair TR[1]

[1]*Agronomy Physiology Laboratory, Department of Agronomy, University of Florida, POB 110965, Gainesville, FL 32611, U.S.A;* [2]*Departamento de Ciencias del Medio Natural, Universidad Pública de Navarra, 31006 Pamplona, Spain. *cesarai@unavarra.es*

Summary
Nodulated soybean plants were subjected to different temperature/air humidity combinations in order to create different vapor pressure deficit (VPD) conditions and monitor their effects on the nitrogen fixation activity of those plants. The results showed a positive linear regression between VPD and nitrogen fixation and suggest a direct involvement of long-distance transport in the regulation of nitrogen fixation.

Introduction

Legumes are essential to improve the soil fertility and quality of agricultural lands, making them crucial for agricultural and environmental sustainability. This ability largely relies on legumes capability of establishing symbiotic relationships with soil bacteria which leads to the initiation of root nodules, where nitrogen fixation (NF) takes place. However, NF in legumes is very sensitive to environmental constraints and drought, in particular. The main metabolic factors that regulate nodule NF are an adequate oxygen balance, the availability of energy-yielding substrates, and the maintenance of an adequate plant N status.

Although the classical view of the inhibition of NF by drought was initially ascribed to a lack of carbohydrate supply to the nodules, it has been shown that BNF is more sensitive than photosynthesis to moderate environmental stresses. Nevertheless, it has been shown recently that a shortage of carbon flux within nodules can occur in response to environmental constraints as a consequence of the down regulation of nodule sucrose synthase activity or the recycling of respiratory CO_2.

As in free-living diazotrophs, it has been suggested that the nitrogen status of the plant may be regulating nodule BNF. The N feedback hypothesis has also been proposed to be involved in the regulation of BNF under different environmental constraints with several candidate N molecules suggested to act as a signal molecule.

These different facts depict a complex network of signals linking the perception of environmental stresses with an eventual decline of NF. However, little evidence is known in relation to long-distance transport in legumes and the response to atmospheric water deficit rather than soil water shortage. The aim of this work has been to elucidate the response of nitrogen fixation in soybean in response to increased water flux in plants as a result of changes in vapor pressure deficit (VPD) around the shoots of plants.

Materials and Methods

Plants of *Glycine max* (L.) Merr. cv. Biloxi were sown into pots made from 0.01 m diameter, 0.03 m tall polyvinyl chloride pipes. The cultivar Biloxi was selected because it has been shown to have steady increase in transpiration rate as VPD is increased (Sinclair *et al.,* 2008). An end cap was glued to the bottom of the pots through which a fitting for introducing gas into the pot was installed for measurements of NF activity. The fitting was left open when NF was not being measured, serving as a drainage hole. The top, open end of the pipes was fitted with toilet flanges to which a lid could be sealed for NF measurements and, at the same time, the VPD chamber can be attached. Each pot was filled with Gardenplus top soil (Lowes Inc., NorthWilkesboro, NC), inoculated with *Bradyrhizobium japonicum* (Nitragin, Brookfield, WI) and supplied with <4 g of osmocote vegetable and bedding slow-release plant fertilizer (14% N–6.1% P–10% K). Plants were raised in a glasshouse of the Agronomy Dept, UF in Gainesville, FL (29º38'N, 82º22'W) and watered every 2–3 days from sowing

until measurements began. The day before measurements, plants were moved into a second glasshouse (800 m away). Each pot was fully watered to dripping. A two-piece methacrylate lid was bolted on to the toilet flange on top of the pot and sealed around the stem of the plant, making the entire pot gas tight and allowing the gas mixture be flowed through the pots. A 0.34 m diameter lid of a food container (Rubbermaid Commercial Products LLC, Winchester, VA) with the centre cut out was attached on top of the methacrylate lid. The following morning the aerial parts of the plants were sealed into a 21 L transparent plastic food container (Rubbermaid) by placing the inverted container over the plant and sealing it into the previously installed lid. These containers extinguished approximately 25% of the solar radiation. The light levels in the greenhouse were approximately 70% of irradiance incident to the greenhouse. Each container was equipped with a 12 V, 0.76 m diameter computer box fan (Northern Tool and Equipment, Burnsville, MN) to mix the air inside the chamber. In addition, a pocket humidity/ temperature pen (Extech Instruments, League City, TX) was mounted through the sidewall of each container. Various humidity levels were achieved by flowing different air sources into these containers via plastic tubing (8 mm outside diameter) to balance the humidifying effect of the transpiring leaves (Fletcher *et al.,* 2007). For each measurement the humidity was set and then the plant and air in the container were allowed to equilibrate for 90 min before NF measurements began. NF was then monitored using a flow-through acetylene reduction assay, flowing a 1:9 volume mixture of acetylene: air through all pots simultaneously at 1 L min^{-1} (Devi *et al.,* 2010). The gas mixture was flowed for 15 min to allow equilibration of the gas mixture in the pot and ethylene production. Three gas samples were collected at the outlet port in the lid using 1 mL syringes. After sample collection, acetylene was removed from the pots. Ethylene concentration in the gas samples was analyzed with a gas chromatograph (Shimadzu GC-9A, Kyoto, Japan). Correlation analysis between VPD and NF was performed using Statview SE + Graphics (Abacus Concepts, Piscataway, NJ).

Results and Discussion

The analysis was performed using 48 measurements, whose VPD ranged from 0.4 to 2.6 kPa. As expected, NF measured by the ARA technique showed quite a large variability among plants, despite a uniform biomass and development. Nevertheless, a linear regression between the two variables resulted in a remarkably positive linear correlation ($r^2 = 0.61$; $p>0.0001$). To our knowledge this is the first report of a link between VPD and NF. Since transpiration rate in Biloxi increases with increased VPD and there is little capacity for water storage in these plants, it is inferred that NF increased in association with increased xylem flow rates. We speculate nodule compounds, including NF products, may be more rapidly removed from the nodules by greater xylem flow under higher VPD, thus resulting in a progressive elimination of N-feedback effects under high VPD. Under optimal environmental circumstances, particularly those dealing with unlimited water supply, and short high-VPD periods, NF will increase. Whether this higher NF rates depends exclusively on the removal of nodule N-compounds and whether the higher xylem flow can also drive a higher phloem flow, in order to support an adequate carbon supply for this enhanced NF over extended time periods, require further investigation.

Acknowledgments

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Systemic regulation of nitrogen fixation by nitrate

De Miguel C, Sanz-Corres X, Gil-Quintana E, Larrainzar E, Arrese-Igor C, González EM

Departamento de Ciencias del Medio Natural, Universidad Pública de Navarra, 31006 Pamplona, Spain.

Summary

Legumes have the ability to acquire nitrogen from the atmospheric N_2, through symbiosis with nitrogen fixing bacteria, but also from inorganic nitrogen forms (nitrate or ammonium). However, the presence of inorganic nitrogen inhibits the nitrogen fixation process. Since nitrogen fixation has the goal of supplying nitrogen for the plant growth, it seems reasonable to think that nitrogen plant status will control symbiotic activity. Thus, different studies demonstrate the regulation of the nitrogen fixation through the nitrogen status of the plant. However, the mechanism responsible for such inhibition is not yet known. The current study aims to ascertain the mechanism involved in the nitrogen fixation inhibition provoked by the presence of mineral nitrogen. By using a split root system in pea plants, a systemic regulation of nitrogen fixation by inorganic nitrogen is clearly shown. In addition, it is shown that this regulation mechanism is completely independent of the nitrate concentration within the nodule tissue.

Introduction

Legumes have the ability to acquire nitrogen from the atmospheric N_2, through symbiosis with nitrogen fixing bacteria, but also from inorganic nitrogen forms (nitrate or ammonium). Although mineral nitrogen is a positive treatment for plants, its presence inhibits the nitrogen fixation process in nodulated legumes (Streeter, 1988). Since nitrogen fixation has the goal of supplying nitrogen for the plant growth, it seems reasonable to think that nitrogen plant status will control symbiotic activity. Thus, different studies demonstrate the regulation of the nitrogen fixation through the nitrogen status of the plant (Streeter, 1988; Parsons *et al.,* 1993; Hartwig, 1998). However, the mechanism responsible for such inhibition is not yet known. The current study aims to ascertain the mechanism involved in the nitrogen fixation inhibition provoked by the presence of mineral nitrogen. By using an split root system in pea plants, the question to be answered is whether such mechanisms work at a local level (nodule, root) or whether a systemic control can be shown. In addition, alterations of the nitrogen metabolite composition within the different parts of the plants will be analysed.

Materials and Methods

Pea plants (*Pisum sativum* L. cv. Sugar Lace) were inoculated with *Rhizobium leguminosarum* biovar. viciae NLV8. Plants grew with an split root system (SRS) for 4 weeks and were watered with N-free nutrient solution (Rigaud & Puppo, 1975). At this time, plants were separated into 3 sets: 1) Control (C) plants: both root sides were maintained as nitrogen fixing plants; 2) Nitrate (N) plants: both root sides were irrigated with nutrient solution containing 5 mM amonium nitrate and 3) Partial Nitrate (NP) plants: one part of the split root was irrigated with N-free nutrient solucion (NP^-) and the other part was irrigated with nutrient solution containing 5 mM ammonium nitrate (NP^+). Apparent nitrogenase activity (Marino *et al.,* 2007) and tissue sampling for analytical determinations were carried out at days 0, 1 and 5 after starting the treatment. Nitrate was determined by ion chromatography in a DX-500 system (Dionex) by gradient separation with a Dionex IonPac AS11.

Results and Discussion

Nitrogenase activity declined dramatically in the N plants (Figure). In the NP plants, nitrogenase activity declined not only in the part of the root treated directly with mineral nitrogen (NP^+) but also in the part of the root irrigated with N-free nutrient solution (NP^-) (Figure). These results back up a previous study presented by Tanaka *et al.* (1985), which showed that nitrogen absorbed by one part of the root move to the other part of the root affecting root development and nitrogen fixation.

Carbon metabolism has been shown to be significantly affected under drought stress and several studies show that carbon metabolism is involved in nitrogen fixation regulation. This carbon regulation is exerted at the level of sucrose synthase activity, which dramatically declines under drought altering the carbon skeleton supply within the nodules (Galvez *et al.,* 2005). However, nitrate inhibition of nitrogen fixation is not related with any carbon metabolism alteration (De Miguel C & Gonzalez EM, unpublished) and thus, the systemic regulation showed previously must be provoked by other kind of mechanism.

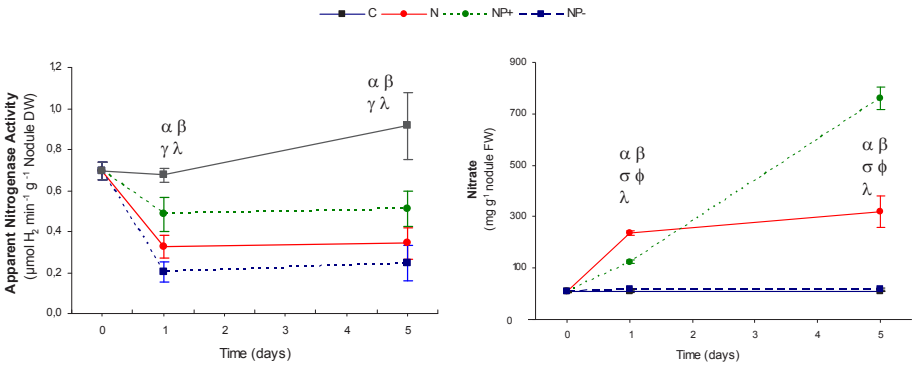

Figure. Apparent nitrogenase activity and nitrate content in nitrogen-fixing, nitrate treated and partially nitrate treated pea plants growing with a split root system. C, Control plants: both root sides were maintained as nitrogen fixing plants; N, Nitrate plants: both root sides were irrigated with nutrient solution containing 5 mM amonium nitrate; NP^+, root side irrigated with nutrient solution containing 5 mM ammonium nitrate of the partial nitrate plants; NP^- root side irrigated with N-free nutrient solution of the partial nitrate plants. Values represent mean ± standard error (n = 3). Significant differences ($p \leq 0.05$) are represented as follow: α (C vs N); β (C vs NP+); γ (C vs NP-); σ (N vs NP+); φ (N vs NP-); λ (NP+ vs NP-).

In this context, the present study examines the alteration of single amino acids, and inorganic nitrogen forms within the different parts of the plant. Nitrate enters only in those nodules directly exposed to exogenous nitrate, whilst nodules of the nitrogen fixing root part keep nitrate content at control values (Figure). In spite of the fact that nitrate did not enter NP^- nodules, nitrogen fixation in this root side declined equally to the NP^+. This result clearly show that nitrate itself does not mediate the nitrogen fixation inhibition caused by inorganic nitrogen. Changes of the single amino acid pattern within the nodules, root and shoot will be discussed in order to better understand the nitrogen fixation regulation exerted by nitrate.

Acknowledgments

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Analysis of sulphur metabolism in *Medicago truncatula* nodules under drought/recovery

Larrainzar E[1*], Wienkoop S[2], Alibert B[3], Limami AM[3], Arrese-Igor C[1], González EM[1]

[1]*Departamento de Ciencias del Medio Natural, Universidad Pública de Navarra, 31006 Pamplona, Spain;* [2]*Department of Molecular Systems Biology, University of Vienna, 1090 Vienna, Austria;* [3]*Physiologie Moléculaire des Semences, University of Angers, 49045 Angers, France.* [*]*estibaliz.larrainzar@unavarra.es*

Summary

Sulphur (S) is essential not only for general plant growth but also for the establishment of an efficient nitrogen-fixing symbiosis. In a previous study nodule S metabolism, particularly plant methionine (Met) synthase, was identified as one of the proteins whose levels declined during drought in *Medicago truncatula*. The objectives of the present work are to analyse the effects of drought and a subsequent recovery treatment on the levels of the main enzymes responsible for the biosynthesis of Met and ethylene in nodules of this model legume, both at the gene expression and protein levels. One of the most relevant results is the detection of a significant down-regulation of one of the isoforms of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase at the transcript level, suggesting a possible link between drought stress and a reduced evolution of ethylene from the root system.

Introduction

Sulphur (S) is one of the six macronutrients necessary for plant growth, particularly required for the biosynthesis of amino acids cysteine (Cys) and methionine (Met), oligopeptides such as glutathione (GSH) and phytochelatins, along with a variety of secondary metabolites. In contrast to animals, plants are able to assimilate S from inorganic forms present in soil. The first step in this assimilation pathway requires the reduction of inorganic sulphate to sulphite and its incorporation into amino acids to form Cys (Figure). Cys is considered the starting point for the biosynthesis of other S-containing compounds such as Met and GSH. The *de novo* synthesis of Met requires the coordinated action of three successive enzymes: cystathionine γ-synthase, cystathionine β-lyase and Met synthase. The major metabolic fates of Met include its incorporation into proteins, adenosylation to form S-adenosylMet (SAM) and methylation to form S-methyl Met. In turn, SAM is precursor of a number of compounds including ethylene.

S metabolism and S-containing compounds play important roles in the establishment and function of a nitrogen-fixing symbiosis. In a previous proteomic study, Met synthase was identified as one of the nodule plant proteins negatively affected by drought stress in *M. truncatula* (Larrainzar *et al.,* 2007). This led us to further investigate the involvement of nodule S metabolism in response to drought stress in this model legume. The objectives of this work are to analyse the changes of gene expression and protein content in absolute terms of the main enzymes involved in the biosynthesis of Met and ethylene in nodules, namely, cystathionine γ-synthase, cystathionine β-lyase, Met synthase, SAM synthetase, 1-aminocyclopropane-1-carboxylic acid (ACC) synthase and oxidase.

Materials and Methods

M. truncatula Jemalong A17 plants were inoculated with *S. meliloti* strain 2011 and grown in symbiotic conditions as described (Larrainzar *et al.*, 2007). Plants were subjected to drought by withholding nutrients/water for 6 days, followed by a two day recuperation period. Nodule and root samples were harvested at day 0, 3, 6 and 2 days after the reestablishment of watering. Quantitative real time PCR analyses were performed on a light cycler ABI Prism 7000 SDS using the SYBR Green PCR Master Mix. Levels of expression of genes in treated plants were calculated relative to those of control plants using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001). Absolute quantification of S metabolism proteins was performed as described (Wienkoop *et al.,* 2008).

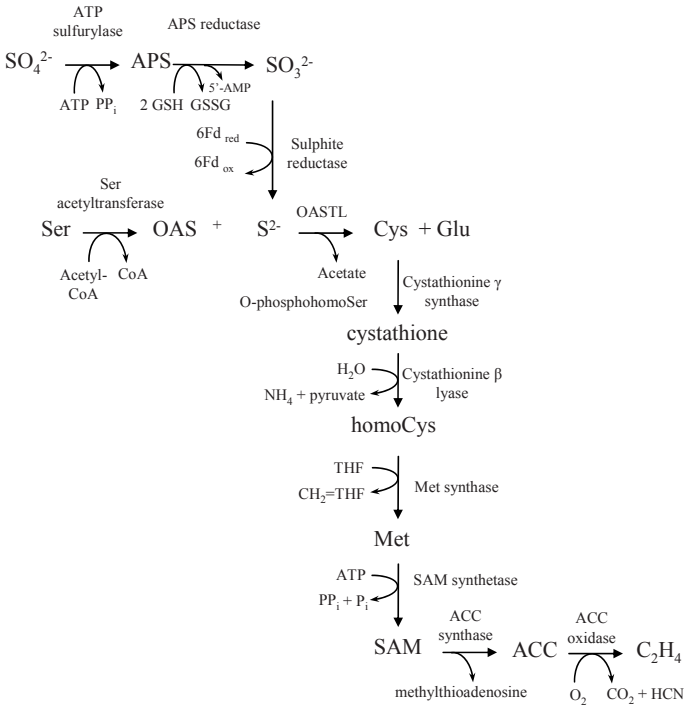

Figure. Scheme of the main pathways involved in S assimilation and ethylene production in plants. Adapted from Hell (1997), Hesse & Hoefgen (2003), and Taiz & Zeiger (2002).

Results and Discussion

S metabolism is known to be affected by abiotic stresses. For instance, in soybean seedlings, root ATP sulphurylase has been shown to be induced by cold treatment (Phartiyal *et al.*, 2006), while tomato plants under salinity stress showed enhanced expression of SAM synthetase both at the transcript and protein levels (Sanchez-Aguayo *et al.*, 2004). In the present work, both the levels of gene expression and the amount of protein in absolute terms of a number of proteins involved in the biosynthesis of Met and ethylene in nodules have been analyzed. In terms of gene expression, one of the most significant changes observed during drought was the down-regulation of one of the isoforms of ACC synthase. These results suggest that the production of ethylene in mature nitrogen-fixing may be altered during drought stress.

Acknowledgments

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Local regulation of nitrogen fixation in drought-stressed soybean plants

Gil-Quintana E, Sanz-Corres X, Larrainzar E, Arrese-Igor C, González EM

Departamento de Ciencias del Medio Natural, Universidad Pública de Navarra, 31006 Pamplona, Spain

Summary

Symbiotic nitrogen fixation (SNF) is very sensitive to a wide range of environmental stresses including drought. Despite the considerable research effort on the subject, neither the mechanisms influencing the regulation of SNF under limited water availability nor the possible origin of the regulatory signal controlling this response are totally understood. The aim of the present work was to determine whether the SNF response to drought is influenced by systemic or local signals in soybean (*Glycine max*) plants. To do so, plants were grown using a split-root system (SRS). Six week-old nodulated soybean plants were randomly separated into three sets. Controls were watered to field capacity at both sides of the SRS, whereas drought was applied by withholding water at both sides. Partial drought plants were irrigated to field capacity at one side of the SRS, whilst water was withheld at the other side. Evapotranspiration (ET), nodule water potential and apparent nitrogenase activity (ANA) were measured at days 0, 2, 4, and 7 after the onset of the drought treatment. Our results support a local regulation of SNF in soybean plants and suggest that a reduced transpiration is not involved in the decline of SNF observed under drought conditions.

Introduction

Although SNF is widely known to be inhibited under drought, the molecular mechanism involved in the regulation of SNF in plants subjected to water deficit remains unclear. Three major factors have been proposed to be involved this inhibition under drought stress: oxygen limitation, carbon shortage and regulation by nitrogen metabolism. Recent work suggests that the regulation of SNF in drought-stressed soybean plants involves nitrogen feedback, along with an impairment of metabolic carbon flux in nodules (Ladrera *et al*., 2007). These results are also consistent with a local nodule-based regulation of SNF in pea plants subjected to drought (Marino *et al*., 2007). Nevertheless, the occurrence of a local regulation of SNF under drought in soybean remains to be experimentally proven. Therefore, the aim of the present work is to determine whether the inhibition of SNF under drought in soybean plants is governed by systemic or local signals.

Materials and Methods

Soybean (*Glycine max* cv. Oxumi) plants were inoculated with *Bradyrhizobium japonicum* strain UPM 752 and grown in a split-root system (SRS) in a controlled environment chamber for 6 weeks. Then, plants were separated randomly into three sets: Control (C), drought (D) and partial drought (PD). C were daily supplied with nutrient solution to field capacity at both sides of the SRS, whereas drought was achieved by withholding water/nutrients from both sides of D plants. PD plants were irrigated to field capacity to one side of the SRS (named PD-C), whilst water/nutrients were withheld at the other side (PD-D). At days 0, 2, 4 and 7 after the onset of drought evapotranspiration (ET), apparent nitrogenase activity (ANA) and nodule water potential measurements were carried out. Following this, leaves, roots and nodules were harvested for further analysis and dry weight determinations.

Results and Discussion

Four days after the onset of the drought treatment, a significant decline of ET in both D and PD plants was observed (Figure, **A**). This fall was progressive for D plants, reaching ET values a 69% lower than those of C plants at drought day 7, whereas ET only partially declined in PD plants (-25%), a decline which was approximately maintained throughout the study period. In contrast, nodule water potential showed a significant decrease after 4 days of

water stress only in D plants, while in PD-D plants a significant reduction was first observed at day 7 (Figure, **B**).

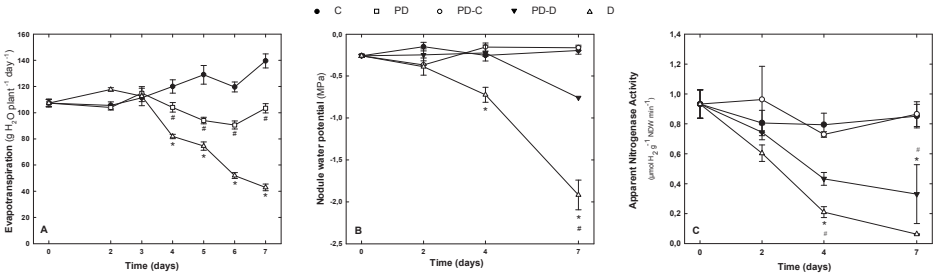

Figure. Effects of drought on ET (**A**), nodule water potential (**B**) and ANA (**C**). In panel **A**, C denotes control, PD partial drought, and D drought plants. In panels **B** and **C**, both root parts of the PD treatment are represented independently: PD-C denotes the irrigated part and PD-D the non-irrigated part of PD plants. Values represent the mean ± SE (n=3). An asterisk (*) represents significant differences ($P \leq 0.05$) between D and C plants and a hash (#) represents significant differences between PD-D and C.

Although, based on nodule water potential values, the root system of PD-D plants exhibited lower levels of water stress compared to those of D plants, ANA significantly declined in both treatments (Figure, **C**). On the contrary, nitrogen-fixing activity in PD-C nodules was maintained at control levels (Figure, **C**). These results support a local regulation of SNF also in soybean plants.

Some authors have proposed that a reduced transpiratory flux would lead to a limitation in the export of N compounds from nodules, provoking an accumulation of these compounds and thus a feedback inhibition of SNF (Walsh, 1990; Serraj *et al.,* 2001). However, results presented here show that nodules of PD-C plants are able to maintain high nitrogenase activity despite a 25% decline of ET. Therefore, the reduced transpiratory flux does not lead to the observed decline in ANA under drought conditions (PD-D).

Further studies focused on the effect of drought on the levels of certain N compounds, namely ureides and amino acids, in different soybean tissues will provide insights into the interactions between these N compounds and the regulation of SNF in this legume plant.

Acknowledgments

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Sulphur starvation effects on nitrogen fixation and nodule performance in pea

Muñoz-Azcárate O*, Aldasoro J, Arrese-Igor C

*Departamento de Ciencias del Medio Natural, Edificio Los Olivos, Universidad Pública de Navarra, Campus de Arrosadia, 31006 Pamplona, Spain. *olaya.munoz@unavarra.es*

Summary

The role of sulphur (S) deficiency in growth, nitrogen fixation and nodule metabolism was investigated in nodulated pea (*Pisum sativum* L.). We tried to elucidate which stages of plant and nodule development are mostly affected by S deprivation. Inoculated pea plants were grown in hydroponic solution and separated into three groups: one group was transferred into a S-free solution the day of transplanting (S0), another group was transferred into that S-free solution seven days after transplanting (S7), and the third one was kept as a control. Physiological traits, such as photosynthesis, N_2 fixation, nodule biomass, and root and shoot length and biomass, were determined. Also, S-content in roots, shoots and nodules were analyzed, as well as main enzymes of nodule metabolism. S starvation caused a decline in BNF and photosynthesis in S0 plants, and a decrease in root, shoot and nodules biomass. In S7 plants, these parameters had no significant differences with control plants. Regarding biochemical analysis, S content decreased in both S-free treatments in shoots, and the activities of nodule enzymes sucrose synthase and isocitrate dehydrogenase seem to decrease in S-deficient conditions. Altogether, these results suggest that S starvation reduces N_2 fixation in legumes by impairing nodule development and functioning.

Introduction

Nitrogen-fixing symbioses between legume plants and rhizobia are the largest natural source of nitrogen for agriculture, and, since the use of nitrogen fertilizer contributes substantially to environmental pollution, this biological alternative has received increasing attention in agriculture in the last few years. These symbioses are very sensitive to abiotic stresses. Among these stresses, S deficiency has been reported with increasing frequency over the last decade, affecting crop yield and quality. In legumes S deprivation limits the synthesis of S-containing amino acids such as methionine and cysteine, diminishing their nutritional value. In spite of this fact, its effect on biological N_2 fixation (BNF) has received little attention to date. A few studies (Zhao *et al.,* 1999; Scherer *et al.,* 2008; Varin *et al.,* 2010) reported a decrease in BNF and nodule biomass as well as a decrease in the activity of nodule-containing proteins in S-deficient conditions. However it is not well established yet whether the effect of lack of S is affecting nodule growth and development or also has an effect on nodule metabolism.

Materials and Methods

Seeds of *Pisum sativum* L. cv *sugar boys* were germinated and then inoculated with a *Rhizobium leguminosarum* strain. Then, plants were transferred to individual pots with an aerated hydroponic solution (Kalia & Drevon, 1985) in a growth chamber with a controlled environment. A group of these plants was transferred into a modified Kalia and Drevon S-free solution the day of transplanting (S0 plants), and another group was transferred into that S-free solution one week after transplanting (S7 plants). Control plants were kept under full nutrients availability.

Four weeks after transplanting, N_2 fixation, photosynthesis, nodule biomass and root and shoot length and biomass were measured. Also, aliquots of roots, shoots and nodules were frozen in liquid nitrogen and kept at -80ºC for biochemical analysis. Total S was extracted by acid digestion from dry shoots, roots and nodules and analyzed by induced coupled plasma spectrometry (ICP). The activities of the main enzymes involved in nodule carbon and nitrogen metabolism were assayed according to Gonzalez *et al.* (1995).

Results and Discussion

Sulphur starvation resulted in a significant yield reduction of shoots, roots and nodules in S0 plants, but no significant effects were found in S7 plants. Also, BNF and photosynthesis in S0 plants showed a significant reduction when compared with control plants, while S7 plants showed no significant differences with control plants.

In both S deficient treatments (S0 and S7), S content in shoots was two-fold decreased compared with control plants. Regarding enzymatic assays, preliminary experiments suggest a decrease in both sucrose synthase (SuSy) and isocitrate dehydrogenase (ICDH) activities upon S starvation. SuSy is the main enzyme involved in carbon supply to nodules, and a downregulation of its activity has been described as a response to abiotic stresses (Gonzalez *et al.,* 1995). Hence, BNF impairment in S-deficient conditions might be also modulated by a decline in SuSy activity. ICDH links carbon and nitrogen metabolism in nodules, providing carbon skeletons to plastids for the synthesis of amino acids. (Galvez *et al.,* 2005). Thus, a decrease in its activity might provoke a decline in nitrogen supply to the whole plant, impairing its performance.

Further biochemical analysis are under way in order to establish the specific mechanism by which S starvation is affecting nodule metabolism, although the results obtained so far suggest that the main effects occur at the early stages of nodule initiation, causing an impaired nodule development.

Acknowledgments

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Response of common bean nodules to salinity and drought

Gozálvez MS, Lluch C, Ocaña A

Departamento de Fisiología Vegetal, Facultad de Ciencias, Universidad de Granada, Spain.

Summary

In order to compare the principles of acclimatization of the *Phaseolus vulgaris–Rhizobium tropici* symbiosis to salt and non-ionic osmotic stress, plants were cultivated under NaCl and drought, respectively. Physiological responses observed in root nodules were different under both types of stress. Thus, drought inhibited plant growth and nodulation in comparison with salt stress, whereas nitrogenase activity was reduced on salt stressed plants compared to the activity observed in plants grown under drought. The accumulation of mannitol, and possibly other solutes, could somehow explain a better osmotic adjustment under salt stress, which allows this legume to acclimate to salinity.

Introducción

Los mecanismos adaptativos al estrés salino e hídrico son variados e incluyen la síntesis y acumulación de ciertos metabolitos como polioles. El mio-inositol, poliol cíclico de seis carbonos, es el precursor de otros polioles asociados al estrés hídrico (Streeter *et al.*, 2001). Otro poliol ampliamente distribuido en las plantas es el manitol asociado a estrés salino (Loescher y Everard, 1996). En este trabajo se comparan parámetros de crecimiento, fijación de nitrógeno, y síntesis y acumulación de algunos polioles en nódulos de la simbiosis *P. vulgaris-R. tropici* de plantas con potencial hídrico (ψ) semejante obtenido por la adición de NaCl a la solución nutritiva o provocando sequía.

Materiales y Métodos

Plantas de *Phaseolus vulgaris* var. Contender inoculadas con *Rhizobium tropici* cepa 899 crecieron en condiciones controladas, y el sustrato (vermiculita/arena 8/2) se regó diariamente con solución nutritiva libre de nitrógeno. El estrés salino se provocó añadiendo a la solución nutritiva 50 mM de NaCl, y el estrés hídrico manejando la intensidad de riego para que el potencial hídrico medido en la última hoja totalmente expandida fuese semejante al de sus homólogas cultivadas en salinidad. Las plantas se cosecharon a los 27 días de edad, y los tratamientos se iniciaron 2, 4 y 7 días antes de la cosecha, no aplicándose a las plantas control. Se determinó: peso seco (PS) de planta y de nódulos, actividad aparente de la nitrogenasa (ANA; Wittin y Michin, 1998), el potencial hídrico en las hojas (Ψ) con un psicrómetro de termopar (Model C-52, Wescor, Inc., Logan, UT, USA). El contenido de polioles se determinó por cromatografía iónica (CS-3000 Dionex). Las actividades manosa 6-P oxidorreductasa (M6PR) y *mio*-inositol 1-P sintasa (MIPS) se valoraron según Loescher *et al.* (1992) y Barnett *et al.* (1970), respectivamente.

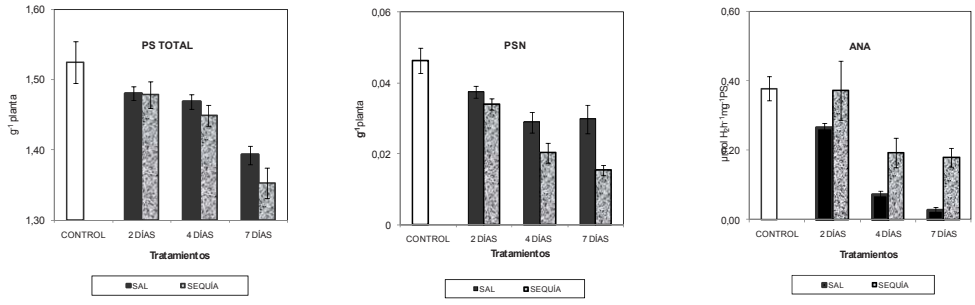

Figura 1: Peso seto total (PST), actividad aparente de nitrogenasa (ANA) y peso seco de nódulos (PSN) en simbiosis *Phaseolus vulgaris-Rizobium tropicci 899* sometidas a 50 mM de NaCl y estrés hídrico equivalente durante 7 días. Valores ± corresponden al ES de 5 de plantas individuales.

Resultados y Discusión

El potencial hídrico de hoja disminuyó progresivamente con el tiempo de tratamiento, igual que el crecimiento de la planta (PST; Fig. 1). El estrés osmótico, al reducir la turgencia disminuye la expansión celular y en consecuencia el PST de los tejidos (Munns, 2002). El efecto iónico de la salinidad intensificaría este proceso. La sequía afecta más al crecimiento y nodulación, mientras que la actividad nitrogenasa se inhibe más por la salinidad.

La actividad MIPS en nódulos (Fig. 2) decrece desde el inicio en ambos tratamientos, y al final se recupera sólo en los nódulos de salinidad. El contenido de *mio*-inositol (MYO) de los nódulos presentó una caída drástica al inicio de la aplicación de sal y se mantuvo por debajo del control en los tratamientos más prolongados, mientras que en sequía no varió. La actividad M6PR mostró incrementos significativos en los tratamientos salinos, y disminuyó con la sequía, al igual que la concentración de manitol (MAN).

Los resultados parecen indicar una mejor tolerancia de esta especie a la salinidad que a la sequía, que podría relacionarse con la acumulación de manitol y la síntesis de otros polioles, como ononitol (datos no mostrados) de los que *mio*-inositol es precursor, lo que explicaría su descenso. Un mejor ajuste osmótico en nódulos puede relacionarse con menor disminución de la masa nodular que compense el descenso de la actividad nitrogenasa.

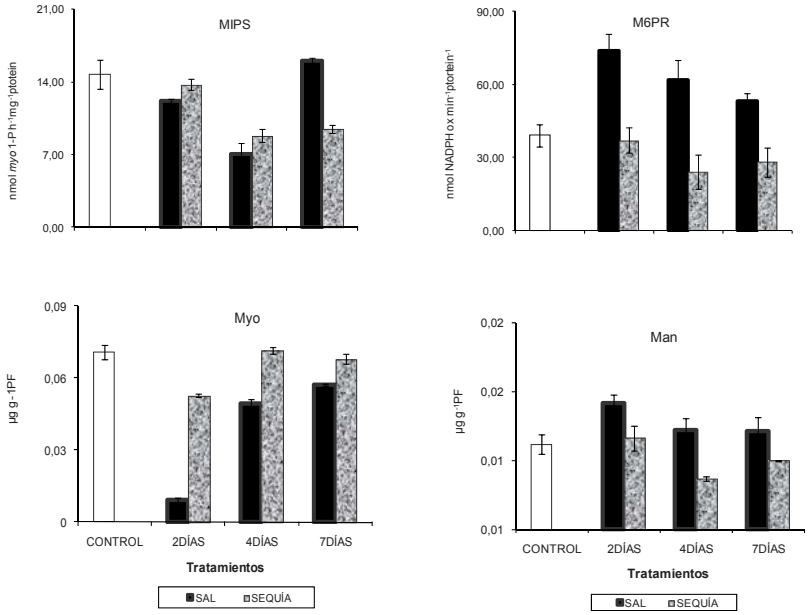

Figura 2. Actividades *mio*-inositol-1-P (MIPS) y manitol-6-P óxido reductasa (M6PR), y contenidos de *mio*-inositol (Myo) y manitol (MAN) en nódulos (*Phaseolus vulgaris*-*Rhizobium tropici*) de plantas tratadas con 50 mM de NaCl o sometidas a estrés hídrico equivalente durante 7 días. Los valores ± corresponden al ES de 5 plantas individuales.

Agradecimientos

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Comparative study of osmotic and NaCl stress effects on the salt sensitive *Medicago truncatula* var. R-108 in symbiosis

Palma FJ*, Herrera-Cervera JA

*Departamento de Fisiología Vegetal, Facultad de Ciencias, Universidad de Granada, 18071 Granada, Spain. *fpalma@ugr.es*

Summary

In this study, a comparison of salt and osmotic stress effects on the salt sensitive *Medicago truncatula* var. R-108 was carried out. Alterations on plant growth, symbiotic nitrogen fixation performance and ammonium-related enzymatic activities were determined at the vegetative and flowering stages under salt (50 and 100 mM NaCl) and mannitol (50 and 100 mM) stress. Differences were found on the accumulation of total soluble sugars and free amino acids under both types of stress. In addition, a correlation was observed on glutamine synthetase, glutamate synthase and glutamate dehydrogenase activities with the dose of stress.

Introducción

La fijación biológica de nitrógeno representa la fuente principal de entrada de nitrógeno en suelos agrícolas, incluyendo las regiones áridas. Los sistemas simbióticos desempeñan un papel significativo en la mejora de la fertilidad y de la productividad de suelos pobres en nitrógeno. La simbiosis *Rhizobium*-leguminosa está afectada por diferentes factores ambientales, entre los que destaca el estrés salino. Los efectos negativos del NaCl son debidos principalmente a la acumulación de niveles tóxicos de iones y a la menor disponibilidad de agua debido al estrés osmótico, provocándose la alteración de la nutrición mineral de la planta (Patemak, 1987). El polietilenglicol y manitol aplicados a la solución nutritiva se usan como medios para inducir estrés hídrico en cultivos de plantas y tejidos (Perez-Alfocea *et al.,* 1996), no obstante, el manitol es considerado como un soluto compatible, que puede acumularse a altas concentraciones en las células vegetales sin afectar al metabolismo celular (Borowitzka, 1981).

Materiales y Métodos

Material biológico y condiciones de crecimiento. Semillas de *Medicago truncatula* var. R-108 desinfectadas y germinadas se transfirieron a bandejas de cultivo con solución nutritiva, y se inocularon con *S. meliloti* GR4. En estas condiciones crecieron hasta el día 35 de cultivo (simbiosis establecida), en ese momento se aplicaron tratamientos isosmóticos de manitol (0, 50 y 100 mM) y NaCl (0, 50 y 100 mM) y se mantuvieron hasta el final del tiempo de experimentación. Las plantas fueron cosechadas coincidiendo con dos estados fisiológicos del desarrollo de la planta: inicio de floración (49 días) y plena floración (56 días).

Fijación de nitrógeno. La actividad nitrogenasa (EC 1.7.9.92) se realizó mediante la medición de la evolución de H_2 en un sistema de flujo abierto (Witty & Minchin, 1998). La actividad nitrogenasa aparente (ANA) era determinada bajo una corriente de N_2:O_2 (80%:20%) y la actividad nitrogenasa total (ANT) bajo Ar:O_2, ambas con un flujo total de 0.4 l/min. La tasa de fijación de nitrógeno (TFN) era calculada como (ANT-ANA)/3.

Actividades glutamina sintetasa (GS), glutamato sintasa (GOGAT) y glutamato deshidrogenasa (GDH). La extracción y cuantificación se realizó según el método descrito por Cordovilla *et al.* (1996). La actividad GS se determinó por la formación de γ-glutamil hidroxamato y las actividades GOGAT y GDH mediante la oxidación del NADH.

Contenido de aminoácidos libres totales, prolina y azúcares solubles totales. El contenido de prolina (Irigoyen *et al.,* 1992) y aminoácidos libres (Yemm & Cocking, 1955) se determinó en extractos de nódulos usando el reactivo ninhidrina. El ensayo de azúcares solubles totales se realizó siguiendo el método colorimétrico de Irigoyen *et al.* (1992).

Resultados y Discusión

El crecimiento y la fijación de nitrógeno se reduce en ambos muestreos con los tratamientos de manitol y NaCl, observándose la inhibición más drástica con la dosis 100 mM de manitol (datos no mostrados), esto puede ser debido a la posible influencia del manitol en el metabolismo secundario de la planta (Tholakalabavi *et al.,* 1994). Las actividades GS y GOGAT disminuyen al inicio de floración con el manitol y el NaCl, sin embargo, en plena floración ambas actividades muestran su valor más elevado con 100 mM de manitol. En general, la actividad GDH incrementa con el manitol y el NaCl en ambos muestreos (Tabla).

Tabla. Efecto del tratamiento con manitol y NaCl en la actividad GS expresada en nmol γ-glutamil hidroxamato mg^{-1} prot min^{-1}, las actividades GOGAT y GDH expresadas en nmol NADH mg^{-1} prot min^{-1}, y el contenido de aminoácidos libres totales (Aa) expresados en mg asparragina g^{-1} PF, prolina (Pro) en mg prolina g^{-1} PF y azúcares solubles totales (Ast) expresados en mg glucosa g^{-1} PF, en nódulos de plantas de *M. truncatula* inoculadas con *S. meliloti* GR4.

Tiempo (días)	Manitol (mM)	NaCl (mM)	GS	GOGAT	GDH	AST	Aa	Prol
	0	0	14.3^{d}	0.036^{b}	0.012^{e}	8.0^{cde}	12.2^{cd}	0.184^{d}
	50	0	12.1^{e}	0.019^{g}	0.018^{c}	6.4^{e}	8.9^{e}	0.377^{b}
49	100	0	11.7^{e}	0.019^{g}	0.027^{a}	7.9^{de}	14.2^{b}	0.519^{a}
	0	50	14.1^{d}	0.030^{c}	0.008^{f}	7.9^{de}	10.8^{cd}	0.214^{d}
	0	100	9.1^{f}	0.019^{g}	0.024^{b}	12.1^{b}	10.7^{d}	0.280^{c}
	0	0	14.9^{c}	0.028^{d}	0.015^{d}	10.2^{bcd}	11.8^{cd}	0.201^{d}
	50	0	11.7^{e}	0.021^{f}	0.015^{d}	10.9^{b}	14.5^{b}	0.379^{b}
56	100	0	23.1^{a}	0.041^{a}	0.024^{b}	9.5^{bcd}	16.9^{a}	0.560^{a}
	0	50	11.7^{e}	0.022^{f}	0.019^{c}	10.6^{bc}	12.3^{c}	0.256^{c}
	0	100	16.4^{b}	0.024^{c}	0.022^{b}	15.7^{a}	14.6^{b}	0.396^{b}
MDS ($P \leq 0.05$)			0.4	0.001	0.002	2.6	1.5	0.041

El contenido en azúcares solubles totales tan sólo aumenta significativamente con 100 mM de NaCl al inicio y plena floración, mientras que en aminoácidos libres totales se detecta que el manitol induce una mayor acumulación que la salinidad. La concentración de prolina aumenta proporcional a la dosis de manitol y NaCl en los dos muestreos, siendo más elevado en los nódulos procedentes de plantas tratadas con manitol (Tabla). La inducción de la acumulación de prolina en nódulos de *M. truncatula* frente al estrés salino ha sido descrito anteriormente (López *et al.,* 2008). Coincidiendo con nuestros resultados, en plantas de *S. portulacastrum* el tratamiento con manitol induce la acumulación de prolina (Slama *et al.,* 2007).

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New approach to the role of boron in the nitrogen-fixing heterocystous cyanobacterium *Anabaena* sp. PCC 7120

Abreu I, Bolaños L, Bonilla I*

*Departamento de Biología, Facultad de Ciencias, Universidad Autónoma de Madrid, Darwin 2, 28049, Madrid, Spain. *ildefonso.bonilla@uam.es*

Summary

Boron (B) was described by our group as essential for maintaining heterocyst envelopes in nitrogen-fixing cyanobacteria. The micronutrient was not required for growth in media containing combined nitrogen but, when *Anabaena sp.* PCC7120 was cultivated in the presence of NO_3^-, which is repressive for heterocyst differentiation, and under B-deficiency, it developed long filaments and started to differentiate heterocysts within a few hours. These results suggest that signals preventing heterocyst differentiation are overtaken under B deficiency. Furthermore, heterocyst differentiation in the absence of nitrogen started under B deficiency earlier than in the presence of B, although B-deficient heterocysts were not functional due to the instability of the envelopes. Observation of filaments showed that vegetative cells in B-deficient media were apparently in continuous cell division. Fluorescence after DAPI staining seemed to confirm a higher DNA content in B-deficient vegetative cells which could be related to accelerated cell division. Because there is evidence that a signal pathway involving cell division independent of the 2-oxoglutarate signal is involved in heterocyst differentiation, we postulated that such a signal is increased due to increase of cell division rates in B-deficient *Anabaena* PCC7120, so that heterocyst differentiation is triggered even when the 2-oxoglutarate signal is inhibited by NO_3^-.

Introduction

El B fue descrito como micronutriente esencial para las plantas hace mas de 80 años, y, sin embargo, aunque se ha visto relacionado con los procesos mas diversos: respiracion, metabolismo de acidos nucleicos y azúcares, regulacion hormonal, etc., no es hasta comienzos de este siglo que se confirma, de entre los propuestos, su papel estructural. En este sentido, se ha demostrado experimentalmente que este bioelemento determina la dimerización de la pectina RGII (rhamnogalacturonano II) mediante enlaces diéster en la pared vegetal (O'Neill *et al.,* 2001), y que estabiliza la molecula-señal de *quorum sensing* AI2 en bacterias (Chen *et al.,* 2002).

Nuestro grupo, en la segunda mitad de los años 80, demostró, trabajando con cianobacterias, la esencialidad del micronutriente para formas filamentosas con heterocistos, fijadoras de nitrógeno (Mateo *et al.,* 1986, Bonilla *et al.,* 1990), en las que la deficiencia de B conducía a la desestabilización de las cubiertas de dichas células fijadoras de N_2 (García-González *et al.,* 1991). No obstante, en los últimos años existen numerosas evidencias en plantas y en animales que relacionan al B con procesos de diferenciación celular y desarrollo (Redondo-Nieto *et al.,* 2008 y citas). Las cianobacterias con heterocistos, y particularmente su estirpe más caracterizada *Anabaena* PCC7120, son modelos sencillos donde estudiar alteraciones en procesos de desarrollo y diferenciación celular. Por tanto, con una estrategia similar, hemos comenzado a investigar la posible relación entre el B y los procesos de señalización celular que tienen lugar durante la inducción en ausencia de nitrógeno combinado o inhibición, en presencia del mismo, de la diferenciación del heterocisto .

Materials and Methods

Anabaena sp. PCC7120 se crecio en medio BG11 (con 5mM NH_4SO_4 ó 17.6 mM $NaNO_3$) o $BG11_0$ (sin N combinado) líquido o sólido (1% agar). La deficiencia de B se consiguió según se describe en Mateo *et al.* (1986) y, para asegurar la eliminación de trazas de B, los medios se incubaron con Amberlita IRA743, resina que específicamente captura ácido bórico e iones borato (Asad et al. 1997). Los cultivos se mantuvieron a 28ºC, con iluminación continua (300 $\mu E\ m^{-2}\ s^{-1}$) y agitación a 140 rpm en agitador orbital en el caso de cultivos líquidos. El crecimiento de los cultivos se siguió mediante espectrofotometría. Para el estudio estructural de los mismos se

usaron técnicas de miicroscopía en campo claro y epifluorescencia. Se realizarón tinciones de polisacáridos mediante Alcian Blue, y tinción de nucleoides con DAPI según el protocolo expuesto en Sakr *et al*. (2006).

Results and Discussion

Los cultivos de *Anabaena sp.* PCC7120 crecidos en medios deficientes en boro mostraron un crecimiento ligeramente retardado y una mayor tendencia a formar agregados que los crecidos en medios suplementados con boro, aunque finalmente alcanzaron un crecimiento similar a estos, salvo los cultivos sin N para los que el B es esencial tal y como se demostró en estudios anteriores (Mateo *et al.,* 1986; Bonilla *et al.,* 1990). Los espectros de absorbancia *in vivo* realizados sobre cultivos líquidos mostraron como única diferencia una mayor proporción de ficobiliproteínas respecto a clorofila a en deficiencia de B. Pese a estas ligeras diferencias, estos resultados vienen a confirmar que el B no supone un factor limitante para el crecimiento de esta cianobacteria en medios suplementados con nitrógeno. Sin embargo, sí que pudimos encontrar diferencias significativas en el desarrollo de los filamentos de *Anabaena* PCC7120 en los diferentes medios ensayados y en función de la presencia o no de B. En medio BG11 suplementado con 5mM NH_4SO_4 los filamentos mostraron ausencia de heterocistos, independientemente de si el medio era o no deficiente en B. En medio inductor de heterocistos, $BG11_0$, como era de esperar, *Anabaena* diferenció estas células, si bien no fueron funcionales en deficiencia de B por la ya comentada desestabilización de sus cubiertas. No obstante, pudimos observar que la diferenciación de heterocistos en medios deficientes en B comenzó antes de las 24h tras el cambio de medio BG11 a $BG11_0$, mientras que demoró más en medios suplementados con B. Aún así, la observación más interesante ocurrió en medio BG11 con nitrato, en el que los cultivos de *Anabaena* en presencia de B no desarrollaron heterocistos, pero sí lo hicieron los deficientes en B entre las 48 y las 72 h. Todos los heterocistos dieron una tinción positiva con Alcian Blue (que tiñe la cubierta de polisacáridos del heterocisto), si bien, los deficientes en B presentaron claras alteraciones estructurales que los hacían no funcionales. La observación microscópica de *Anabaena sp.* 7120 reveló también que, crecida en cualquiera de los medios deficientes en B, presentaba un elevado grado de asincronía en las divisiones de las células que constituían los filamentos, apareciendo regiones de los mismos con una aparentemente muy elevada tasa de división. La tinción de DNA con DAPI dio mayor señal de fluorescencia en estas regiones, lo cual apoyaba la hipótesis de una elevada tasa de división celular en deficiencia de B.

Aunque preliminares, estos resultados apuntan a que el B en cianobacterias, además de un papel estructural en los heterocistos, puede estar relacionado con rutas de regulación del ciclo y la diferenciación celular, como parece ocurrir en células eucariotas. De hecho, además de la señalización de la diferenciación de heterocistos por incremento del metabolito 2-oxoglutarato (Laurent *et al.,* 2005), parece existir otra independiente de esta, controlada por la tasa de división celular (Sakr *et al.,* 2006), que podría ser la relacionada con la ausencia de B.

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Physiological and cell structural variations produced by NaCl in *Vigna unguiculata* and *Phaseolus vulgaris* inoculated with rhizobia isolated from Cuban saline soils

Gómez E[2], Ruiz-Díez B[1], Alarcón K[2], Fajardo S[1], Morcillo C[1], López R[2], Fernández-Pascual M[1*]

*[1]Departamento de Fisiología y Ecología Vegetal, Centro de Ciencias Medioambientales, CSIC, 28006 Madrid, Spain; [2]Universidad de Granma, Bayamo, Cuba. *mfernandezp@ccma.csic.es*

Summary

Salt tolerant and sensitive varieties of *Vigna* and *Phaseolus* have been inoculated with *Rhizobium* strains isolated from Cuban saline soils and the effect of different concentrations of NaCl on the development and ultrastructure of leaves and nodules has been analyzed. Inoculations improve the production of biomass in stressed plants in both types of legume varieties. In the tolerant variety of *Vigna* (86-D-715), there was no decrease in nitrogenase activity with the application of salt stress, whereas in the tolerant variety of *Phaseolus* (VAM-14) the salt treatment generated a decrease in nodule volume and nitrogenase activity, although biomass remained unaffected. With respect to the ultrastructure of nodule cells, only alterations with the higher NaCl concentrations used were observed. In nodules from VAM-14, symbiosomes are degraded, with some peribacteroidal membranes being broken and a high number of vesicles in infected cells being detected. In nodules from 86-D-715 salt affects mainly uninfected cells where degradation of cellular organelle and high vesiculation can be observed.

Introducción

El grave problema a nivel mundial que supone la salinización de los suelos alcanza en Cuba cifras alarmantes en cuanto al número de hectáreas afectadas. De los 6.7 millones de has de superficie agrícola existentes en el país, aproximadamente un millón están afectadas por este problema. Las zonas más representativas se encuentran en la provincia Granma (suroeste cubano) con alrededor de 228.000 has afectadas, de las cuales el 11-28% están fuertemente salinizadas. Estas razones hacen que la búsqueda de variedades tolerantes a la salinidad de alubias (*Phaseolus vulgaris*) y frijol vigna (*Vigna unguiculata*) se haya convertido en una prioridad dentro de las líneas de investigación agrícola en ese país. En el marco de un proyecto bilateral España-Cuba, subvencionado por la AECID, nos hemos propuesto probar el efecto de la salinidad y de varias cepas cepas de rhizobia aisladas de suelos salinos del suroeste cubano (Ruiz-Díez *et al.*, 2009a) sobre el desarrollo y la estructura de hojas y nódulos en las leguminosas anteriormente mencionadas.

Materiales y Métodos

Variedades tolerantes y sensibles de *V. unguiculata* y *P. vulgaris* fueron inoculadas con sus respectivas cepas (VIBA-1 y PHABA-1) aisladas de suelos salinos de Jiguani y Babiney (Granma, Cuba). Las cepas fueron caracterizadas fenotípica y genotípicamente siguiendo los métodos de Ruiz-Díez *et al* (2009b). Las plantas se crecieron en condiciones controladas de luz y temperatura y se regaron con solución nutritiva libre de nitrógeno, añadiendo 80, 120 y 150 mM de NaCl en los tratamientos con sal. A los 45 días de la siembra se recogieron las plantas y se tomaron muestras de hojas y nódulos para su procesamiento para microscopía óptica y electrónica, según de María *et al.* (2005). Se determinó la biomasa de hojas y nódulos, la actividad nitrogenasa y el contenido de proteínas en los diferentes tratamientos.

Resultados y Discusión

Las cepas aisladas de los suelos salinos cubanos han resultado infectivas y muy efectivas. La inoculación de *Vigna*, favorece la producción de biomasa en las plantas estresadas, tanto en la variedad susceptible como en la tolerante. En la variedad tolerante 86-D-715) no se produce descenso de la actividad nitrogenasa con la aplicación del estrés salino. En la ultraestructura

de los nódulos de 86-D-715, se puede observar como la sal afecta fundamentalmente a las células no infectadas en las que se observan signos de senescencia precoz como la alta vesiculación. Esta característica se observa también en las células infectadas de los nódulos de VAM-14 (Figura). La inoculación de la VAM-14 produce un número elevado de nódulos, que incluso se ve aumentado con el tratamiento de sal. La salinidad no afecta al desarrollo de la parte aérea ni al peso de los nódulos, sin embargo el volumen de los nódulos y la actividad nitrogenasa disminuyen significativamente a medida que aumenta la concentración de NaCl. Los datos indican que las variedades de *Vigna* y *Phaseolus* seleccionadas son altamente resistentes al estrés salino en condiciones controladas.

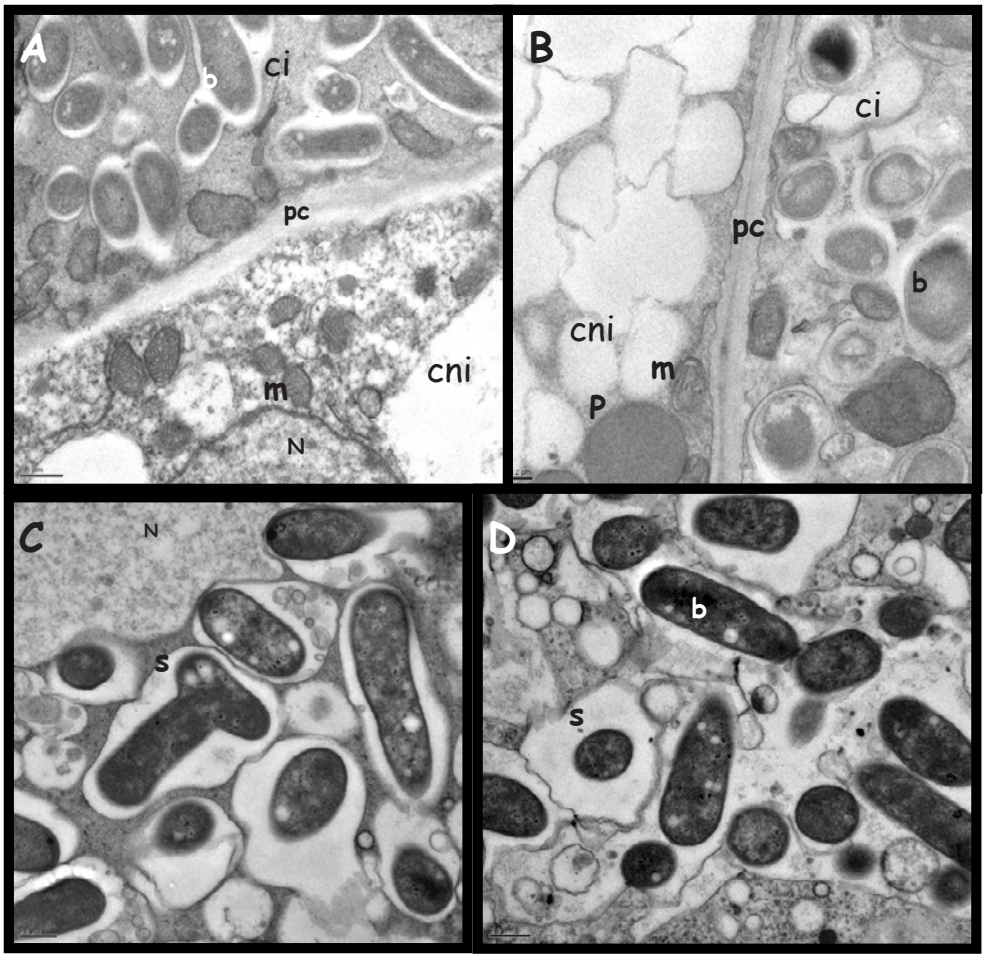

Figura. Nódulos de *Vigna* control (**A**) y tratados con NaCl (**B**). Nódulos de *Phaseolus* control (**C**) y tratados con NaCl (**D**). b, bacteroides; Ci, célula infectada; Cni, célula no infectada; m, mitocondria; N núcleo; P peroxisoma; Pc, pared celular; S, simbiosoma.

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Legume and rhizobial aldehyde oxidases in the *Medicago* sp.-*Sinorhizobium meliloti* symbiosis

Coba de la Peña T[1], Torres MJ[2], Paradela C[1], Rincón A[1], Fajardo S[1], Morcillo CN[1], García de la Torre VS[1], Pueyo JJ[1], Delgado MJ[2], Lucas MM[1*]

[1]*Departmento de Fisiología y Ecología Vegetal, Instituto de Recursos Naturales, Centro de Ciencias Medioambientales, CSIC, Serrano, 115bis, 28006 Madrid, Spain;* [2]*Microbiología del Suelo y Sistemas Simbióticos, Estación Experimental del Zaidín, CSIC, Apartado 419, 18080 Granada, Spain.*
**mlucas@ccma.csic.es*

Summary
Aldehyde oxidase (AO) catalyzes the last step of the biosynthesis of indole-3-acetic and abscisic acids. Two putative *Medicago truncatula AO* genes were identified by database mining. The expression patterns of both homologues, *MtAO1* and *MtAO2*, were determined by quantitative real-time PCR in leaves, roots and nodules. Leaves displayed higher *MtAO1* expression than roots or nodules, whereas *MtAO2* expression was similar in all three organs. The presence of *AO* genes in the genome of *Sinorhizobium meliloti* 1021 was also investigated. Comparison to AO protein sequences from *Methylobacillus* sp. KY4400 allowed us to identify three putative *AO* genes in *S. meliloti* that clustered in the same transcriptional order found in *Methylobacillus* sp. Mutants in the first gene of the transcriptional unit were constructed by marker exchange. The symbiotic phenotype of one of the mutant strains was investigated. It elicited nitrogen-fixing nodules in *M. sativa* that displayed similar nitrogenase activity to nodules elicited by wild-type *S. meliloti*. The mutant appeared more efficient in nodulation (percentage of nodulated plants and number of nodules per plant) than wild-type and had a negative effect on the number of secondary roots.

Introduction
Phytohormones such as indole-3-acetic acid (IAA), synthesized by both symbionts, and abscisic acid (ABA) play important roles in nodule development and functioning. In plants, the enzyme AO is involved in the biosynthesis of ABA as well as in the synthesis of IAA (*via* the tryptophan-dependent indole-3-pyruvic acid pathway). Recently, AOs have also been described in some microorganisms, including rhizobia (Yasuhara *et al*., 2005; Fedorova *et al.,* 2005). To date, little is known about the biosynthesis of these hormones in nodules. AO is most probably involved, as we showed the presence of AO in nodules of *Medicago truncatula* and *Lupinus albus* (Fedorova *et al.,* 2005).

Materials and Methods
Seeds of *Medicago truncatula* Gaertn. R-108-1 (c3) and alfalfa (*M. sativa* cv Aragón) were sterilized and germinated as described previously (Verdoy *et al.,* 2006). Two days after sowing, *M. truncatula* was inoculated with *Sinorhizobium meliloti* strain 41 and alfalfa with strain 1021 or with an *AO* mutant. Plants were grown in vermiculite or in hydroponic culture under controlled conditions.

Search of *AO* homologs was performed in *M. truncatula* and *S. meliloti* 1021 genomes by using nucleotide and protein databases. Expression patterns of putative *M. truncatula AO* genes were determined by quantitative real-time PCR, using methodology described previously (Verdoy *et al.,* 2006). A bacterial mutant in the first gene of an *AO* transcriptional unit was constructed by marker exchange. Alfalfa nodule structure and nitrogenase activity were analysed according to Shvaleva *et al.* (2010).

Results and Discussion
We found that at least two *AO* homologs are present in the *M. truncatula* genome. The first homolog (*MtAO1*) has *c.* 97% nucleotide identity to pea (*Pisum sativum*) aldehyde oxidase 2 (*PsAO2*), and the second homolog (*MtAO2*) has *c.* 95% nucleotide identity to pea aldehyde oxidase 3 (*PsAO3*) (Zdunek-Zastocka, 2008). Both putative translated proteins contain typical functional domains of AO proteins: Fe-S and FAD binding domains, AO and xanthine

dehydrogenase domains, and a molybdopterin-binding domain. In order to determine the expression levels of both homologs in different organs, transcript accumulation levels were analyzed in roots and shoots of *M. truncatula* seedlings (3-days old) and in roots, leaves and nodules of 24-days post inoculation (dpi) plants. In seedlings, the highest *MtAO1* expression level was recorded in shoots, *c.* 4-fold higher than in roots. In 24-dpi plants, leaves displayed higher *MtAO1* expression than roots (*c.* 9-fold) and nodules (*c.* 18-fold). *MtAO2* expression was similar in all organs. The putative *AO* gene, *MtAO1*, showed similar expression pattern to the *PsAO2* homolog in pea seedlings. *PsAO2* is highly expressed in young leaves, but not in mature leaves of adult plants. It is not known whether *PsAO2* is involved in the formation of any dimeric PsAO isoform (Zdunek-Zastocka, 2008). *MtAO2* displayed different pattern expression that its homolog *PsAO3*, which is mainly expressed in aging leaves. It has been suggested that *PsAO3* might encode a homodimeric PsAO-γ isoform that participates in stress-induced ABA biosynthesis (Zdunek-Zastocka, 2008). Further characterization of *MtAO1* and *MtAO2* expression will be performed in order to elucidate possible functions for these putative aldehyde oxidases.

A search in the genome sequence of *S. meliloti* 1021 using the protein sequences corresponding to the small, medium and large subunits, AodS, AodM and AodL, of the AO enzyme (Aod) from *Methylobacillus* sp. KY4400 (Yasuhara *et al.,* 2005) was performed. We have identified three genes in *S. meliloti* 1021, SM_b21556, SM_b21557, and SM_b21558, clustered in the same transcriptional order found for *Aod* genes in *Methylobacillus* sp. In order to investigate the involvement of these genes in nodulation, a mutant in the first gene of the transcriptional unit (SM_b21556) was constructed. The symbiotic phenotype of this mutant was characterized in alfalfa. No significant differences were found in nitrogenase activity between wild-type and mutant nodules. In nodulation kinetics experiments, the mutant produced higher number of nodules than the wild-type strain, with the highest significant difference at 5-dpi. Futhermore, the number of plants nodulated by the mutant was higher as compared to plants nodulated by the wild-type strain. Plants inoculated with the mutant displayed a significant lower number of secondary roots than plants inoculated with the wild-type strain. These results suggests that the lack of AO in *S. meliloti* has a differential effect on nodulation and secondary root formation during the first days after inoculation, probably due to altered IAA levels and/or hormone imbalance.

Acknowledgments

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Mercury accumulation in legume plants inoculated with rhizobia isolated from mercury-contaminated areas

Quiñones MA[1*], Ruiz-Díez B[1], Fajardo S[1], López MA[2], Higueras PL[2], Fernández-Pascual M[1]

*[1]Departamento de Fisiología y Ecología Vegetal, Centro de Ciencias Medioambientales, CSIC, 28006 Madrid, Spain; [2]Departamento de Ingeniería Geológica y Minera. Escuela Universitaria Politécnica de Almadén, Universidad de Castilla-La Mancha, 13400 Almadén, Ciudad Real, Spain. *maquinones@ccma.csic.es*

Summary

Three different locations near Almadén (Ciudad Real) with high (Almadenejos), medium (Las Cuevas) and low (San Quintín) soil mercury (Hg) concentrations were selected for plant and soil sample collection. Trap plants [lupin (*Lupinus albus*), chickpea (*Cicer arietinum*) and common bean (*Phaseolus vulgaris*)] were planted in all collected soils to determine their Hg absorption and accumulation capacity and to isolate rhizobia. The results showed that Hg accumulation was particularly high in roots and nodules from lupin plants grown in Almadenejos soil. Therefore, lupin was chosen to study the effect of different concentrations of Hg in the watering solution of plants inoculated with two strains of *Bradyrhizobium canariense*, L-3 (Hg-sensitive) and L7AH (Hg-tolerant). The results showed that Hg affected the growth of lupin plants decreasing the mass of their aerial parts and the number and weight of their nodules. The L7AH Hg-tolerant strain seemed to confer lupin plants some resistance regarding their nitrogenase activity, since it remained constant through the different Hg treatments.

Introducción

La contaminación por metales pesados está llegando a ser un grave problema para los ecosistemas terrestres. En el distrito minero de Almadén (Ciudad Real), se ha explotado durante más de 2000 años el que es uno de los mayores yacimientos de cinabrio del mundo para la extracción de mercurio, así como otros yacimientos menores, dispersos en un área total de unos 120 km^2. Esto ha provocado la difusión de dicho metal y la contaminación de los suelos de los alrededores de las áreas de explotación hasta niveles extremadamente altos (Molina *et al.,* 2006). En los últimos años, un aumento en la preocupación por la toxicidad del mercurio llevó al cierre de las minas y a la realización de gran número de estudios sobre la distribución de mercurio en los suelos y su acumulación en la flora de Almadén (Higueras *et al.,* 2003), así como sus efectos sobre las poblaciones humana y animal. Sin embargo, no se conoce cómo afecta la contaminación por mercurio a la diversidad de los microorganismos del suelo, o su efecto sobre las interacciones beneficiosas que algunos de ellos, los rizobios, establecen con las leguminosas. Éste es el objetivo del presente estudio.

Materiales y Métodos

Se recogieron muestras de los primeros 15 cm de suelo de tres localidades cercanas a Almadén (Ciudad Real): Almadenejos (concentración alta de Hg), Las Cuevas (concentración media de Hg) y San Quintín (concentración baja de Hg). Dichos sustratos se distribuyeron en macetas de plástico donde se sembraron semillas de lupino (*Lupinus albus*), garbanzo (*Cicer arietinum*) y judía (*Phaseolus vulgaris*), que se regaron con agua esterilizada cada 2 días durante 1 mes en un invernadero. Transcurrido ese tiempo se recogieron las plantas y se separaron las hojas, los tallos, las raíces y los nódulos (no todas las plantas nodularon), así como muestras del suelo, para analizar su contenido en Hg, utilizando un Analizador Lumex RA-915. Para el experimento sobre el efecto del Hg en plantas de lupino, se esterilizaron semillas de la variedad G1 y se plantaron en macetas con vermiculita esterilizada, en una cámara de crecimiento (16 h de luz, temperatura día/noche 25/20ºC). Los botes se dividieron en 3 grupos que se regaron con soluciones nutritivas sin N_2 que contenían 0, 5 ó 25 μM de Cl_2Hg. Cada grupo se dividió a su vez en otros dos que se inocularon con las cepas de *Bradyrhizobium canariense*, L-3 (sensible al Hg, usado como control) o L-7AH (tolerante al Hg, procedente de Almadenejos). Transcurridas 5 semanas, se recogieron las plantas y (1) se midieron y pesaron las partes aéreas, (2) se midió la actividad nitrogenasa, (3) se separaron y pesaron los nódulos y (4) se prepararon muestras de las hojas, los tallos, las raíces y los nódulos, así como de vermiculita, para el posterior análisis de su contenido en Hg y para su estudio microscópico.

Resultados y Discusión

Los suelos con los diferentes niveles de contaminación por Hg (Tabla), se utilizaron para crecer plantas trampa (judía, garbanzo y lupino). Se estudió así la capacidad acumuladora de Hg de dichas plantas y se aislaron rhizobios de sus nódulos (ver comun. de Ruiz-Díez *et al*.). Los resultados mostrados en la Tabla ponen de manifiesto que las plantas de lupino crecidas en el suelo de Almadenejos (máxima conc. de Hg) acumulan enormes concentraciones de este metal en sus raíces y nódulos. Asimismo, las judías crecidas en el mismo suelo acumulan también gran cantidad de Hg en sus raíces, aunque no en los nódulos. Las diferencias entre los resultados obtenidos con dicho suelo y los de Las Cuevas y San Quintín pueden deberse no sólo al mayor contenido en Hg del suelo de Almadenejos, sino también a que dicho metal esté más biodisponible.

Tabla. Concentración de mercurio en los suelos y acumulación en los órganos de las tres plantas trampa utilizadas. Todos los valores están en ppm y son la media de, al menos, 5 medidas.

	Suelo	**Judía**			**Garbanzo**			**Lupino**		
		Hojas	Raíces	Nódulos	Hojas	Raíces	Nódulos	Hojas	Raíces	Nódulos
San Quintín	212	6	163	nn	9	80	nn	6	23	6
Las Cuevas	970	3	213	105	10	97	nn	24	97	nn
Almadenejos	3870	20	999	54	ng	ng	ng	54	2190	1620

nn, no nodulada; ng, no germinada

Por otra parte, se plantaron semillas de lupino en vermiculita, se inocularon con la cepas de *B. canariense* tolerantes (L7AH) o sensibles (L-3) al Hg y se regaron con soluciones nutritivas con 0, 5 ó 25 μM de Cl_2Hg. Transcurridas 3 semanas de experimento, se empezaron a notar los primeros signos de estrés (hojas maduras cloróticas) en las plantas regadas con soluciones nutritivas con Hg. Sin embargo, todas las plantas sobrevivieron las 5 semanas del experimento en buenas condiciones. Finalizado éste, se pudo comprobar que el mercurio (tanto a 5 como a 25 μM) afectaba al crecimiento de las plantas, disminuyendo el peso de la parte aérea (Figura), el número de nódulos y el peso de éstos (no mostrado), no habiendo diferencias significativas entre las dos cepas utilizadas. Por otro lado, la cepa L7AH parecía conferir a las plantas de lupino una cierta resistencia al Hg, ya que la actividad nitrogenasa se mantenía constante mientras que en plantas inoculadas con la cepa L-3 disminuía al aumentar la concentración de Hg en la solución nutritiva (Figura). A la vista de estos resultados, se prevé aumentar las concentraciones de Cl_2Hg en la solución nutritiva para comprobar el grado de tolerancia a Hg que confiere la cepa L7AH.

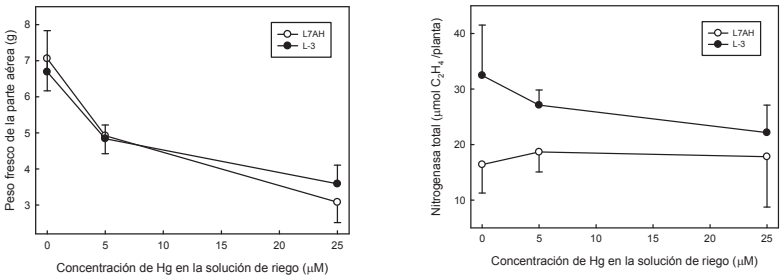

Figura. Efecto del Hg sobre el peso de la parte aérea y la actividad nitrogenasa total en plantas de lupino crecidas en vermiculita, inoculadas con las cepas L7AH (tolerante a Hg) y L-3 (sensible a Hg) de *B. canariense* y regadas con solución nutritiva con 0, 5 ó 25 μM de Cl_2Hg. Los resultados son la media ± desviación standard de 3 o más medidas.

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Trehalose synthesis in *Rhizobium etli* and carbon metabolism of common bean nodules under drought conditions

Hidalgo-García A[1*], Hinojosa MB[1], Larrainzar E[2], González EM[2], Iturriaga G[3], Delgado MJ[1]

*[1]Estación Experimental del Zaidín, CSIC, Apartado 419, 18080 Granada, Spain; [2]Dpto. Ciencias del Medio Natural, Universidad Pública de Navarra, Pamplona, Spain; [3]Centro de Investigación en Biotecnología, Universidad Autónoma del Estado de Morelos, Cuernavaca, Mexico. *alba.hidalgo@eez.csic.es*

Summary

In this work, carbon metabolites (sucrose, glucose and fructose) and activity of sucrose synthase (SS) and glucose-6-P-dehydrogenase (G6PDH) were analyzed in nodules of common bean plants subjected to drought and inoculated with *Rhizobium etli* CFN42 (WT) and two *R. etli* CFN42 derivatives that overexpress the trehalose-6-phosphate synthase (TPS) gene from *R. etli* (*otsA*$^+$) and the TPS-TPP genes (*BIF*) from *Saccharomyces cerevisiae*. Sucrose content in nodules increased in response to drought, consistently with a decline in SS activity, although this effect was less dramatic in those nodules from plants inoculated with *otsA*$^+$ strain than in WT nodules. In nodules of plants inoculated with the *BIF* strain, SS activity was lower and sucrose content higher than in those inoculated with the parental strain CFN42, under both normal irrigation and drought conditions. Glucose and fructose levels also increased more significantly in *otsA*$^+$ nodules than in WT nodules in response to drought. Furthermore, G6PDH activity was induced in nodules from plants inoculated with *otsA*$^+$ strain under severe drought compared to control plants. Nitrogen fixation rate measured as total N content of shoots was higher in plants inoculated with *otsA*$^+$, and lower in those inoculated with *BIF* strain, respectively, than that of plants inoculated with WT under either control or stressed conditions. These results suggest that TPS overexpression stimulates carbon metabolism and nitrogen fixation in nodules, whereas TPS/TPP overexpression would have the opposite effect.

Introducción

La trehalosa (α-D-glucopyranosyl-1,1-α-D-glucopyranoside), es un disacárido no reductor ampliamente distribuido en la naturaleza, que en determinados organismos juega un papel crucial como osmoprotector frente a estreses abióticos (Iturriaga *et al*., 2009). La ruta de síntesis más común utiliza UDP-glucosa (UDPG) y glucosa-6-fosfato (G6P) para producir trehalosa-6-fosfato (T6P) por la acción de la trehalosa-6-fosfato sintasa (TPS), codificada por el gen *otsA* en bacterias, y a continuación la T6P es desfosforilada hasta trehalosa, por la acción de la trehalosa-6-fosfato fosfatasa (TPP). Recientemente, se ha demostrado que la inoculación de plantas de judía con una cepa de *Rhizobium etli* que sobreexpresa el gen *otsA* mejora la tolerancia de las mismas a sequía (Suárez *et al.,* 2008). En este trabajo se ha analizado la interacción de la sobreexpresión en *R. etli* de genes que codifican las enzimas TPS y TPP con el metabolismo carbonado de los nódulos de plantas de *Phaseolus vulgaris* en condiciones de sequía.

Materiales y Métodos

Se han utilizada las cepas CFN42 de *R. etli* (WT), *otsA*$^+$ (Suárez *et al.,* 2008), y la *BIF* (TPS/TPP). Para la construcción de esta cepa, la misma construcción quimérica TPS/TPP proveniente de *Saccharomyces cerevisiae*, e introducida previamente en *Azospirillum brasilense* (Rodriguez-Salazar *et al.,* 2009), se introdujo en *R. etli* CFN42 (Gabriel Iturriaga, *comun. pers.*). Se inocularon plantas de *P. vulgaris* var. Negro Jamapa con las tres cepas de estudio, en una mezcla 1:1 de vermiculita:arena estéril. Se dejaron crecer en el invernadero, regándose a su capacidad de campo alternativamente con agua y solución nutritiva libre de nitrógeno. A los 21 días post-inoculación, se suspendió el riego en parte de las macetas, mientras que el resto se regó normalmente. Se recolectaron las plantas y los nódulos a los 5-7 días (sequía moderada) y 10-12 días (sequía severa) de sequía. En nódulos se cuantificó la concentración de sacarosa, glucosa y fructosa según Sekin (1978) y se determinaron las actividades SS y G6PDH según Gálvez *et al.* (2005).

Resultados y Discusión

Tras someter las plantas a una sequía moderada, se observó una disminución de la actividad SS respecto a las condiciones control, que fue más significativa en los nódulos de plantas inoculadas con la cepa WT (34%) que en los de plantas inoculadas con la cepa *ots*A^{+} (18%, Figura, **B**). Esta disminución de SS resultó en un aumento de la concentración de sacarosa en los nódulos de la cepa WT (35%) y en los nódulos de la cepa *otsA*$^{+}$ (24%) (Figura, **A**). Estos resultados sugieren que la inoculación de judía con la cepa *otsA*+ alivia el efecto negativo de la sequía sobre la actividad SS de los nódulos. En el caso de la cepa *BIF*, la actividad SS de los nódulos fue inferior y la concentración de sacarosa mayor a la de los nódulos WT en condiciones de riego y sequía (Figura, **B**).

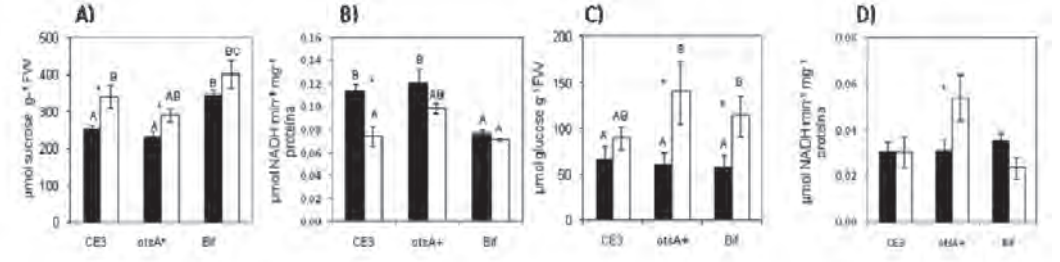

Figura. **A)** Contenido de sacarosa y **B)** actividad SS en nódulos sometidos a una sequía moderada. **C)** contenido en glucosa y **D)** actividad G6PDH en nódulos sometidos a una sequía severa. Condiciones control (barras negras) y de sequía (barras blancas). Diferentes letras denotan diferencias significativas entre plantas inoculadas con las diferentes cepas, y el asterisco (*) denota diferencias significativas entre el control y la sequía dentro de la misma cepa (test Tukey, $p < 0.05$). Los datos son resultado de 3 experimentos independientes

Tras someter las plantas a una sequía severa, se acumuló glucosa (Figura, **C**) y fructosa (no se muestran los resultados) en los nódulos de las tres cepas, siendo este efecto más significativo en los de la *otsA*$^{+}$, en los cuales también se observó una inducción de la actividad G6PDH en respuesta a la sequía. La enzima G6PDH cataliza el primer paso de la parte oxidativa del ciclo de las pentosas fosfato (OPPP). Como parámetro indicativo de la fijación de nitrógeno se analizó el contenido total de nitrógeno de las plantas (datos no mostrados) que fue mayor en las plantas inoculadas con la cepa *otsA*$^{+}$ y menor en aquellas inoculadas con *BIF*, respecto a las inoculadas con WT tanto en condiciones control como en sequía. Considerados en conjunto, estos resultados confirman los previamente publicados por Iturriaga *et al*. (2008) y sugieren que la inoculación de plantas de judía con *otsA*$^{+}$, produce una activación del metabolismo carbonado de los nódulos y una mayor fijación de nitrógeno en respuesta a sequía. Sin embargo, los nódulos de las plantas inoculadas con *BIF* son menos eficientes.

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The implication of *Rhizobium etli cbb$_3$* oxidase in the response of common bean to drought

Talbi C[1*], Sánchez C[1], Hidalgo-Garcia A[1], Girard L[2], Bedmar EJ[1], Delgado MJ[1]

*[1]Estación Experimental del Zaidín, CSIC, Apartado 419, 18080 Granada, Spain; [2]Centro de Ciencias Genómicas, UNAM, Cuernavaca, México. *chouhra29@hotmail.com*

Summary

To investigate the involvement of *Rhizobium etli cbb$_3$* oxidase in the response of common bean (*Phaseolus vulgaris*) to drought, plants were inoculated with a *R. etli* strain CFNX317 that overexpresses this oxidase in bacteroids and were subjected to moderate and severe drought. After a moderate stress, a decrease in plant dry weight, nitrogen content, and nitrogenase activity of the nodules was observed compared to irrigated plants. This decrease was more significant in plants inoculated with the wild type strain than in those inoculated with strain CFNX713. Bacteroids produced by CFNX317 strain and isolated from nodules of control or stressed plants showed higher respiratory capacity than those produced by the parental strain. Similarly, *cbb$_3$* oxidase expression was higher in bacteroids of the CFNX317 strain than in those of the wild type strain in all experimental conditions. The decline of sucrose synthase activity and increase of sucrose content provoked by a moderate drought was more significant in nodules from plants inoculated with the parental strain than in those inoculated with CFNX317 strain. Taken together, these results suggest that a major expression of the *cbb$_3$* oxidase in the bacteroids, which enhances their respiratory capacity, increases the tolerance of the common bean-*R. etli* symbiosis to drought. We propose that a high efficiency in oxygen consumption by bacteroids could be involved in the response of sucrose synthase activity to drought.

Introduction

The effect of drought on symbiotic nitrogen fixation (SNF) has been widely reported (Zahran, 1999) as it is considered to be the most important environmental factor limiting crop production. Limitation of nodule permeability to O_2 was initially suggested to be the cause of the inhibition of SNF under drought. Regulation of SNF has also been related to a reduced C supply (mostly in the form of malate) for bacteroid respiration due to the down-regulation of sucrose synthase in nodules (Gálvez *et al.,* 2005). In bacteroids, the high-energy demand for nitrogen fixation is fulfilled through the expression of a high oxygen affinity terminal oxidase, the *cbb*$_3$-type oxidase, encoded by the *fixNOQP* operon (Delgado *et al*., 1998). Very recently, it has been demostrated that inoculation of common bean plants with a *Rhizobium etli* strain CFNX317, which overexpresses the *cbb$_3$* oxidase, improves nitrogen fixation capacity (Granados-Baeza, 2007). In this work, we have demostrated that inoculation of plants with the *cbb*$_3$ overexpressing *R. etli* strain CFNX317 increases respiratory capacity of the bacteroids and improves the tolerance of the common bean-*R. etli* symbiosis to drought. The involvement of bacteroidal respiration in nodule C metabolism has also been investigated.

Materials and Methods

Common bean (*Phaseolus vulgaris* cv. Negro jamapa) plants were inoculated with *R. etli* CFN42 (WT) or CFNX713 (*cbb$_3^+$*) strains, and grown in pots containing 1:1 (v/v) vermiculite:sand under controlled-environment conditions. After 21 days, plants were subjected to drought by withholding irrigation until the plants reached a leaf Ψ_W of about -1.75 MPa (moderate drought, 5-7 days) and -2.5 MPa (severe drought, 10-12 days). Control plants had a Ψ_W of -1 MPa. At harvesting, bacteroids were isolated, oxygen consumption was measured using a Hansatech electrode, and expression of *cbb$_3$* was analyzed as previously described (Granados-Baeza, 2007). Sucrose synthase activity and sucrose content (González *et al.,* 2005) were measured in the nodule cytosol.

Results and Discussion

A moderate drought provoked a decrease in plant dry weight, nitrogen content, and nitrogenase activity of the nodules being more significant in plants inoculated with the WT

strain that in those inoculated with cbb_3^+ (not shown). Similarly, plants inoculated with cbb_3^+ strain were more tolerant to a severe drought that plants inoculated with WT strain (not shown). Bacteroids of strain cbb_3^+ possess major respiratory capacity than WT bacteroids after their isolation from plants grown either under control or stress conditions (Figure, a). A moderate drought decreased respiratory capacity of WT bacteroids, but not of cbb_3^+ bacteroids (Figure, a). A severe drought reduced about 40% and 20%, respiratory capacity of WT and cbb_3^+ bacteroids, respectively (Figure, a). After heme *c* staining analyses, two bands of 32, and 27 kDa were detected in membranes of bacteroids (Figure, b). These proteins were previously identified as the FixP and FixO components of the cbb_3 oxidase of R. *etli* (Granados-Baeza, 2007). Bacteroids of cbb_3^+ strain showed increased expression of FixP and FixO that those of WT strain, independently of the treatment (Figure, b). A moderate drought caused a slight increase in FixP and FixO expression in bothWT and cbb_3^+ bacteroids (Figure, b). Moderate drought stress caused a more significant decline in sucrose synthase activity in nodules produced by the WT strain (49%) than in those produced by the cbb_3^+ strain (15%). Similarly, WT nodules accumulated more sucrose than cbb_3^+ nodules (37% versus 15%) in response to a moderate drought. Our results suggest that respiratory capacity of the bacteroids contributes to the tolerance of SNF to drought and is involved in the regulation of C metabolism in common bean nodules. Supporting our observations, it has been recently proposed that inhibition of SNF in *M. truncatula* under drought stress appears to be related to an impairment of bacteroid metabolism and N_2-fixing capacity rather than to a limitation of C respiratory substrate to fuel bacteroid nitrogenase (Larrainzar *et al*., 2009).

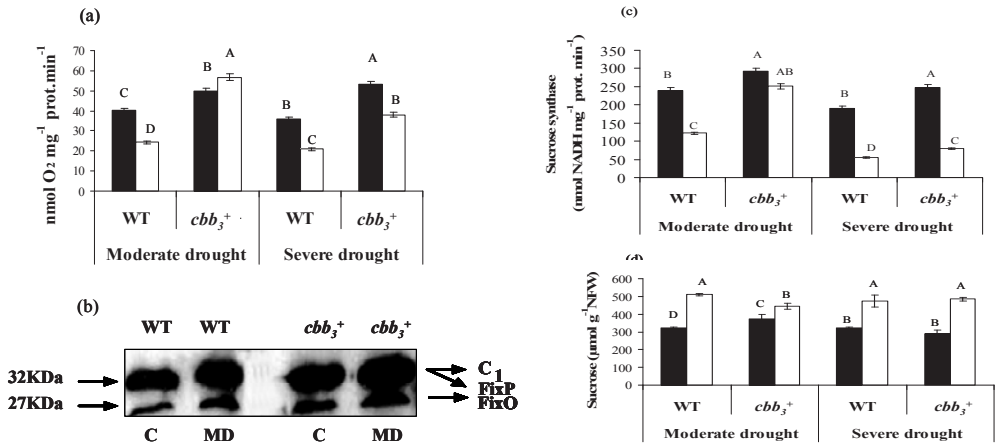

Figure. (a) Oxygen consumption and **(b)** FixP and FixO expression in membranes of bacteroids from nodules of common bean inoculated with *R. etli* CFN42 (WT) and CFNX317 (cbb_3^+) strains. **(c)** Sucrose synthase activity and **(d)** Sucrose content of nodules. Data are means of two replicates. For each treatment (moderate and severe) bar with different letters are significantly different Tukey HSD test ($P \leq 0.05$). ■ control □ drought; C: control, MD: moderate drought.

Acknowledgments

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Changes in the antioxidants of mitochondria during natural senescence of legume nodules

Matamoros M*, Loscos J, Becana M

*Departamento de Nutrición Vegetal, Estación Experimental de Aula Dei, CSIC, Apartado 13034, 50080 Zaragoza, Spain. *manumat@eead.csic.es*

Summary

Legume nodules have elevated respiratory rates in mitochondria and bacteroids to meet the energy requirements for N_2 fixation. However, respiration inevitably produces reactive oxygen species (ROS) such as superoxide radicals and hydrogen peroxide (H_2O_2). We have studied the changes associated with natural senescence (aging) in the antioxidant system of mitochondria purified from common bean (*Phaseolus vulgaris*) nodules. During nodule aging, the concentrations of lipid peroxides and oxidized proteins increased in mitochondria, but not in the cytosolic fraction. Also, the activities of three enzymes involved in ascorbate synthesis and regeneration were down-regulated. By contrast, qRT-PCR analysis, immunoblots, and enzyme activity assays reveal a significant up-regulation of manganese superoxide dismutase (MnSOD) activity during aging, whereas the protein level of peroxiredoxin IIF (PrxIIF) remained unchanged. Overall, our results suggest that, in legume nodules, natural senescence is associated with a decline in the capacity of mitochondria to synthesize and regenerate ascorbate, and that this organelle is a preferential target of oxidative stress.

Introduction

N_2 fixation requires high amounts of ATP generated in the mitochondria through oxidative phosphorylation. As a consequence, mitochondria are a major source of ROS (Dalton *et al.*, 1995; Rhoads *et al.*, 2006). Nodule mitochondria, as the rest of cellular compartments, contain a vast array of antioxidants that finely regulate ROS concentration (Figure). These include the MnSOD, ascorbate-glutathione pathway enzymes, and PrxIIF (Iturbe-Ormaetxe *et al.*, 2001; Groten *et al.*, 2006). Antioxidants prevent oxidative damage of mitochondrial components and allow the participation of ROS in the signaling of the mitochondrial redox status to the rest of the cell (Rhoads *et al.*, 2006).

In a previous work, we described the antioxidant system of bean nodule mitochondria and proposed a model for ROS metabolism in this organelle (Iturbe-Ormaetxe *et al.*, 2001). Here, we report the aged-related changes in the antioxidant system and determine the relative contribution of mitochondria to the aging of legume nodules.

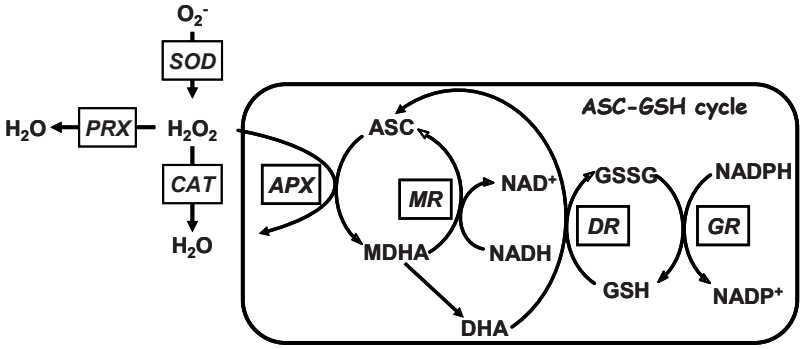

Figure. Antioxidants in legume nodules. *Abbreviations:* APX, ascorbate peroxidase; ASC, ascorbate; CAT, catalase; DHA, dehydroascorbate; DR, dehydroascorbate reductase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; MDHA, monodehydroascorbate; MR, monodehydroascorbate reductase; PRX, peroxiredoxin; SOD, superoxide dismutase.

Materials and Methods

Nodulated common bean plants (*Phaseolus vulgaris* cv. 'Contender' x *Rhizobium leguminosarum* bv. *phaseoli* 3622) were grown in a controlled environment chamber as described (Loscos *et al.,* 2008). Nodules were harvested from plants at three different stages of development: young (27-28 d old plants without flowers), mature (39-40 d old plants at the late flowering-early fruiting stage), and senescent (53 d old plants with fully developed pods). Nodule mitochondria were purified by differential centrifugation and Percoll density gradients (Iturbe-Ormaetxe *et al.,* 2001). Enzyme activities were assayed in mitochondria following conventional protocols (Loscos *et al.,* 2008; Matamoros *et al.*, 2010). Leghemoglobin (Lb) was quantified as described (Loscos *et al.,* 2008). The oxidative damage of lipids was measured by HPLC as the content of malondialdehyde after its reaction with thiobarbituric acid (Matamoros *et al.,* 2010). The oxidative damage of proteins was estimated as the content of total carbonyl groups using immunoblots, following instructions given in the OxyBlot Protein Oxidation Detection kit (Chemicon, Temecula, CA, USA).

Results and Discussion

The objective of this study was to investigate the effects of aging on some major antioxidants of bean nodule mitochondria. In addition, Lb content and oxidation products of lipids and proteins were measured as markers of nodule activity and oxidative stress, respectively, at the three developmental stages of nodules. Compared to young nodules, Lb concentration in mature nodules did not vary significantly, but decreased by 86% in senescent nodules. However, the amounts of lipid peroxides and oxidized proteins did not change significantly, suggesting that oxidative stress in the cytosolic fraction of nodules is not involved in the degradation of Lb and in the general loss of nodule activity. By contrast, nodule aging induced an oxidative stress in mitochondria, as estimated by the accumulation of oxidatively-modified lipids and proteins. Therefore, we decided to study the effects of aging on some key antioxidants of mitochondria. The four enzyme activities of the ascorbate-glutathione pathway were determined in mitochondria purified from young, mature, and senescent nodules. APX and GR activities remained unaltered. However, mitochondrial MR was reduced by 47% in senescent nodules, whereas the activity of DR, the other enzyme involved in ascorbate regeneration, declined slightly (15%) in mitochondria from both mature and senescent nodules. Furthermore, the activity of L-galactono-1,4-lactone dehydrogenase, the last enzyme of the main ascorbate biosynthetic pathway in plants (Wheeler *et al.,* 1998; Loscos *et al.,* 2008), decreased by 30% in senescing nodules. MnSOD and PrxIIF, along with the ascorbate-glutathione pathway, participate in the control of H_2O_2 levels in mitochondria. Our results show an increase of MnSOD activity during aging, whereas the PrxIIF protein level remains constant. These results suggest that aging of legume nodules is associated with a decline in the capacity of mitochondria to synthesize and regenerate ascorbate, and underscore the importance of this organelle as a preferential target of senescence-induced oxidative stress.

Acknowledgments

This work was supported by the MICINN (AGL2008-01298) and Gobierno de Aragón (group A53).

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Localization of manganese superoxide dismutase promoter activity in nodules of *Lotus japonicus*

Rubio MC*, Becana M

*Departamento de Nutrición Vegetal, Estación Experimental de Aula Dei, CSIC, Apartado 13034, 50080 Zaragoza, Spain. *mcr@eead.csic.es*

Summary

We have performed a preliminary analysis of the promoter activity of the manganese superoxide dismutase gene (*LjMnSOD*) during nodule development in the model legume *Lotus japonicus*. The putative promoter region was amplified by PCR and cloned into a binary vector containing *GUS* (β-glucuronidase) as the reporter gene. Transgenic roots and nodules were produced by infection with *Agrobacterium rhizogenes* LBA1334 using the 'hairy roots' system. Histochemical analysis of GUS activity showed that the *LjMnSOD* promoter is active in the vascular tissue of the nodule, particularly at the base, where nodules are connected to the subtending root. Promoter activity increased during nodule development. These data suggest an important role of MnSOD in the nodule vascular tissue, which could be related to the high respiratory activity of the vascular bundle cells.

Introduction

Reactive oxygen species (ROS) are potentially damaging molecules but also perform useful functions in stress signaling, depending on their concentration, localization, and chemical composition. For this reason, steady-state ROS concentrations need to be kept under tight control and this is mainly achieved by antioxidant metabolites and enzymes. Amongst the most important antioxidant enzymes are the superoxide dismutases (SODs), a family of metalloenzymes that catalyze the dismutation of superoxide radicals to oxygen and hydrogen peroxide. There are three types of SODs in plants, differing in their metal cofactor at the active site: CuZnSODs, FeSODs, and MnSODs. The three types of SODs are present in nodules, although they are localized in different subcellular compartments. Thus, the CuZnSODs and FeSODs are localized in the cytosol and plastids, while MnSODs are localized in the mitochondria and bacteroids (Moran *et al.,* 2003; Rubio *et al.,* 2007). In this work, we have studied the spatio-temporal expression pattern of the *LjMnSOD* promoter during nodule development and natural senescence (aging) in *Lotus japonicus*.

Materials and Methods

The putative promoter region (2,989 bp) of *LjMnSOD* was amplified by PCR using specific primers, and sequenced. The fusion construct between the *LjMnSOD* promoter and GUS (*gusA::int*) was made using the binary vector pMP7101. The *CaMV 35S-gusA::int* was used as a positive control during hairy root transformation. The pMP7101 vector was used as a negative control. The resulting binary vectors were introduced into *Agrobacterium rhizogenes* strain LBA1334 by triparental mating. Induction of transgenic roots was performed by the 'hairy roots' method described by Diaz *et al.* (2006). Transgenic hairy roots were transferred to pots containing vermiculite, inoculated with *Mesorhizobium loti* R7A, and grown under conditions described elsewhere (Rubio *et al.,* 2007). Emergent (5-7 days post inoculation; dpi), young (13-15 dpi), mature (34-37 dpi), and old (50-56 dpi) nodules were harvested for histochemical GUS staining, which was performed as described by Quaedvlieg *et al.* (1998) with some modifications. Some nodule material was embedded in 0.5% (w/v) agar and sectioned (60 µm) with a vibratome (Leica VT1000S). GUS expression was examined with a stereomicroscope (Leica M165FC) connected to a digital camera Leica DFC420C.

Results and Discussion

The aim of this work was to study the localization of the *LjMnSOD* promoter activity during nodule development in *L. japonicus*. To this end, the *LjMnSOD* promoter was fused to the reporter gene *gusA::int* using the binary vector pMP7101. The construct was inserted into *A.*

rhizogenes LBA1334 and induction of transgenic roots and nodules of *L. japonicus* was performed by the 'hairy roots' method.

Different stages of nodule development (emergent, young, mature, and old nodules) were histochemically analyzed for GUS activity. During the early stages (emergent nodules), GUS staining was not detectable (Figure, **A**). However, some GUS staining was observed in the base of young nodules (**B**). Subsequently, GUS expression spread to the vascular tissue, particularly in the base of mature nodules where they were connected to the subtending root (**C, D, F, G**). In some cases, a weak GUS staining was also observed in the vascular bundles in the cortex of mature nodules (**D**). In old nodules, GUS activity was enhanced in the vascular tissue (**E, H**). By contrast, we could not observe GUS activity in the infected zone of nodules (**F, H**). Additionally, the *LjMnSOD* promoter was active in the vascular tissue of the roots. No GUS expression was observed in nodules transformed with the binary vector as a negative control (data not shown).

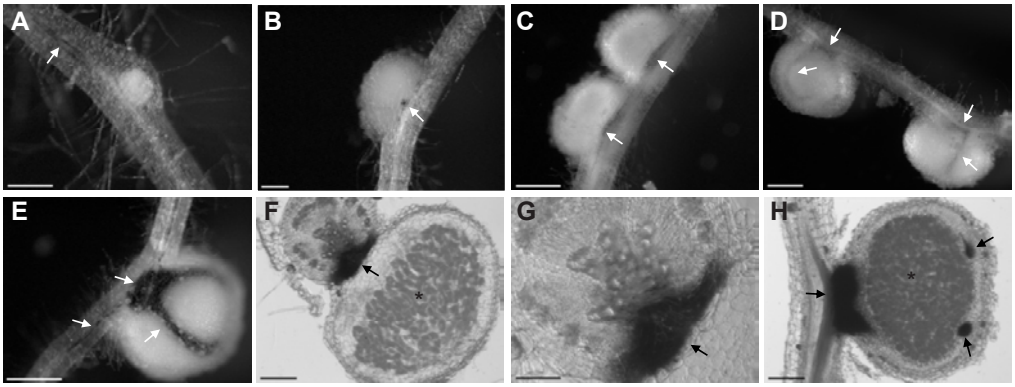

Figure. Histochemical localization of GUS activity during nodule development in transgenic hairy roots of *L. japonicus* expressing *LjMnSODp-gusA::int*. **A-E,** Different stages of nodule development: emergent (**A**), young (**B**), mature (**C-D**), and old (**E**) nodules. **F-G,** Transverse sections of a mature nodule. **H,** Longitudinal section of an old nodule. The infected tissue (**F, H**) is indicated by an asterisk. Arrows indicate GUS staining activity. Bars: 100 µm (**G**), 200 µm (**A-B, F, H**), 500 µm (**C-E**).

The strong expression of the *LjMnSOD* promoter in the vascular tissue of the nodule is consistent with the high levels of MnSOD protein and superoxide radicals detected in the vascular tissue of stem and root nodules of *Sesbania rostrata* (Rubio *et al.,* 2009). This suggests an important role of MnSOD in the nodule vascular tissue, which could be related to the high respiratory activity of the vascular bundle cells.

Acknowledgments

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Peroxiredoxin genes of *Lotus japonicus*: expression analysis in plants and hormone effects

Tovar-Méndez A[1*], Bustos-Sanmamed P[1], Sato S[2], Dietz K-J[3], Cejudo FJ[4], Tabata S[2], Becana M[1]

*[1]Estación Experimental de Aula Dei, CSIC, Apartado 13034, 50080 Zaragoza, Spain; [2]Kazusa DNA Research Institute, 2-6-7 Kazusa-Kamatari, Kisarazu, Chiba 292-0818, Japan; [3]Biochemistry and Physiology of Plants, W5-134, Bielefeld University, 33501, Bielefeld, Germany; [4]Instituto de Bioquímica Vegetal y Fotosíntesis, Universidad de Sevilla y CSIC, 41092 Sevilla, Spain. *tovarmendeza@eead.csic.es*

Summary

We have identified nine genes encoding peroxiredoxins (Prxs) in the model legume *Lotus japonicus*. The genes belong to the typical four classes of Prxs found in plants: 1Cys (*1C-Prx*), 2Cys (*C2-PrxA* and *C2-PrxB*), PrxQ *(PrxQ1*, *PrxQ2*, and *PrxQ3*), and type II *(PrxIIB/C, PrxIIE,* and *PrxIIF).* All genes were found to be transcribed, except *PrxQ3*. The expression pattern among the various plant organs and tissues is gene specific, with some overlapping in genes of the same class. Additionally, we have assessed the response of *Prx* genes to treatments with classical hormones and related stress and signaling compounds in roots of hydroponically-grown plants. Most *Prx* genes, especially *1C-Prx*, were responsive to indole-3-acetic acid (IAA) or cytokinins (CKs). There were limited and specific changes of *Prx* expression after plant treatment with abscisic acid (ABA), 1-aminocyclopropane-1-carboxylic acid (ACC), gibberellic acid (GA), or jasmonic acid (JA). The unique expression pattern for each *Prx* gene within the plant and the specific response of *Prx* expression to hormones support a non-redundant role for most of these genes.

Introduction

In plants, reactive oxygen and nitrogen species (RONS) are produced during normal plant cell metabolism and are generated in the chloroplasts, mitochondria, peroxisomes, and apoplast (del Río *et al.,* 2002; Lamattina *et al.,* 2003). Overproduction of RONS is potentially toxic, although these molecules, at low steady-state concentrations, also fulfill essential functions in plant development and redox signaling. Thus, RONS need to be tightly regulated by antioxidant metabolites and enzymes. Among these, Prxs are ubiquitous, small proteins (17-29 kDa) that are able to reduce hydrogen peroxide, alkylhydroperoxides, and, in some cases, peroxynitrite (Rhee *et al.*, 2005; Tripathi *et al.,* 2009). Prxs themselves are also peroxide and redox sensors (Dietz *et al.,* 2006). In this work we have focused on the role of Prxs in symbiotic nitrogen fixation because nodules are a major site of RONS production (Besson-Bard *et al.,* 2008). The first step was to identify the complete suite of *Prx* genes and to determine their expression patterns in the model legume *Lotus japonicus*. Because hydrogen peroxide can act as a messenger in hormone signaling pathways (Kawano, 2003, Apel & Hirt, 2004), we have investigated also the effects of hormones on *Prx* expression levels in the roots.

Materials and Methods

Lotus japonicus (Regel) Larsen cv. MG20 plants were nodulated after 7 d from germination with *Mesorhizobium loti* strain R7A and grown for another 35 d in hydroponic cultures without nitrogen supply. The profiles of *Prx* expression were determined by qRT-PCR, and the Prx proteins were immunodetected with polyclonal antibodies raised against *Arabidopsis thaliana* (PrxQ, 2C-Prx, PrxIIC, and PrxIIF) or *Triticum aestivum* (1C-Prx) proteins. Plants were treated with 50 μM of several phytohormones for 48 h.

Results and Discussion

Screening of gene and EST databases of *L. japonicus* allowed us to identify nine *Prx* genes of this model legume (Table). These genes encode the four typical classes of Prx proteins known to occur in plants: 1Cys (1C-Prx), 2Cys (2C-PrxA and 2C-PrxB), PrxQ (PrxQ1, PrxQ2, and

PrxQ3), and type II (PrxIIB/C, PrxIIE, and PrxIIF). All genes were found to be actively transcribed, except *PrxQ3*, whose message was not detected under physiological conditions. The *2C-PrxA*, *2C-PrxB*, *PrxQ1*, and *PrxQ2* genes were transcribed in all organs tested, but mRNA levels were lower in pollen, roots, and nodules. The *1C-Prx* mRNA level was only abundant in embryos and, to a lesser extent, in seeds. The *PrxIIB/C* and *PrxIIF* genes were highly expressed in pollen and embryos respectively, with low basal expression in all other organs examined. The *PrxIIE* gene was expressed at a low constitutive level in all plant organs. At the protein level, 1C-Prx and PrxQ (1+2) were exclusively detected in embryos and leaves, respectively. The 2C-Prx proteins were observed in all photosynthetic organs, but not in roots, nodules, and pollen. The PrxIIB/C and PrxIIF proteins were detected in all tested organs but both proteins showed the highest abundance in pollen and embryos. The unique pattern of expression of the different Prx types suggests a non-redundant role for these genes.

We also evaluated *Prx* gene expression in response to hormone treatments in hydroponic cultures. Treatment with IAA caused induction of all *Prx* genes in roots, *1C-Prx* being the most responsive. In contrast, CKs only enhanced expression of *1C-Prx*, but reduced that of all other *Prx* genes. There were limited and rather specific changes of *Prx* gene expression after treatment with ABA, ACC, GA, or JA. These findings suggest a role for the *L. japonicus* Prx enzymes in modulating the concentration of hydrogen peroxide produced as a result of hormone supply.

Table. The *Prx* genes of *L. japonicus*. Localization of mRNAs and proteins in plant organs.

Gene	# ESTs[a]	Predicted protein subcellular localization	mRNA expression	Protein expression
1C-Prx	4	nucleus	Embryo (seed)	Embryos
PrxQ1	11	chloroplast	Green organs mainly	Leaves
PrxQ2	10	chloroplast	Green organs mainly	Leaves
PrxQ3	-	chloroplast	Not detected	-
2C-PrxA	48	chloroplast	Green organs mainly	Leaves (LCP[c])
2C-PrxB	62	chloroplast	Green organs mainly	Leaves (LCP[c])
PrxIIB	14	cytosol	Pollen and embryos (LCE[b])	Pollen and embryos (LCP[c])
PrxIIE	21	chloroplast	Constitutive expression	Not determined
PrxIIF	16	mitochondria	Embryos (LCE[b])	Pollen and embryos (LCP[c])

[a]Number of expressed sequence tags. [b]Low constitutive expression levels in other organs. [c]Low constitutive protein levels in other organs.

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Hemoglobins of *Lotus japonicus*: differential gene expression in plant organs and in response to hormones in nodules

Bustos-Sanmamed P[1], Tovar-Méndez A[1], Crespi M[2], Sato S[3], Tabata S[3], Becana M[1*]

[1]*Departamento de Nutrición Vegetal, Estación Experimental de Aula Dei, CSIC, Apartado 13034, 50080 Zaragoza, Spain;* [2]*Institute des Sciences du Végétale, CNRS, Gif-sur-Yvette, France;* [3]*Kazusa DNA Research Institute, 2-6-7 Kazusa-Kamatari, Kisarazu, Chiba 292-0818, Japan* *becana@eead.csic.es

Summary

In plants, three distinct types of hemoglobins (Hbs) are known: symbiotic, nonsymbiotic, and truncated. Nonsymbiotic Hbs are further divided into class-1 and class-2 based on primary sequences and oxygen-binding properties. Symbiotic Hbs are well-characterized, whereas the functions of the other two types of Hbs are not fully understood. We have identified five genes encoding two class-1 (*GLB1-1*, *GLB1-2*), one class-2 (*GLB2)*, and two truncated (*GLB3-1*, *GLB3-2*) Hbs in *Lotus japonicus*. *GLB1-2* and *GLB3-2* are expressed in all plant organs, whereas *GLB1-1*, *GLB2*, and *GLB3-1* are expressed abundantly only in nodules. We have assessed also the response of *GLB* genes in nodules to hormone treatments. *GLB1-1* was up-regulated by abscisic acid (ABA), 1-aminocyclopropane-1-carboxylic acid (ACC), and polyamines (PAs), whereas *GLB2* and *GLB3-1* were drastically down-regulated by cytokinins (CKs). *In situ* RNA hybridization showed that the *GLB1-1* and *GLB1-2* mRNAs are mainly localized in the cortex, vascular bundles, and fixation zone, whereas *GLB2* and *GLB3* mRNAs are mainly confined to the cortex and slightly to the vascular bundles. *GLB3-2* promoter activity was localized by GUS staining in the vascular and endodermis/sclerenchyma cells in the cortex of mature nodules.

Introduction

Hemoglobins (Hbs) are ubiquitous proteins in all organisms. In plants, three types of Hbs have been described: symbiotic, nonsymbiotic, and truncated. Nonsymbiotic Hbs are widespread in plants and are expressed in roots, leaves, stems, seeds, and legume nodules. A general function of GLB1 proteins is to modulate nitric oxide concentration (Dordas *et al.,* 2003; Hebelstrup *et al.,* 2006). Much less is known about GLB2 and GLB3, although it has been suggested that they may be involved in suppressing the defense response during the onset of symbioses with *Rhizobium*, *Frankia*, or mycorrhiza fungi (Vieweg *et al.,* 2005). A molecular study of the three types of Hbs is necessary in order to gather information on the expression and function of the *Hb* genes in the symbiotic interaction and nodule development.

Materials and Methods

Seedlings of *Lotus japonicus* (Regel) Larsen cv. MG20 were nodulated when 7 days old with *Mesorhizobium loti* strain R7A and grown for another 35 days in hydroponic cultures without nitrogen supply. The profiles of *GLB* expression were determined by real-time quantitative-PCR. Plants were treated with 50 μM of several hormones for 48 h. For *in situ* hybrydization, nodules were fixed in 4% formaldehyde, included in paraffine with Paraplast X-tra, and sectioned (10 μm). Probes were amplified by PCR from cDNA obtained from nodule RNA. All protocols have been published (Válóci *et al.,* 2006). The signal was visualized with substrate solution (nitroblue tatrazolium chloride, 5-bromo-4-chloro-3-indolyl phosphate toluidine). After stopping the reactions, sections were counter-stained with Alcian Blue, mounted with DPX, and examined with an inverted compound microscope. For *GLB3-2* promoter::GUS fusion constructs, a fragment of 1955 bp upstream from the ATG start codon was amplified by PCR. The product was cloned and the promoter was subcloned into the *Pst*I and *Nco*I sites of the binary vector pGFPGUS+. Hairy root transformation of *L. japonicus* with *Agrobacterium rhizogenes* LBA1334 carrying the pGUS-GLB3-2 vector was performed as described (Díaz *et al.,* 2005).

Results and Discussion

Screening of gene and EST databases of *L. japonicus* allowed us to identify five genes encoding two class-1 (*GLB1-1*, *GLB1-2*), one class-2 (*GLB2)*, and two truncated (*GLB3-1*, *GLB3-2*) Hbs. The *GLB1-2* and *GLB3-2* genes were found to be transcribed in all organs examined, whereas significant *GLB1-1*, *GLB2*, and *GLB3-1* mRNA levels were found only in

roots and more abundantly in nodules (Figure 1). *In situ* hybridization showed that *GLB1-1* and *GLB1-2* mRNAs are localized in the cortex, vascular bundles, and fixation zone, whereas *GLB2* and *GLB3* mRNAs are mainly confined to the cortex and slightly in vascular bundles (data not shown). Fusion constructs of the *GLB3-2* promoter with *GUS* confirmed gene expression in the vascular tissue and endodermis/sclerenchyma cells in the cortex of mature nodules (Figure 2).

Changes of *GLB* expression in response to hormone treatments were also examined. In nodules *GLB1-1* was upregulated in response to ABA, ACC, and PAs; however, *GLB2* was downregulated by PAs, and *GLB2* and *GLB3-1* mRNAs were undetectable after CK treatment. There were no changes in expression of the other *GLB* genes for any of the hormone treatments.

Our results show that *Hb* mRNA levels are highly dependent on plant organs and tissues, suggesting distinct roles for each gene. In particular, the strong expression of *GLB1-1*, *GLB2*, and *GLB3-1* in nodules may be associated to a role of the corresponding proteins during the rhizobia-legume interaction. Localization of *GLB2* and *GLB3* mRNAs in the nodule cortex but not in the infected zone, and complete down-regulation of *GLB2* and *GLB3-1* with CKs, point to a role of these proteins in nodule development rather than (directly) in N_2 fixation.

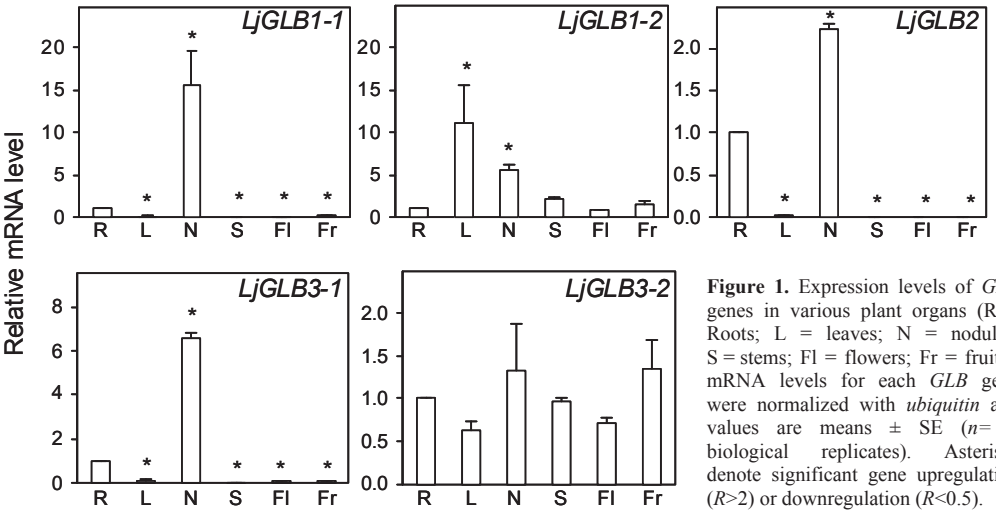

Figure 1. Expression levels of *GLB* genes in various plant organs (R = Roots; L = leaves; N = nodules; S = stems; Fl = flowers; Fr = fruits). mRNA levels for each *GLB* gene were normalized with *ubiquitin* and values are means ± SE (n= 4 biological replicates). Asterisks denote significant gene upregulation (R>2) or downregulation (R<0.5).

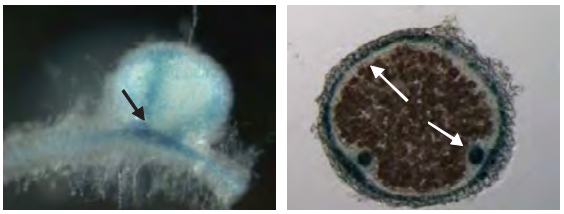

Figure 2. *GLB3-2* promoter activity. GUS staining was localized in vascular tissue and endodermis/sclerenchyma cells in the cortex of mature nodules.

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Session 5

Applications in agriculture and environment

Plant growth promotion, biological control and bioremediation mechanisms in novel plant-associated nitrogen-fixing *Burkholderia* species

Caballero-Mellado J*, Martínez-Aguilar L, Onofre-Lemus J, Wong-Villareal A, Castro-González R, Gómez-Hernández N, Ramírez-Trujillo A, Estrada-de-los-Santos P

*Centro de Ciencias Genómicas, Universidad Nacional Autónoma de México, Apartado Postal No. 565-A, Cuernavaca, Morelos, México. *jesuscab@ccg.unam.mx*

Summary

The genus *Burkholderia* includes 58 species isolated from a wide range of environmental niches and can be divided in two major clusters. The first cluster includes plant and animal pathogens and 17 species of the *Burkholderia cepacia* complex (Bcc) isolated from patients with cystic fibrosis. The other major cluster comprises 29 non-pathogenic species, which in most cases are found in association with plants, and many are N_2-fixing and legume-nodulating species. The N_2-fixing plant-associated *Burkholderia* species possess large genomes (7.0-10 Mb) structured in 2-5 replicons, usually chromosomes. These features in the diazotrophic *Burkholderia* could explain their great ability to colonize the rhizosphere and endophytic environments of many host plants. Moreover, it could account for their ability to produce biologically active compounds involved in plant growth promotion, such as indoleacetic acid (IAA) and 1-aminocyclopropane-1-carboxylate (ACC). The siderophore synthesis, characteristic in several N_2-fixing *Burkholderia*, seems to be involved in their antagonistic ability against plant pathogens. Some N_2-fixing plant-associated *Burkholderia* are able to grow on pollutant aromatic hydrocarbons as the sole carbon source. Transmissibility factors identified among clinical isolates of Bcc have not been detected in any of the plant-associated N_2-fixing *Burkholderia*. Certainly, the N_2-fixing plant-associated *Burkholderia* species represent a great potential for agro-biotechnological applications, which could lead towards using consortia of these species as plant growth promoting rhizobacteria (PGPR) and, concomitantly, in rhizoremediation and for biological control of plant pathogens.

Until recently, the genus *Burkholderia* included forty validly described species (Compant *et al.,* 2008), but this number has increased to 58 at present. *Burkholderia* species are widely distributed in the natural environment (Coenye & Vandamme, 2003), but they are found in the hospital environment as well, especially the *B. cepacia* complex (Bcc) species isolated from patients with cystic fibrosis (Mahenthiralingam *et al.,* 2005). Phylogenetic trees, based on 16S rRNA sequence analysis, show clearly the separation of the genus *Burkholderia* in two major clusters (Caballero-Mellado *et al.,* 2007), one of them mainly represented by plant, animal and Bcc species (Coenye & Vandamme, 2003). The second major cluster, phylogenetically largely distant from the Bcc, is formed exclusively by novel environmental non-pathogenic species described later than the year 2000, which include mainly plant associated, rhizospheric and/or endophytic bacteria, many of which are nitrogen-fixing (Caballero-Mellado *et al.,* 2004; Goris *et al.,* 2004; Perin *et al.,* 2006b; Reis *et al.,* 2004) and legume-nodulating species (Chen *et al.,* 2006; Elliott *et al.,* 2007; Vandamme *et al.,* 2002).

Historically, the ability to fix N_2, the reduction of atmospheric N_2 to ammonia, in bacteria of the genus *Burkholderia* was identified only in the species *B. vietnamiensis* (Gillis *et al.,* 1995), a member of the Bcc (Mahenthiralingam *et al.,* 2005). *B. kururiensis*, a trichloroethylene degrader (Zhang *et al.,* 2000), was soon after identified as a diazotrophic species (Estrada-de los Santos *et al.,* 2001). Nevertheless, analysis of important crop plants grown under field conditions revealed the genus *Burkholderia* to be rich in unknown diazotrophs (Estrada-de los Santos *et al.,* 2001). Subsequently, many N_2-fixing isolates were recovered from different plants, e.g., rice, maize, sugarcane, sorghum, coffee and tomato, or

from their rhizospheres. At present 9 diazotrophic plant-associated *Burkholderia* species have been described validly, for example, *B. unamae* (Caballero-Mellado *et al.,* 2004), *B. xenovorans* (Goris *et al.,* 2004), *B. tropica* (Reis *et al.,* 2004), and *B. silvatlantica* (Perin *et al.,* 2006b), as well as the legume-nodulating species *B. tuberum*, *B. phymatum* (Vandamme *et al.,* 2002), *B. mimosarum* (Chen *et al.,* 2006) and *B. nodosa* (Chen *et al.,* 2007). "*B. brasilensis*" strain M130 (Baldani *et al.,* 1997), a plant-associated species never described validly, has been re-classified as *B. kururiensis* (Caballero-Mellado *et al.,* 2007). $^{15}N_2$ isotope dilution assays revealed unambiguous diazotrophy in these novel species, and *nifH* gene sequence analysis, often used to determine phylogenetic relatedness between diazotrophs, show a tight clustering among *Burkholderia* species, which are clearly distinct from those of other diazotrophs (Martínez-Aguilar *et al.,* 2008). Although the absence of plasmids seems to be a rule more than an exception in strains of N_2-fixing *Burkholderia* species, the *nif* genes and the nitrogen-fixing ability in *B. tropica* strains has been showed to be located on a 450-600 kb plasmid, while in the other diazotrophic *Burkholderia* species such *nif* genes are located on chromosome (Martínez-Aguilar *et al.,* 2008).

The novel nitrogen-fixing plant-associated *Burkholderia* species possess large genomes, usually greater than 7.0 Mb, structured in 2 to 5 replicons, usually chromosomes (Martínez-Aguilar *et al.,* 2008). The large genomes and replicon multiplicity in the novel diazotrophic *Burkholderia* species could explain their great ability to colonize the rhizosphere and endophytic environments of a wide range of taxonomically unrelated host plants. Moreover, it could account for their ability to produce many biologically active compounds involved in promoting the plant growth, and for their metabolic versatility in using diverse aromatic hydrocarbons.

In addition to the potential diazotrophic role of the *Burkholderia* species, the 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity, involved in promoting root elongation, is expressed, for example, by the endophytic species *B. unamae*, and by many *Burkholderia* species, including the legume nodulating *B. tuberum* and *B. phymatum*, but not by *B. tropica* (Onofre-Lemus *et al.,* 2009). In *Burkholderia* species the *acdS* gene sequences are highly conserved (76-99% identity). Phylogenetic analysis of *acdS* gene sequences in *Burkholderia* showed a tight clustering among the Bcc species, which are clearly distinct from N_2-fixing plant-associated *Burkholderia*. ACC deaminase activities suggest a potential ability of most *Burkholderia* diazotrophic species to modulate ethylene levels and enhance plant growth as showed in tomato plants inoculated with an *acdS*-knockout mutant of *B. unamae*, and a transcriptional *acdSp-gusA* fusion (Onofre-Lemus *et al.,* 2009).

Indoleacetic acid (IAA), the main auxin involved in the plant growth promotion, has been detected in cultures from several N_2-fixing plant-associated *Burkholderia* species, for instance in *B. unamae*, *B. tropica*, and by the legume nodulating *B. phymatum* and *B. mimosarum*; the IAA production seem to be to through different biosynthetic pathways (unpublished results). Interestingly, some diazotrophic *Burkholderia* species are able to use IAA, both as sole carbon and nitrogen sources. Although the ecological role of bacteria that are capable of synthesizing and to degrade IAA is unknown at present, it could play a role as recyclers of IAA in association with plants.

Mineral phosphate solubilization appears to occur in most diazotrophic *Burkholderia* species (Caballero-Mellado *et al.,* 2007). Seed inoculation with efficient phosphate solubilizing bacteria is known to increase solubilization of fixed soil phosphorus and immobilized phosphates in the soil after the application of mineral fertilizers, resulting in higher crop yields. This remarkable ability to convert insoluble mineral phosphorus to an available form is an important trait for allowing these diazotrophic species to be defined as a PGPR.

Production of siderophores by PGPR is considered to be important in the suppression of deleterious microorganisms and soil-borne plant pathogens, and in some cases appear to trigger induced systemic resistance (ISR). The production of hydroxamate-type siderophores by *B. unamae* and *B. tropica* strains is a characteristic feature in these and other N_2-fixing species (Caballero-Mellado *et al.,* 2007). The synthesis of siderophores and/or unknown volatile compounds seems to be involved in the antagonistic ability of *B. unamae* and *B. tropica* against plant pathogenic fungi.

Several diazotrophic *Burkholderia* species, e.g., *B. unamae* and *B. kururiensis*, are able to grow on benzene and phenol as sole carbon sources, and the ability to grow on phenol confirmed with the detection and sequencing of phenol monooxygenases genes by PCR (Caballero-Mellado *et al.,* 2007). The ability of *B. unamae* to grow using aromatic hydrocarbons, as well as its widespread association with different plant species suggest it could be suitable for applications in rhizosphere remediation of common soil pollutants. In addition, taking into consideration the host plant range of *B. unamae* and its endophytic character, it would be of great interest to know the natural ability of this species to improve phytoremediation of volatile organic pollutants.

Transmissibility factors such as the *cblA* gene, encoding giant cable pili, and the epidemic strain marker regulator or *esmR* gene necessary for virulence and part of a genomic island, have been identified among clinical and environmental isolates of opportunistic pathogens of *B. cenocepacia* and other Bcc species, but have not been detected in many of the plant-associated N_2-fixing *Burkholderia* isolates analyzed (Caballero-Mellado *et al.,* 2007; Perin *et al.,* 2006; Wong-Villarreal & Caballero-Mellado, 2010).

Certainly, the nitrogen-fixing *Burkholderia* species associated with plants represent a great potential for agro-biotechnological applications, which could lead towards using consortia of these species as plant growth promoting rhizobacteria and, concomitantly, in rhizoremediation and/or for improving phytoremediation, and for biological control of plant pathogens.

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Climatic change and nitrogen fixation

Olivares J*

*Departamento de Microbiología y Sistemas Simbióticos, Estación Experimental del Zaidín, CSIC, Apartado 419, 18080 Granada, Spain. *jose.olivares@eez.csic.es*

Summary

Climatic change is understood to be the alteration in climate caused directly or indirectly by human activities which modify the global composition of the atmosphere, and/or to natural variations in the climate which occur during more or less long periods of time. The greenhouse effect derives from the accumulation of gases in the atmosphere that avoid the liberation of part of the infrared radiation released by the earth surface by natural causes or by the effect of human activity. Nitrous oxide (N_2O) is one of the principal contaminant gases and mainly derives from the denitrification of nitrogen fertilizers. Its impact on climatic change is important taking into account its global warming potential (GWP), which is 310-fold higher than that of CO_2. The best way to diminish the effect of agricultural practices on climatic change is to promote the efficient use of nitrogen fertilizers and to expand the field of biological nitrogen fixation (BNF) to plants other than legumes by manipulating them to either fix nitrogen *per se* or to be able to establish a *Rhizobium*-legume like symbiosis.

Como "cambio climático" se entiende una alteración del clima debida bien directa o indirectamente a las actividades humanas que modifican la composición global de la atmósfera, o/y a la variabilidad natural del clima durante períodos de tiempo más o menos largos. Que estemos en una situación u otra es motivo de controversia, aunque los datos de los parámetros considerados apoyan la influencia antropogénica en el cambio actual por el paralelismo existente entre la modificación de las constantes y la creciente actividad humana contaminante. Este hecho se hace evidente a partir de la industrialización con el uso masivo del carbón, petróleo y otros productos perjudiciales. La importancia de la atmósfera es fundamental para el desarrollo de la vida. Esta capa protectora de radiaciones nocivas mantiene una temperatura media en la superficie de la Tierra de unos 15ºC, lo que permite que el agua se encuentre en forma de poder ser usada por los seres que habitan el planeta. El efecto invernadero se deriva de la acumulación en la atmósfera de gases que impiden la salida de parte de la radiación infrarroja emitida por la superficie de la Tierra por causas naturales o por efecto de la mano del hombre.

Los principales gases contaminantes son el CO_2, CH_4, N_2O y los fluorocarbonados cuya concentración en la atmósfera ha crecido paralelamente a la industrialización y al aumento de la temperatura del aire en la superficie terrestre. Aunque los datos disponibles sobre los incrementos de la concentración de estos gases son esclarecedores sobre sus efectos sobre el calentamiento global, no hay acuerdo general, sin embargo, sobre la no existencia de causas naturales, independientes de la acción del hombre. De hecho, el clima no se ha mantenido estable a lo largo de cientos de miles de años e incluso en tiempos más cercanos, como son el periodo cálido medieval o la pequeña edad del hielo, en la Baja Edad Media y en la Edad Moderna, respectivamente. No todos los gases tienen el mismo potencial de GWP, que es la cantidad de calor atrapado por unidad de masa del gas contaminante con respecto al CO_2 tomado como uno, y por tanto, su efecto invernadero. Aunque todas las miradas se dirigen hacia el CO_2, por mayoritario, el papel de los otros no es nada despreciable teniendo en cuenta su elevado GWP. La principal fuente de N_2O en la atmósfera son los fertilizantes nitrogenados que se aplican masivamente, y muchas veces de forma improcedente, para obtener el máximo rendimiento de los cultivos. Una parte del fertilizante aplicado se pierde por lixiviación, y otra pasa a la atmósfera como nitrógeno molecular o en forma de óxidos

derivados de la actividad desnitrificante de los microorganismos del suelo. Estos óxidos, de gran poder contaminante, están implicados en la lluvia ácida, en la destrucción de la capa protectora de ozono y, como en el caso del N_2O, en el efecto invernadero, con un GWP de 310.

La fertilización nitrogenada presenta otros efectos colaterales ya que la obtención industrial de amonio da lugar a la producción de elevados niveles de CO_2. Por tanto, hay dos procesos contaminantes de la atmósfera en el caso del nitrógeno. Por un lado, la propia obtención del fertilizante y, por otro, su destino una vez aplicado a los cultivos, aparte de los problemas derivados de su lixiviación. Mientras que la aplicación de fertilizante nitrogenado de síntesis ha seguido una tendencia creciente desde el inicio de su producción industrial hasta hoy día que llega a las 100 x 10^6 Tm, la FBN se ha mantenido prácticamente constante. El cultivo de las leguminosas, que en simbiosis con *Rhizobium* suponen la principal vía de incorporación de N_2 molecular a la biomasa, apenas ha variado en un sentido u otro. Fuentes importantes de nitrógeno fijado, como las cianobacterias y otras bacterias fijadoras libres o en asociación con plantas se mantienen prácticamente constantes.

Globalmente, la emisión de gases con efecto invernadero debida a la agricultura supone el 25,5% del total (9,2% para la Europa de los 27). La reducción de estas cifras es un reto importante desde el punto de vista ambiental, industrial y energético. La European Fertilizar Manufacture Association (EFMA) promueve técnicas encaminadas al manejo productivo del suelo y al uso eficiente de los fertilizantes minerales para el control efectivo del impacto sobre el clima. Como era de esperar, la fijación biológica de nitrógeno no es tenida en cuenta entre las medidas a tomar para disminuir dicho impacto. Las emisiones de CO_2 derivadas del manejo del suelo no son muy altas en Europa, mientras que el N_2O y el CH_4 son más relevantes. Este último proviene principalmente de la ganadería y el N_2O del nitrógeno aplicado al suelo y de la producción de fertilizantes. Según el Intergovernmental Panel Climate Change (IPCC) se puede considerar que el 1% del nitrógeno aplicado pasa a la atmósfera como N_2O lo que supone alrededor de un millón de Tm año equivalentes a 310 x 10^6 Tm de CO_2.

La mejor forma de aminorar el efecto de las prácticas agrícolas en relación con el cambio climático es promover el uso eficiente de los abonos nitrogenados y el adecuado manejo del suelo, por lo que habría que evitar la sobreaplicación de fertilizante, usarlo en el momento oportuno o añadir inhibidores de la nitrificación cuando se utilicen formas químicas distintas al nitrato. La actuación en este sentido ha hecho que la eficiencia en el uso del nitrógeno haya crecido sobre un 45% desde 1985. El posible impacto de la fijación de N_2 libre o simbiótica en la emisión de gases de efecto invernadero no es superior a la que supone la incorporación de materia orgánica al medio. Como los altos rendimientos actuales de los diferentes cultivos dependen de la aplicación masiva, y muchas veces incontrolada, de los fertilizantes nitrogenados de síntesis, el camino más limpio lleva a ampliar el campo de actuación de la FBN con la extensión de esta facultad a cultivos distintos de las leguminosas, bien manipulando las plantas para que sean capaces de fijar el nitrógeno requerido o para establecer simbiosis del tipo *Rhizobium*-leguminosa. De todas formas, con estas actuaciones quedaría un importante problema por resolver ya que las plantas son más eficientes a la hora de utilizar nitrógeno combinado del suelo que el hipotético N_2 molecular.

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Climate change affects taproot reserves of nodulated alfalfa

Erice G[1]*, Sanz-Sáez A[1], Aranjuelo I[2], Irigoyen JJ[1], Aguirreolea J[1], Avice J-C[3], Sánchez- Díaz M[1]

*[1]Departamento de Biología Vegetal, Sección Biología Vegetal (Unidad Asociada al CSIC, EEAD, Zaragoza e ICVV, Logroño), Facultades de Ciencias y Farmacia, Universidad de Navarra, Pamplona, Spain. [2]Unitat de Fisologia Vegetal, Facultat de Biologia, Universitat de Barcelona, Barcelona, Spain. [3]INRA, UMR INRA / UCBN 950, EVA (Ecophysiologie Végétale & Agronomie, Nutritions N, C, S), Institut de Biologie Fondamentale et Appliquée, Université de Caen Basse-Normandie, 14032 Caen CEDEX, France. *gerisor@unav.es*

Summary

Growth responses to elevated CO_2 are dependent on sink-source status and it is generally accepted that photosynthetic down-regulation occurs in situations with insufficient C sink plant capacity. Lower rubisco activity results in decreased N demand from shoots and the fall of N_2 fixation by a N feedback mechanism related to increased N compounds in the vascular tissue. Alfalfa (*Medicago sativa*) management involves cutting of shoots altering source-sink relationship and hence photosynthetic behaviour. As growth rate decreased at the end of pre-cut vegetative growth, nodulated alfalfa plants showed photosynthetic down-regulation at elevated CO_2. However, during regrowth, after defoliation, acclimation to elevated CO_2 disappeared. Shoot harvest also leads to drop in C translocation to the roots resulting in N_2 fixation reduction due to the dependence on photosynthate supply to support nodule function. Therefore, the production of new shoots during the first days following cutting requires the utilization of reduced C and N compounds previously stored in reserve organs. Water stress conditions reduced shoot dry matter, but proteins stored in the taproot were enhanced, which may lead to better plant growth in the following cut/regrowth cycle.

Photosynthetic down-regulation under long-term exposure to elevated CO_2

The initial increase in photosynthesis in response to elevated CO_2 is followed by a drop without significant decrease in leaf conductance. Growth responses to elevated CO_2 depend on the ability of plants to develop new sinks or expand the existing ones. In this sense, many studies suggest that down-regulation is the consequence of an insufficient sink plant capacity (Ainsworth *et al.,* 2004). Because of the narrow relationship between C and N cycles, nitrogenase activity is regulated by photosynthesis, N availability and N demand. Besides, the repression of genes involved in photosynthesis is more severe in N limited plants via carbohydrate accumulation and sugar-mediated regulation.

However, some studies suggest that photosynthates increase does not result in improved nodule activity. Besides, plant and bacteroide soluble proteins and metabolic activities decline

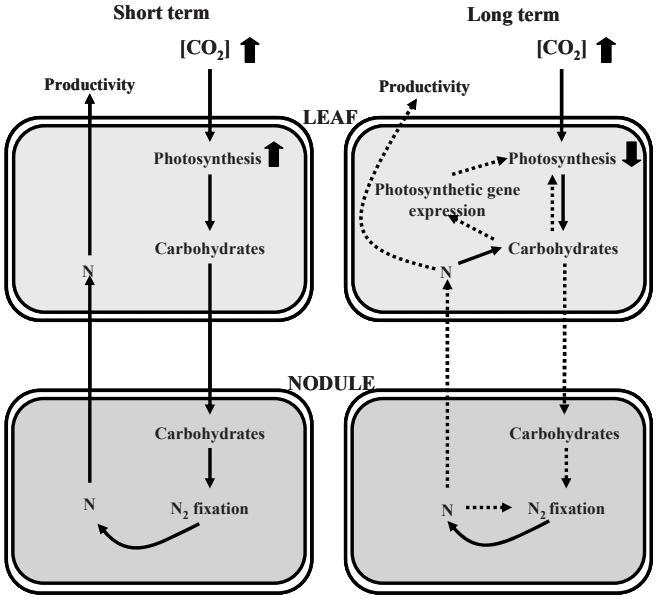

Figure. C and N mobilization between leaf and nodule after short or long term exposure to elevated CO_2. Solid lines indicate non-limited relationships whereas dotted lines indicate limited relationships.

in high CO_2 grown plants (Aranjuelo *et al.,* 2008), associated to the lower leaf N demand as a result of reduced rubisco activity (Almeida *et al.,* 2000). In a similar way to the imbalance between leaf C sources and sinks, it has been stated an imbalance for leaf N demand and its supply from nodules leading to the inhibition of nitrogenase activity in the bacteroids (Aranjuelo *et al.,* 2008) (Figure). In fact, the addition of increased doses of mineral N caused the disappearance of photosynthetic acclimation as C/N ratio was recovered in plant tissue.

Alfalfa management alters N_2 fixation and CO_2 plant response

Periodical shoot cutting leads to the rapid change in source/sink ratio due to the disappearance of source tissues and the formation of new sinks with developing shoots. Results with alfalfa are consistent with the source-sink balance hypothesis of photosynthetic down-regulation occurring in long-term elevated CO_2 treated plants (Ainsworth *et al.,* 2004). Photosynthesis acclimation at the end of pre-cut growth, measured as rubisco *in vivo*, *in vitro* and Vcmax, disappeared during regrowth, a very active growing phase. Defoliation causes the reduction of C supply to the roots and as a consequence nodule activity declines (Ta *et al.,* 1990). After cutting, N_2 fixation drops coupled to increased nodule protease activity and the disorganization and bacteroid loss in nodule cortical cells. However, 13 days after cutting, protease activity decreases, increasing soluble protein content as well as nitrogenase activity and new nodules are formed (Vance *et al.,* 1979). Shoot cutting impacts drastically photosynthetic rate and N acquisition. Therefore the production of new shoots during the first days following the cutting requires the utilization of reduced C and N compounds, being the availability of organic reserves a critical factor during regrowth.

Nitrogen reserves from taproot as key organic nutrients for alfalfa regrowth

During the first days following defoliation an active mobilization of endogenous C and especially N reserves in the taproot of nodulated alfalfa occurred. In leguminous forages like alfalfa several studies have demonstrated that availability of N reserves, particularly the Vegetative Storage Proteins (VSP), is closely related to the potential of shoot growth (Justes *et al.,* 2002). Plants showing the highest VSP accumulation exhibit faster growth of buds and higher shoot production. VSP accumulation is mediated by methyl jasmonate and ABA also promotes N allocation towards the roots. Alfalfa plants subjected to drought showed 30% reduction in biomass but 78% increase in VSP taproot concentration (Erice *et al.,* 2007). Therefore, in alfalfa subjected to water deficit, induction of VSP may be mediated by ABA. The N reserve increase in the taproot can be an important adaptive trait to withstand low water availability. This situation could occur in Mediterranean areas under climate change scenario, allowing plants to grow in better conditions during the following cutting/regrowth cycle.

Acknowledgments

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Plant Growth Promoting Rhizobacteria for protection against drought

Narváez-Reinaldo JJ, Vílchez JI, Oliver-Jacobo A, SantaCruz-Calvo L, Picazo-Espinosa R, González-López J, Manzanera M*

*Department of Microbiology and Institute of Water Research, University of Granada, Spain 18071. *Manzanera@ugr.es*

Summary

A collection of desiccation tolerant microorganisms has been obtained based on a new technology developed in our laboratory. This technology combines the use of selective agents, with a quick test for the counter-selection of spore forming bacteria. The survival rates to desiccation of the different isolates were tested and those with more than 20% survival were selected for further experiments. This collection was tested for the protection of plants with agriculture interest including pepper (*Capsicum annuum*) and tomato (*Lycopersicon esculentum*) plants. The different microorganisms were taxonomically characterized by 16S rRNA sequencing and by DNA-DNA hybridization and all different isolates were proposed as members of the *Actinobacteria* class. We have proven that some of these isolates promote plant growth and some others protect plants against drought. The ability to use polycyclic aromatic hydrocarbon (PAHs) have been tested as well for their potential use in bioremediation of arid and semiarid regions.

Introduction

Plant growth and development is deeply affected by lack of water, defining the productivity of major crops and efficiency of rhizoremediation programs in most regions of the world (Boyer, 1982; Tollenaar & Wu, 1999). Plant growth promoting rhizobacteria (PGPR) are defined as those bacteria which can colonize plant roots and improve plant growth. These so-called PGPR often prevent the negative effects of abiotic stressors from the environment such as extreme temperatures or hyperosmotic conditions (Kloepper & Schroth, 1978; Bai *et al.,* 2003). In our group we have previouly shown that desiccation sensitive *Pseudomonas putida* KT2440 can be artificially made desiccation tolerant (Vilchez *et al.,* 2008). Using a similar technology we have developed a new method for the isolation of a collection of natural desiccation tolerant microorganisms from the rhizosphere of plants.

Materials and Methods

For the isolation of desiccation tolerant bacteria one gram of air-dried soil was mixed with 10 ml selective agent and thoroughly mixed. After the incubation time, the mixtures were transferred onto sterile glass plates and incubated in sterile conditions to allow for the complete evaporation of the agent. Then the soil was mixed with water, after the soil particles settled, serial dilutions were made and a 100 µl aliquot from each dilution were plated on TSA medium plates. After 48 h of incubation at 30°C, individual colonies were randomly picked and streaked out to obtain pure cultures. To determine survival rates, a colony of each pure culture containing 10^7–10^9 cells was resuspended in 1 ml of M9 minimal medium. Aliquot volumes (100 µl) were placed on sterile Petri dishes and dried under a current of sterile air for 24 h. Cells were reconstituted in 1 ml of sterile M9 buffer, and serial dilutions of the cell prior and after drying were plated on TSA plates. The survival rate (%) was calculated as the rate of cells/ml after drying with reference to cells/ml before drying. The assays were performed in triplicate. Plants material and growth conditions were based on the protocol established by Mayak *et al.* (2004). Data were analysed by analysis of variance (ANOVA), and pairwise comparisons were done using a student's *t* test. All hypotheses were tested at the 95% confidence level.

Results and Discussion

The first aim of this work was the isolation of a collection of desiccation-tolerant, non-sporulating microorganisms to study their effect on tomato and pepper plants subjected to drought stress (Figure 1). Then, we tested 13 desiccation-tolerant strains for their potential for growth promoting of pepper and tomato plants.

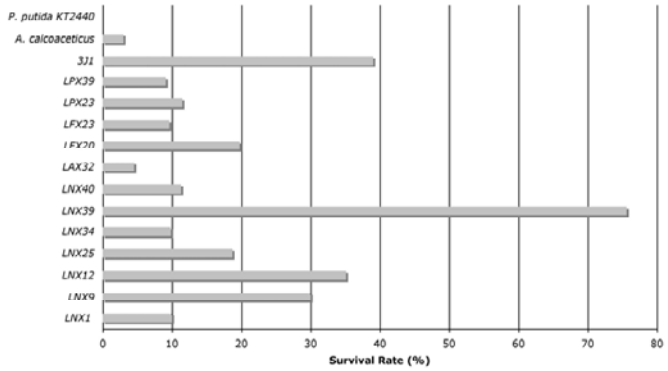

Figure 1. Survival rate after desiccation protocol of the different isolates. Values in percentage and name of the isolates, are shown the x and y-axes respectively. *P. putida* KT2440 and *A. calcoaceticus* were used as negative and positive controls respectively.

The protective effect of two of these isolates that showed the highest level of desiccation tolerance (a *Microbacterium* sp. and an *Arthrobacter* sp) was extended over pepper plants. As a positive control we included *Pseudomonas putida* KT2440, which is known as a saprophytic bacterium and has been used as a model system for studying the biodegradation and interactions of a nonsymbiotic microorganism with plants. As negative control same volume of cell free incolum was used (Figure 2). Similar results were found on tomato plants. A parallel experiment was set up under watering conditions and showed that the *Microbacterium* sp. strain but not the *Arthrobacter* sp one promoted stronger plant growth than *P. putida* KT2440 in these conditions.

Figure 2. Effect of different isolates over pepper plants after 33 days of drought. From left to right pepeer plants incolated with a *Microbacterium* sp., with an *Arthrobacter* sp., with *P. putida* KT2440 and a non-inoculated plant.

Acknowledgments

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Root colonization and growth promotion of tomato and pepper seedlings by *Rhizobium leguminosarum* isolated from peat

García-Fraile P[1], Robledo M[1], Peix A[2], Igual JM[2], Mateos PF[1], Rivas R[1], Velázquez E[1*]

*[1]Departamento de Microbiología y Genética, Universidad de Salamanca; [2]Instituto de Recursos Naturales y Agrobiología de Salamanca, Consejo Superior de Investigaciones Científicas (IRNASA-CSIC), Salamanca, Spain. *evp@usal.es*

Summary

In the present work several strains were isolated from effective nodules of common bean (*Phaseolus vulgaris*) cultivated in peat. These strains were identified on the basis of their *rrs, atpD* and *recA* gene sequences and 16S-23S intergenic spacer (ITS) as *Rhizobium leguminosarum*. The analysis of the *nodC* genes showed they belong to the biovar phaseoli of this species and they were very effective as common bean endosymbionts. From the results of symbiotic effectiveness on this legume, we select the strain TPV08 for GFP labelling in order to analyse its ability to colonize the roots of tomato (*Lycopersicum esculentum*) and pepper (*Capsicum annuum*) by fluorescence and confocal microscopy. The results obtained showed that this strain is able to colonize the roots of both plants and thus the ability of the strain TPV08 as PGPR for tomato and pepper was analysed. For this we inoculated with this strain the seeds of both plants maintaining them four weeks in a growth chamber. The seedlings obtained were significantly more developed than those uninoculated and have a dry weight almost three times higher than the control in the case of tomato and two in the case of pepper.

Introduction

Pepper and tomato are two of the most economically important crops in Spain that are mainly carried out in integrated greenhouse with controlled intake of nutrients. The high cost of these farming systems translates into higher prices for the consumer who demands the highest quality in the final product that includes the reduction of chemical fertilizers. The plant growth promotion by microorganisms is one of the most attractive alternatives to chemical fertilizers enabling the elimination or at least the reduction of phytochemicals. Rhizobia are able to fix nitrogen in legume symbiosis but also to promote the plant growth of lettuce and cereals (Chabot *et al.*, 1996; Peix *et al.*, 2001) and therefore they are promising for use as inoculants for both legumes and non-legume plants (Bhattacharjee *et al.*, 2008).

Material and Methods

The strains were isolated using common bean var. 'Pinta' as trap plant and a commercial peat as substrate. We selected only the nodules highly effective formed in five different plants and the strains were isolated on yeast mannitol agar. The plasmid profile analysis and effectiveness studies were carried out as was recently descibed (Ramírez-Bahena *et al.*, 2009). The identification of strains were performed by sequencing of different chromosomal markers as was previously described (Ramírez-Bahena *et al.*, 2008). The GFP labelling was done according to Cheng (1998) and the labelled strains were inoculated on 5 days germination seedlings in water-agar plates. Non-labelled strains were also inoculated into seeds and they were germinated in peat, watered with commercial Hoagland's solution and maintained in growth chamber during 4 weeks. After that they were removed and drying to obtain the weights of plants.

Results and Discussion

The strains isolated from nodules of common bean plants cultivated on peat presented a 16S rRNA gene identical to that of the type strain of *Rhizobium leguminosarum* USDA 2370^T as was already observed for other strains isolated from this legume in several Spanish soils (García-Fraile *et al.*, 2010). However, despite that all of them were close to *R. leguminosarum*, they do not belong to the same phylogenetic group when the *recA* and *atpD* genes were analysed. The strains presented four different plasmids profiles from which a representative strain was selected from effectiveness studies in *P. vulgaris*. These studies

showed that the more effective strains were TPV05 and TPV08 and from them, strain TPV08 was selected for GFP labelling and inoculation of tomato and pepper experiments.

The labelled strain TPV08 was able to colonize both tomato and pepper roots after 48h contact with them as was observed by fluorescence and confocal microscopy.

When seeds of these both plants were inoculated and maintained during four weeks in growth chamber significant differences were found between inoculated and non-inoculated plants. The inoculated plants have a dry weight almost three times higher than the control in the case of tomato and two in the case of pepper (Table).

Table. Weight of seedlings after four weeks of inoculation.

Treatment	Shoot dry weight	Root dry weight
Tomato		
Uninoculated control	13,7a	6,7a
TPV08	33,8c	14,2b
Pepper		
Uninoculated control	14,3a	8,3a
TPV08	22,7b	19,0b

Values followed by different letter are significantly different from each other at P=0.05 according to Fisher's Protected LSD (Least Significant Differences).

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Survey of diazotrophic bacteria associated to annual ryegrass in the "montado" ecosystem

Fareleira P*, Delgado Rodríguez AI, Ferreira E

*L-INIA/INRB, IP, Quinta do Marquês, Av. da República, 2784-505 Oeiras, Portugal. *paula.fareleira@inrb.pt*

Summary

Two soils (granite and schist) from "montado" were surveyed for enumeration and isolation of putative diazotrophic bacteria associated to annual ryegrass, either in the root external environment and plant tissues. Different N-free culture media were used, allowing for the selection of distinct metabolic types: aerobic heterotrophs, microaerophilic malate-users, and diazotrophic *Burkholderia*-like bacteria. Heterotrophic aerobes were the largest population in all samples, with particularly high counts in rhizosphere and rhizoplane samples. Considerable numbers of microaerophilic malate-users and azelaic acid-users were also obtained in most samples. Approximately 300 isolates were recovered from each culture media and are currently being characterized for nitrogen fixing activity, as well as other plant growth promoting activities.

Introduction

Several cereal crops, such as rice, maize or wheat, were already recognized as having the ability to establish associative interactions with either free-living or endophyte nitrogen-fixing microorganisms, and diazotroph-based inoculants for these crops start to be envisaged as a plausible way of supplying a significant part of their nitrogen requirements. However, the lack of adaptability of inoculant diazotrophs to local environmental conditions seems to be a common cause of low performance. This can be particularly significant in marginal lands, where the survival of introduced strains will mostly depend on saprophytic competence and ability to face local conditions. In such regions, the impact of high input rates of nitrogen fertilization can be severely aggravated by diminished soil structure. Thus, the introduction of biofertilizers containing adaptable strains of highly effective diazotrophs is an issue of utmost importance for vulnerable regions. Here we describe the very first steps of a starting project towards the development of a nitrogen-fixing biofertilizer for annual ryegrass, a forage crop that is extensively used in poor-productive areas dedicated to the exploitation of cork and holm oaks (the "montado" or "dehesa" ecosystem) in southern Portugal. Our initial strategy was to trap native ryegrass-associated diazotrophs from representative local ecosystems. Population sizes were evaluated and an assortment of isolates from both the rhizosphere soil and plant tissues was constituted, aiming at obtaining representatives of each free-living and endophytic diazotrophs; this will be the starting material for a series of characterization-selection steps for good colonizing ability and growth-promotion of this plant.

Materials and Methods

Surface soils were collected from natural pastures associated to "montado" in two representative spots of the main geological types at Alentejo, schist and granite (December 2009). Both sites were located in the same agricultural exploitation (Herdade do Zambujeiro, Crato). Seeds of annual ryegrass (*Lolium multiflorum* L.) were surface disinfected and transferred to flasks containing the soils. After incubation for 8 weeks in a plant growth chamber, three groups of 6-8 plants from each soil were selected and used for enumeration and isolation of diazotrophic associated bacteria using described procedures (Estrada-de los Santos *et al.,* 2001). The root-adherent soil fraction was recovered, as well as rhizoplane-associated bacteria. Roots, stems and leaves were pooled and sectioned, surface sterilized with 3% sodium hypochlorite and homogenized. Tenfold dilutions of rhizosphere soils, rhizoplane bacterial suspensions and plant tissues homogenates were inoculated on N-free culture media supplemented with 20 mg/L cycloheximide. Burk's solid medium (Martinez-Toledo *et al.,* 1985) was used for colony forming units (CFU) counts of aerobic populations; single colonies were randomly selected from each sample and transferred to fresh agar plates until purity was established. N-free semi-solid malate medium (NFb) (Döbereiner *et al.,* 1976) was used for Most Probable Number (MPN) estimations and isolation

of microaerophilic bacteria. Additionally, N-free semi-solid azelaic acid medium (BAz) (Estrada-de los Santos *et al.,* 2001) was used for selection of diazotrophic *Burkholderia*. In either case, positive tubes showing a subsurface pellicle were transferred 1-2 times in the same media and then streaked out on N-supplemented NFb or BAc (azelaic acid-citrulline) agar plates, respectively; single colonies were purified as described above.

Results and Discussion

Two soil types (granite and schist) from "montado" were surveyed for enumeration and isolation of diazotrophic bacteria associated to annual ryegrass. Previous results obtained in our lab indicated that significant levels of diazotrophic populations and nitrogen fixation associated to this plant could be often observed in such ecosystems, suggesting a good potential for isolation of high-performing diazotrophic strains (Ferreira *et al.,* 2005). In view of this, we used different N-free culture media to target diazotrophs in either the root external environment and plant tissues, allowing for the selection of distinct metabolic types: aerobic heterotrophs, microaerophilic malate-users, and diazotrophic *Burkholderia*-like bacteria able to grow with azelaic acid as only carbon source. The results are presented in the Figure.

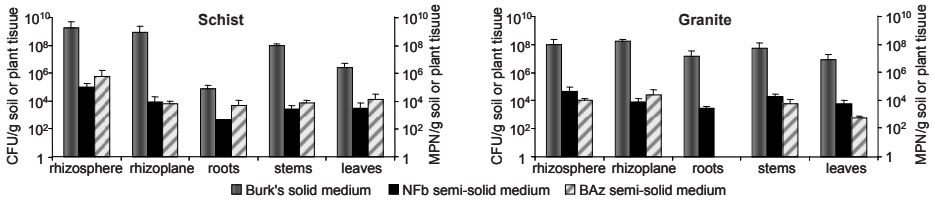

Figure. Levels of putative diazotrophic bacterial populations associated to the root external environment and tissues of annual ryegrass plants grown in schist and granite soils from "montado". CFU of aerobic diazotrophs were determined in N-free Burk's solid medium. MPN counts of microaerophilic populations were evaluated in N-free malate (NFb) and azelaic acid (BAz) semi-solid media. Values are means ± SE of 3 replicate groups of 6-8 plants.

Both soils presented high counts for putative diazotrophs, although with some variability among replicates. Heterotrophic aerobes were the largest population in all samples, the highest CFU counts being found in the rhizosphere and rhizoplane of plants from both soils. Recovered isolates (*c.* 300) presented 6 to 7 colony types, varying in shape, colour, transparency and viscosity. Microaerophilic diazotrophs, either malate- and azelaic acid-users, were also found in most samples, including the root external environments and plant tissues; the exception was the root homogenates of plants grown in granitic soil, where no azelaic acid-utilisers were detected (lower detection limit: 6.5×10^2 cells/g tissue). Approximately 300 isolates were recovered from each semi-solid medium (NFb and BAz). Sub-surface pellicles formed in semi-solid azelaic acid medium varied in colour (white-yellowish), depth (1-2 mm or up to 10 mm below surface), and thickness; at least three colony types could be distinguished in solid medium. All isolates are currently being surveyed for nitrogenase activity, as well as other biochemical traits potentially beneficial for plant growth. These assays will be the first steps to select a smaller set of " potentially interesting" isolates, which after identification by molecular approaches will be fully characterized in what concerns invasiveness, persistence upon inoculation, N_2-fixing activity and other growth-promoting effects on annual ryegrass.

Acknowledgments

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Relationship between photosynthetic capacity, nitrogen assimilation and nodule metabolism in alfalfa grown with sewage sludge

Fiasconaro ML, Sánchez-Díaz M, Antolín MC*

*Dpto. Biología Vegetal, Sección Biología Vegetal, Unidad Asociada al CSIC (EEAD, Zaragoza e ICVV, Logroño), Facultades de Ciencias y Farmacia, Universidad de Navarra, Pamplona, Spain. * cantolin@unav.es*

Summary

Sewage sludge has been used as N fertilizer because it contains some inorganic N, mainly as nitrate and ammonium ions. However, sewage sludge addition to legumes could result in impaired nodule metabolism due to the presence of inorganic N from sludge. A greenhouse experiment was conducted to examine the effects of sewage sludge on growth, photosynthesis, N assimilation and nodule metabolism in alfalfa (*Medicago sativa*). The experiment included three treatments: (1) plants inoculated with rhizobia and amended with sewage sludge at rate of 10% (w/w) (RS); (2) plants inoculated with rhizobia without any amendment (R); and (3) non-inoculated plants fed with ammonium nitrate (N). N_2-fixing plants had lower growth but higher photosynthesis than nitrate-fed plants. Sewage sludge-treated plants evidenced lower photosynthetic capacity and N_2 fixation efficiency than untreated plants. Besides, sewage sludge addition did no affect nodulation but induced a decrease in nodule enzyme activities involved in C and N metabolisms which may lead to accumulation of toxic N-compounds.

Introduction

Sewage sludge has been used as N fertilizer in legumes (Fernández-Luqueño *et al.,* 2010; Antolín *et al.,* 2010). However, combined N (especially nitrate) can inhibit nodulation and nodule activity (Luciński *et al.,* 2002). The aim of this study was to compare the effects of sewage sludge and mineral fertilizer on growth, photosynthesis and N assimilation of alfalfa. Specifically we sought to determine the impact of sewage sludge addition on nodule activity.

Materials and Methods

The experimental design included three treatments: (1) Plants of alfalfa (*Medicago sativa* cv. Aragón) inoculated with *Sinorhizobium melioti* strain 102F34 and amended with sewage sludge (RS) at rate of 10% (w/w), which is equivalent to *c.* 30 Tm dry matter (DM) ha^{-1}; (2) plants inoculated with rhizobia without any amendment (R); and (3) non-inoculated plants fed with ammonium nitrate (N) as a control for comparison.

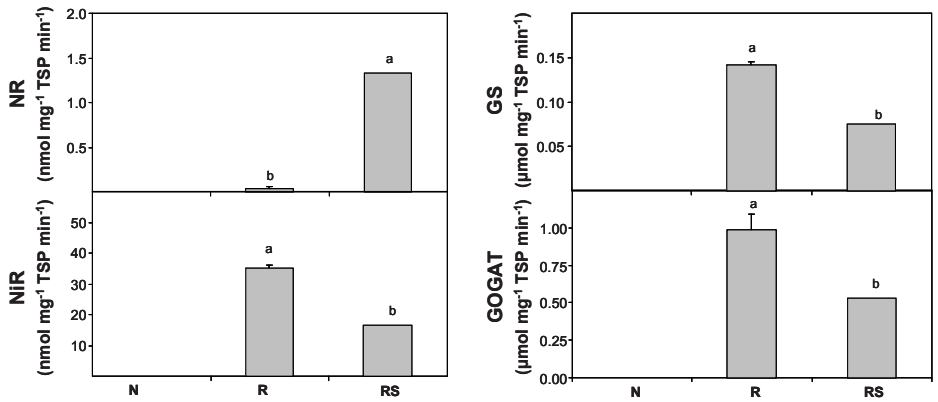

Figure. N assimilation enzyme activities in nodules of N_2-fixing alfalfa plants grown in soils amended with sewage sludge (RS) or in untreated soils (R). Values represent means (n=5); bars indicate standard error (S.E.) of the mean. Different letters indicate significant differences ($p \leq 0.05$) between treatments according to the Tukey's test. *Abbreviations:* NR, nitrate reductase; NiR, nitrite reductase; GS, glutamine synthetase; GOGAT, glutamate synthase.

Results and Discussion

N_2-fixing alfalfa plants had lower growth and higher photosynthesis than nitrate-fed plants because they compensated carbon cost of the rhizobia (Table). Plants amended with sewage sludge did no depend exclusively on N_2 fixation and could utilize combined N from soil. Thus, sewage sludge-treated plants evidenced a loss of carbon sink strength due to N_2 fixation by means of decreased photosynthetic capacity, leaf chlorophylls and N concentration in comparison to untreated plants. Sewage sludge application did no affect nodulation ability but data provide evidence for low N_2 fixation efficiency, which was characterized by decreased nodule enzyme activities involved in C (Table) and N (Figure) metabolisms. These changes could lead to accumulation of toxic N-compounds, which could contribute to nodule damage (Becana *et al.,* 1989).

Table. Growth, plant and nodule activity of nitrate-fed (N) and N_2-fixing alfalfa plants grown in soils amended with sewage sludge (RS) or in untreated soils (R). DM: dry matter. TSP: total soluble proteins. Values are means (n=5). *Abbreviations:* DM, dry matter; GOT, glutamate-oxaloacetate transaminase; PEPC, phospho*enol*pyruvate carboxylase; MDH, malate dehydrogenase.

Measurement	N	R	RS
Plant growth			
Plant DM (g plant^{-1})	5.44 a	3.10 b	3.55 b
Nodule DM (g plant^{-1})	0 b	0.17 a	0.15 a
Photosynthetic parameters			
Leaf chlorophylls (mg m^{-2})	348.76 b	586.97 a	379.96 b
Leaf N (mg g^{-1} DM)	55.28 a	48.52 a	35.80 b
Net photosynthesis (µmol m^{-2} s^{-1})	2.52 b	7.90 a	7.26 a
J_{max} (µmol m^{-2} s^{-1})	180.11 ab	256.8 a	119.30 b
V_{cmax} (µmol m^{-2} s^{-1})	42.59 b	61.02 a	38.26 b
Nodule activity			
PEPC (µmol mg^{-1} TSP min^{-1})	--	7.98 a	1.82 b
MDH (mmol mg^{-1} TSP min^{-1})	--	0.073 a	0.021 b
Nitrogenase (µmol mg^{-1} TSP min^{-1})	--	0.023 a	0.032 a
GOT (µmol mg^{-1} TSP min^{-1})	--	6.98 a	0.58 b

Acknowledgments

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Alfalfa nitrogen fixation under climate change depends on *Sinorhizobium* strain and growth season

Sanz-Sáez A*, Erice G, Irigoyen JJ, Aguirreolea J, Sánchez-Díaz M

*Departamento de Biología Vegetal, Sección Biología Vegetal, (Unidad Asociada al CSIC, EEAD, Zaragoza e ICVV, Logroño) Facultades de Ciencias y Farmacia, Universidad de Navarra, c/Irunlarrea 1, 31008 Pamplona, Spain. *assaezdejau@alumni.unav.es*

Summary

Future climatic conditions of increasing CO_2 and temperature may alter the efficiency of legume N_2-fixing symbiosis. *Rhizobium* strains are known to be differently efficient according to ambient conditions. The aim of the study was to analyze the effect of CO_2 and temperature on the yield and Apparent Nitrogenase Activity (ANA) of alfalfa (*Medicago sativa*) inoculated with different *Sinorhizobium meliloti* strains and developed in two experimental periods (summer and autumn) with a similar degree-days accumulation. Plants were grown in Temperature Gradient Greenhouses (TGG) and were inoculated with *S. meliloti* strains 102F78, 102F34 or 1032GMI. The interaction between elevated CO_2 and increased temperature enhanced plant production and ANA per plant in the two seasons. The most efficient strain was 102F78 in summer and 102F34 in autumn, confirming that the N_2 fixing efficiency of the strains is a key factor for yield. The elevated CO_2 enhanced N availability and thus plant production by increasing nodule mass, but not nodule specific activity.

Introduction

The Intergovernmental Panel on Climate Change prediction (IPCC, 2007) includes the rising of atmospheric CO_2 concentration up to 700 ppm at the end of the present century leading to a temperature increase of 4ºC. The primary effect on plants of these new conditions is the photosynthesis enhancement and, consequently, productivity. However, this higher growth in response to CO_2 may be limited by N availability. In this context, legumes are of particular interest due to their symbiotic relationship with N_2 fixing bacteria which provides N autonomy. The interaction between *S. meliloti* strain and alfalfa genotype has been demonstrated to alter the plant's response to CO_2 (Bertrand *et al.,* 2007). For that reason, when considering the response of alfalfa to future climate conditions, the selection of the more efficient combination *S. meliloti* strain / alfalfa genotype should be taken into account. Therefore, the objective of the present work was to study, under simulated climate change conditions, the N_2-fixing efficiency of different strains of *S. meliloti* in symbiosis with alfalfa developed in two growing seasons (summer and autumn) with similar degree-days accumulation.

Material and Methods

Alfalfa (*Medicago sativa* cv. Aragón) plants (20 plants per pot in 13 L pots containing perlite) were inoculated with *S. meliloti* (102F78, 102F34 or 1032GMI strains) and grown in TGG in Pamplona (42.80N, 1.66W; Spain). Plants were divided into 12 treatments: two CO_2 levels (ambient, 350 ppm, and 700 ppm), two temperature regimes (ambient and ambient + 4ºC) and three *S. meliloti* strains. Plants were irrigated with Evans N-free nutrient solution (Evans, 1974). The experiment was carry out in two different seasons, with similar degree-days accumulation (around 750), which means eight weeks growth during summer and nine in autumn. In both cases the harvest was made just before flowering. At the end of the experiment, dry matter (DM) and Apparent Nitrogenase Activity (ANA) were determined by using an electrochemical H_2 sensor according to Witty & Minchin (1998).

Results and Discussion

In summer plant growing in the TGG under elevated CO_2 and temperature showed increased production regardless of the *Sinorhizobium* strain (Fig. 1). Nevertheless, 102F78 was the only one which increased DM under elevated CO_2 at ambient temperature. In autumn, elevated CO_2 interacted positively with temperature in 102F34 and 1032GMI strains, whereas 102F78

increased DM by elevated temperature regardless of the CO_2 conditions (Fig. 1). Contrariwise to that observed in summer, in autumn the most efficient strain was 102F34, reaching similar values to those observed for 102F78 in summer (Fig. 1).

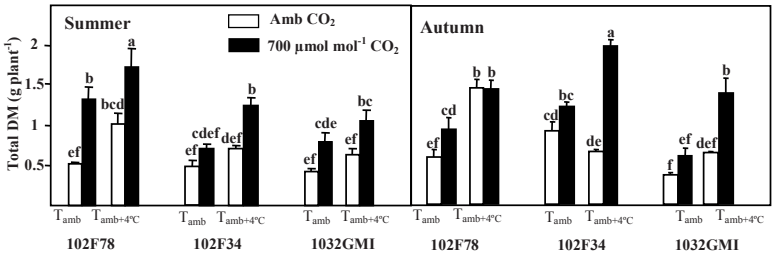

Figure 1. Effect of CO_2 and temperature on total dry matter (DM) in alfalfa nodulated with three *S. meliloti* strains after growing in the TGG in summer and autumn. Bars followed by the same letter are not significantly different (P<0.05).

At elevated CO_2 and temperature, ANA per plant (Fig. 2A) was significantly higher in autumn with all strains, and mainly with 102F78 in summer. These results are in accordance with DM values, except in the case of 102F78. However, when ANA was expressed on a nodule weight basis, CO_2 as a single factor induced a decrease with all strains, both in summer and autumn, and no interactive effect with temperature was observed. The close relation between total DM and ANA may indicate better nodule activity in autumn, with cooler temperatures, as also observed Aranjuelo *et al.* (2007), who showed that N_2 fixation was inhibited by elevated temperature. As a consequence of the enhanced N availability under high CO_2, nodule mass increased (data not shown), without a concomitant increase in specific activity (Fig. 2B). The elevated CO_2 enhanced N availability and thus plant production by increasing nodule mass, but not nodule specific activity.

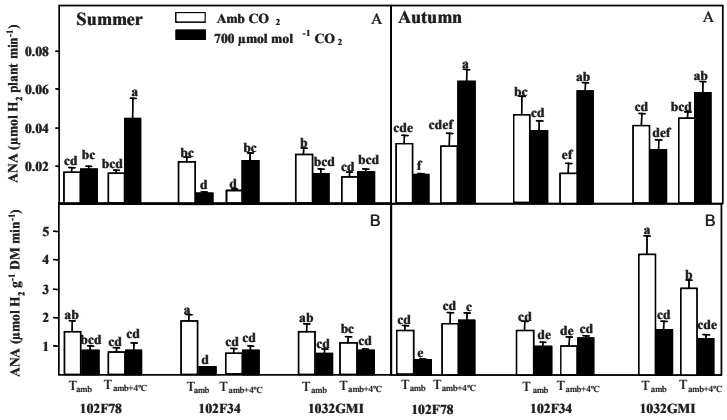

Figure 2. Effect of CO_2 on ANA, expressed on a plant (**A**) or nodule DM (**B**) basis, in alfalfa nodulated with three *S. meliloti* strains after growing in the TGG in summer and autumn.

Acknowledgments

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Medicago truncatula germplasm screening for cadmium and mercury tolerance

García de la Torre VS, Coba de la Peña T, Lucas MM, Pueyo JJ*

*Department of Plant Physiology and Ecology, Instituto de Recursos Naturales, Centro de Ciencias Medioambientales, CSIC, Serrano 115-bis, E-28006 Madrid, Spain. *pueyo@ccma.csic.es*

Summary

Cadmium (Cd) and mercury (Hg) tolerance were determined for 118 *Medicago truncatula* accessions obtained from the USDA National Plant Germplasm System using a hydroponic culture system. Differences in relative root growth (RRG) allowed ranking of the tested accessions into three levels of tolerance: sensitive, intermediate and tolerant. Cd and Hg tolerances were compared and a significant correlation was found, suggesting the prevalence of certain common mechanisms involved in the tolerance to both toxic metals. *M. truncatula* accessions with elevated metal tolerance to either Cd or Hg, as well as accessions displaying significant tolerance to both metals were identified.

Introduction

Cadmium (Cd) and mercury (Hg) are environmental pollutants that pose an important risk for human health and crop production. Heavy metals cannot be degraded, but can be removed and stored or else stabilized in the soil. Phytoremediation is an emerging technology that uses plants and sometimes their associated rhizospheric microorganisms to remove contaminants from waters and soils. Phytoremediation constitutes a cost-effective environmentally-friendly alternative technology for soil detoxification (Zhao & McGrath, 2009). It requires plant species or varieties with high metal tolerance. Several methods have been used to assess metal tolerance in plants, such as tissue culture bioassays, relative growth in metal contaminated soils or accumulation levels (Sanitá di Toppi & Gabrielli, 1999). However, germplasm screening requires fast, accurate and reliable methods.

Hydroponic culture offers a good system to measure relative root growth and other parameters, and provides a reproducible and stable growth set-up for obtaining uniform plants allowing tolerance assessment. Metal concentrations can be carefully controlled and the method can be used to select a subset of plants for testing in soils (Sledge *et al.,* 2005). Barrel medic (*Medicago truncatula*) is a forage crop and also a model legume. *M. truncatula* constitutes an appropriate system to study heavy metal toxicity and resistance mechanisms in legumes (Chandran *et al.,* 2008). In this work we carried out the screening of *M. truncatula* germplasm accessions for metal tolerance in hydroponic culture as an initial step to assess their potential use in phytoextraction and/or phytostabilization of Cd and Hg in contaminated soils, in combination with tolerant bacterial inoculants that favor the establishment of the plants and facilitate heavy metal absorption and accumulation.

Materials and Methods

Seeds of 118 accessions of *M. truncatula*, obtained from the Western Regional PI Station at Pullman, Washington, USA were sterilized and germinated in the dark. Seedlings were transferred to a hydroponic culture system consisting of a glass lidded cuvette furnished with a perforated 3 mm-thick polystyrene film that floated on Hoagland nutrient solution (Hoagland & Arnon, 1938) and provided support to the seedlings. Seedlings were acclimatized for 24 h in the cuvette under growth chamber conditions (150 µmol photon m^{-2} s^{-1}, 25ºC/19ºC, 16 h/8 h, photoperiod) before treatment with Cd or Hg, which were supplied to the nutrient solution as $CdCl_2$ or $HgCl_2$, respectively. To set up the experimental conditions to be used in the tolerance screening assay *M. truncatula* cv. Parabinga was tested at several Cd and Hg concentrations and exposure times. Relative Root Growth (RRG) was used as a measure of metal tolerance (Sledge *et al*., 2005): RRG=root length increment $(cm)_{metal}$/root length increment $(cm)_{control}$.

Results and Discussion

The metal concentrations and time of exposure to perform the screening assay were determined on the basis of the response of *M. truncatula* cv. Parabinga, by testing different metal concentrations at different times. Seedlings were exposed to 0, 2.5, 5, 7.5, 10, 15, 25 and 40 µM $CdCl_2$ and to 0, 1, 2, 2.5, 3, 3.5, 4, 5, 6 and 7 µM $HgCl_2$ for 48, 72 and 96 h. The selected concentrations and time of exposure were 10 µM $CdCl_2$ and 4 µM for $HgCl_2$, both at 48 h of treatment. Under these conditions, RRG for this cultivar, which was considered as sensitive to both metals, was *c.* 20-30%.

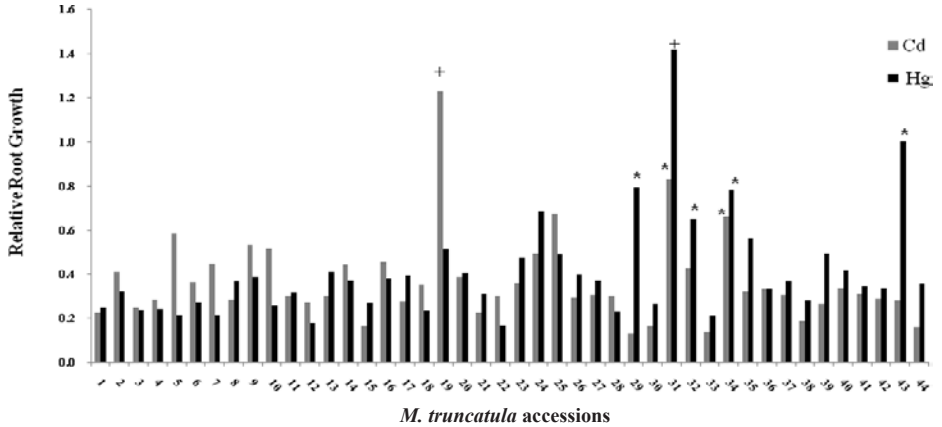

Figure. Relative root growth, the ratio of seedling root length grown with metal to seedling root length grown without metal, of *M. truncatula* accessions exposed to 10 µM $CdCl_2$ (grey bars) or 4 µM for $HgCl_2$ (black bars) for 48 h. Accession 1: *M. truncatula* cv. Parabinga. Values are means (n=10). * indicates no significant differences between treated and untreated seedlings. + indicates significant differences between treated and untreated seedlings with RRG>1.

The screening results showed a range of RRG (Figure) that allowed classification of the accessions in three categories according to their metal tolerance, based on this parameter. Varieties with RRG less than 0.6 were considered sensitive, those with RRG between 0.6-0.7 were considered intermediate and the tolerant category included those with RRG higher than 0.7. As a result of the screening assay ten cultivars were selected which showed no significant differences or positive significant differences between treated and untreated seedlings. A comparative analysis between Cd and Hg RRGs was performed that showed a significant correlation indicating similar tendency for both Hg and Cd stresses. This concordance might indicate the prevalence of common mechanisms involved in tolerance to both toxic metals. No correlation was found when comparing our results with previous data on aluminum tolerance (Sledge *et al.,* 2005). Following this first approach, additional physiological, biochemical and molecular studies will allow identifying new mechanisms and genes in metal-tolerant accessions, which could eventually be of use in phytoremediation.

Acknowledgments

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Positive response of common bean in field conditions to the inoculation with local *Rhizobium leguminosarum* strains in the P.G.I. 'La Bañeza–León' (Spain)

Mulas D[1*], Casquero P[1], Velázquez E[2], González-Andrés F[1]

*[1]Instituto de Medio Ambiente, Recursos Naturales y Biodiversidad, Universidad de León, Av Portugal 41, E24071, León, Spain; [2]Departamento de Microbiología y Genética, Univesidad de Salamanca, P. Doctores de la Reina, E37007 Salamanca, Spain. *dmulg@unileon.es*

Summary

A field experiment was carried out in the Protected Geographic Indication (P.G.I.) '*La Bañeza-León*' (Spain) with the local bean variety 'Riñón' in order to test the performance of three rhizobial strains used as inoculants (*Rhizobium leguminosarum* LCS0306, LBM1123 and ZBM1008) under three tillage systems (no tillage, conventional tillage and conventional tillage followed by a rye mulching buried before sowing). The experiment was settled in two different locations in 2008 and 2009 (three environments) with different native rhizobial populations. There was an increase in yield as a consequence of inoculation with each of the three strains compared to the uninoculated control, with no differences between inoculation and fertilization with ammonium nitrate to fulfil the theoretical N extractions. Among tillage systems, no tillage produced the lowest yield. Consequently, inoculation with the local selected rhizobia replacing N fertilization is recommended.

Introduction

Common bean (*Phaseolus vulgaris*) was an important crop in the province of León for centuries and even now it is the first producer, but the food habits of today consumers, the imports of south-american beans, and the global situation of the agriculture as a consequence of the European agricultural policy has led to a difficult situation for beans crop León. The designation of institutional quality brands has appeared as one possibility to maintain traditional crops improving the quality perception by consumers. The common bean from 'La Bañeza – León' was recognized in 2005 with a Protected Geographical Indication (P.G.I.). On the other hand, the new European scenario of agrochemicals reduction, is a major determining factor for the reduction of such inputs. The N fertilisers in agriculture are a major energetic cost, with a risk of water contamination by lixiviation, that has severe health risks (de Felipe, 2006). The susbstitution of N fertilisers by rhizobial N fixation is a chief alternative for the reduction of synthetic N fertiliser, with important effects in the reduction of production costs. Since 2005, several autochthonous rhizobial strains from the PGI 'La Bañeza León' were isolated, identified as *Rhizobium leguminosarum* by genetic analysis, and sequentially selected by their N fixation capacity in hydroponic medium and in microcosmos (Mulas *et al.,* 2008). The aim of the present work was to assess the grain yield response of 'Riñón' beans to the inoculation with three elite rhizobial strains, under three different tillage systems.

Materials and Methods

The experiment was carried out with the local variety of common bean 'Riñón' in two locations, Sueros and ESTIA, during the seasons of 2008 (only ESTIA) and 2009 (both soils), resulting in three different environments. Sueros showed 3.10^4 bean nodulating rhizobia per gram of soil (Most Probable Number) whereas ESTIA had no rhizobia. The experiment was designed as a split plot, in which the main plot was the tillage system: conventional tillage (CT), conventional tillage followed by a rye cover crop, burried prior to sowing, (RC) and no tillage (NT). The sub-plot corresponded to the inoculation treatment, which included each of the isolates tested in the experiment (*Rhizobium leguminosarum* LCS0306, LBM1123 and ZBM1008) as well as two uninoculated controls, without and with N fertilizer supply to fulfil the N requirements of the crop. Plants received P and K fertilizers prior to sowing as needed based on soil contents and extraction levels. Each subplot included four rows of plants separated from each other 0.5 m between rows and 0.15 m between plants in the same row. Localized irrigation was settled and the experiment was watered to keep a minimum soil humidity of

80% of the useful water. Weeds were manually removed twice during the growing season. From all the parameters analysed: total aeral biomass, yield, harvest index and yield components, the yield is showed in the present work. The parameters were statistically analysed by ANOVA (test LSD). SPSS 17.0 was used.

Results and Discussion

For the grain yield, there were significant differences among environments (p<0.001) and among treatments: tillage (p<0.001) and inoculation treatement (p<0.001). About interactions, there was only a moderately significant interaction between environment and tillage (p<0.05) (Table). The climatic conditions of the year 2009 were better thant those of 2008 for grain yield, and the enviroment of Sueros (climate * soil conditions) better than ESTIA environment for 2009 (Table). The conventional tillage (CT) produced the highest yield with no significant differences with conventional tillage followed by a rye cover, whereas the no tillage, was the worst for yield (Table). The inoculation with any of the elite rhizobial strains, specially strain LCS0306, yielded the highest grain production, although there was no significant difference with the non-inoculated control fertilised with nitrogen (Table). Although the response of beans to the inoculation has traditionaly been considered low or moderate, there are some other works that described a positive response similar to ours (Hungria *et al*., 2003). Therefore, inoculation with autochthonous strains can be recommended, replacing N fertilisation. The interaction between tillage and environment was due to the fact that CT was the less affected by the detrimental environment of ESTIA2008 due to the higher number of pods in this treatment (not shown).

Table. Analysis of Variance of the grain yield for common bean var. 'Riñón' in 3 environments in NW Spain and 2 treatments (tillage and inoculation with selected autochthonous *Rhizobium leguminosarum* strains). Means followed by the same letter did not significantly differ.

	Mean square	F	p
Environment	328926.545	1257.582	0.000
Repetition	324617.140	1189.093	0.000
Analysis of the main plot			
Tillage	327916.673	1241.001	0.000
Environment*Tillage	4533.764	3.706	0.011
Analysis of the sub-plot			
Inoculation	195486.614	723.209	0.000
Environment*Inoculation	381.929	1.485	0.158
Tillage*Inoculation	501.987	1.937	0.051
Environment*Tillage*Inoculation	149.319	0.369	0.974

(1) Least Significant Difference

	Mean	LSD[1]
ENVIRNOMENT		
Sueros2009	33.19	c
ESTIA2009	30.78	b
ESTIA2008	24.51	a
TILLAGE		
NT	26.02	a
CT	33.36	b
RC	27.60	b
INOCULATION		
Control without N	25.43	a
Control with N	29.20	b
LCS0306	31.22	b
ZBM1008	29.66	b
LBM1123	31.02	b

Acknowledgments

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Assessment of the effect of actinobacteria isolated from alfalfa nodules as Plant Growth Promoting Rhizobacteria

Martínez-Hidalgo P[1*], Morales R[2], Trujillo ME[1], Egido J[3], Martínez-Molina E[1]

*[1]Departamento de Microbiología y Genética, Universidad de Salamanca, 37007 Salamanca; [2]Departmento de Construcción y Agronomía, Universidad de Salamanca, 37007 Salamanca; [3]Departmento de Estadística, Universidad de Salamanca, 37007 Salamanca, Spain. *martinezhp@usal.es*

Summary

The studies of new interactions that allow us to design efficient biofertilizers in order to reduce the use of pesticides and chemical fertilizers are very important nowadays. Endophytic actinobacteria have a very high biotechnological potential for agriculture and environment preservation. The activity of endophytic actinobacteria can affect: (a) the development of plants in a direct way; (b) their nutrition, making it easier to get essential elements like nitrogen, iron and phosphor; (c) the plant's health of by means of direct mechanisms, like antibiotic or siderophore synthesis or inducing plant defense respones; and (d) improving tolerance to adverse enviromental factors. In this work, we study the effects of PGPR of actinobacteria from the *Micromonospora* genus which were isolated from alfalfa nodules. Strains that promote plant growth and improve nutrient absortion and nodulation were selected.

Introducción

En los últimos años, se han descrito actinobacterias endofitas pertenecientes a diversos géneros como *Streptomyces, Strepstosporangium, Microbispora, Micromonospora* etc. aislados de raíces de plantas sanas. Estos endofitos, obtienen de ésta nutrientes y protección, a cambio, como PGPR, estimulan su desarrollo mediante la producción de ciertos metabolitos bioactivos, la protegen frente a patógenos o le confieren ventajas para la adaptación al medio. En este sentido las actinobacterias endofitas de raíces y los aislados de nódulos son PGPR de gran potencial en agricultura y podrían ser en el futuro, una alternativa a los fertilizantes y pesticidas químicos. Este potencial se basa en su capacidad para: (1) colonizar activamente las raíces y nódulos de diferentes leguminosas, (2) facilitar a la planta nutrientes como N_2, P y K, (3) producir nuevas moléculas con potencial biotecnológico, (4) inducir los mecanismos defensivos de las plantas, (5) incrementar la nodulación en la asociación de Rhizobia con leguminosas (Rhizobia helper bacteria) y promover el crecimiento vegetal, (6) incrementar la resistencia a la sequía y (7) la combinación de varios modos de acción, que de acuerdo con los resultados de investigaciones paralelas con *Azospirillum*, dan lugar a un mayor efecto sobre el crecimiento vegetal (hipótesis aditiva).

Los problemas causados por fertilizantes y pesticidas hacen necesario desarrollar métodos alternativos de fertilización y de control más respetuosos con el medio. En este contexto tienen gran importancia el estudio de nuevas asociaciones, como la que se produce entre *Micromonospora* y raíces de plantas.

Materiales y Métodos

Material vegetal. Se utilizaron semillas comerciales de alfalfa (*Medicago sativa* cv. Aragón) esterilizadas en superficie. Las semillas estériles se germinaron en semilleros y se transplantaron a macetas con suelo estéril cuando las plántulas tuvieron la primera hoja verdadera. Las plantas fueron inoculadas con diferentes cepas de *Micromonospora* y *Sinorhizobium meliloti*. Se recolectaron al inicio de la floración (10% de plantas con flor) y se analizaron los siguientes parámetros. En la parte aérea: longitud, peso, clorofila, corona, entrenudos, A/L de foliolos y elementos minerales (C, S, N, B, Ca, Cu, Fe, K, Mg, Mn, Na, P y Zn). En la raíz: número de nódulos, longitud, y peso. El contenido de N y C se determinó mediante un analizador LECO. El resto de elementos se midieron con ICP-OES (inductively coupled plasma optical emission spectrometry).

Microorganismos. *Micromonospora:* Se seleccionaron 18 cepas tras su caracterización mediante BOX-PCR y análisis filogenético de las secuencias completas del gen que codifica para el ARNr 16s. *Sinorhizobium:* se utilizó la cepa 1021 de *S. meliloti.* Ambos se cultivaron en medio M65 modificado.
Estadística. El análisis estadístico de los resultados está representado mediante un HJ-biplot y las comparaciones se hicieron mediante ANOVA.

Resultados y Discusión

Se observó un incremento en el crecimiento de las plantas inoculadas con *Micromonospora*, tanto en las que estaban inoculadas únicamente con *Micromonospora* como en las que tenían un coinóculo de Sinorhizobium con *Micromonospora*.Los resultados a destacar son: (1) La acción como PGPR de *Micromonospora* cuando es inoculada sola es más visible que en el coinóculo (según los resultados, estadísticamente significativos, de los parámetros analizados); (2) hay cuatro cepas de entre las 18 estudiadas que tienen un efecto como PGPR mayor que el resto. (ALFb4, ALFpr18c, ALF2, ALFb5); (3) estas cepas, de acuerdo con los resultados de caracterización molecular se asocian con las siguientes especies ya descritas: ALFpr18c con *M. lupini*, ALFb5 con *M. aurantiaca*, ALFb4 con *M. echinospora*, pero es claramente diferente y ALF2 con *M. olivasterospora* aunque las diferencias, en ambos casos, parecen indicar que se traten de especies diferentes; (4) los parámetros con resultados estadísticamente significativos para las plantas inoculadas solo con *Micromonospora* se encuentran recogidos en la Tabla 2; y (5) el análisis estadístico biplot nos muestra las diferencias entre los distintos tratamientos con respecto a los parámetros estudiados y como covarian dichos parámetros (Figura).

Tabla. Cepas de *Micromonospora* seleccionadas por su acción como PGPR (p <0.05).

	Alf-B4	Alf-PR18C	Alf2	Alf-B5
TALLO LONG	0,004		,022 U	,049 U
CLOROFILA	0,009	0,001	0,002	0,021
LONG.RAIZ	0,001	0	0,002	0,001
ENTRENUDOS	0,022			
PESO TALLO	0,043	0,029		
TALLO %C	,041 U	0	0,001	0
TALLO %S	0,044	,023 U	0	0
TALLO %N	0	0	0,004	
% K	,049 U			
Mn (ppm)	0,002	0,006		0,008
% S		0,011	0,032	0,004
Zn (ppm)		0,042		
N total		0,034	0,012	
C/N	0	0	0	0,006
P/N	0,029	0	0	

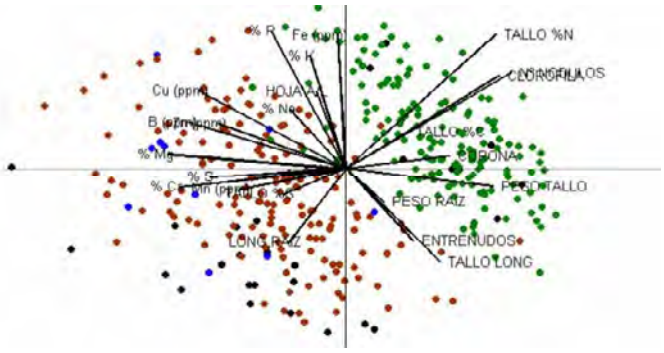

Figura. Representación HJ biplot de todas las cepas y parametros estudiados: ✱ *Micromonospora.* ✱ *Micromonospora* con *S. meliloti.* ✱ y ✱ controles

Agradecimientos

Este trabajo se ha realizado gracias a las ayudas del Ministerio de Ciencia y Tecnología y de la Junta de Castilla y León (grupo GR 49). P.M-H ha recibido una beca predoctoral (Programa JAE) del CSIC.

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Localisation of copper and iron in nodules of copper-stressed soybean plants determined by energy-dispersive X-ray microanalysis

Sánchez-Pardo B*, Carpena RO, Zornoza P

*Departamento de Química Agrícola, Facultad de Ciencias, Universidad Autónoma de Madrid, 28049 Madrid, Spain. *beatriz.sanchezpardo@uam.es*

Summary

Soybean (*Glycine max*) plants were grown hydroponically on perlite in a nutrient solution with different copper (Cu) concentrations (1.6, 48, 96 and 192 μM). Changes in plant growth, nodulation and the localisation of Cu and iron (Fe) in nodules as a result of Cu uptake were studied in 35 day-old plants. A significant reduction was seen in leaf and stem growth with increasing Cu concentration. Root growth and nodule biomass only diminished with Cu concentrations > 48 μM. Moreover, Cu was only found in nodule tissues from plants treated with the highest Cu dose; Fe, in contrast, was found in nodules of plants treated with 1.6 or 192 μM Cu. A Cu concentration gradient was detected in the nodules, with concentrations falling from the outer to inner nodular tissues. Fe showed the opposite trend.

Introduction

Contaminated soils are generally low in nutrients and organic matter. Leguminous species are often included in strategies designed to remediate contaminated soils, a consequence of their capacity to increase soil N content, thus enhancing its fertility (Frérot *et al.,* 2006). N_2 fixation is, however, sensitive to heavy metal pollution. Although soybean is by far the most economically important grain legume, few studies have analysed the response of N_2 fixation to trace element pollution. Some studies have reported that high levels of aluminium (Alva *et al.,* 1987), arsenic (Reichman, 2007) and cadmium (Balestrasse *et al.,* 2001; Chen *et al.,* 2003) inhibit nodulation in this species. This study examines the effect of increasing Cu concentration on the growth and nodulation of hydroponically-grown soybean plants. The microlocalisation (EDXMA) of Cu and Fe in nodule tissues was also studied.

Materials and Methods

Soybean (*Glycine max* L. cv. Williams) plants were inoculated twice with a suspension of *Bradyrhizobium japonicum* strain ISJ78 and grown hydroponically in pots filled with perlite in a glasshouse according to Zornoza *et al.* (2002). Cu was added 10 days after sowing as $CuSO_5{\cdot}5H_2O$, to provide concentrations of 1.6 (control), 48, 96 and 192 μM Cu (4 replicates). After 35 days the plants were divided into leaves, stems, roots and nodules, and weighed. Cu and Fe concentrations were determined by atomic absorption spectrophotometry. Plant material for electron microscopy (Zeiss digital scanning microscope, DSM 960) was prepared as reported by Vázquez *et al.* (2007). EDXMA was performed in conjunction with LTSEM according to Wierzchos & Ascaso (1996). Differences between the means of plant growth variables, and differences in Cu and Fe localisations, were analysed by one-way ANOVA. Means were compared using the Duncan test (P<0.05). All calculations were made using SPSS 17.0 software.

Results and Discussion

Compared to the control plants, significant reductions in leaf and stem FW were seen with increasing Cu concentration (Figure 1a). The root weight of the 48 μM Cu-treated plants was higher than that of the controls, although it decreased with Cu doses >48 μM. Compared to the control plants, nodule biomass was smaller in plants exposed to >48 μM Cu; in contrast, mean nodule weight only decreased in plants treated with the highest Cu dose (Figure 1b). Growth inhibition and a reduction in root biomass, often accompanied by changes in root morphology, are the main symptoms of Cu toxicity (Fernandes & Henriques, 1991).

Nodulation is thought to be more sensitive to Cu excess than plant growth due to the reduction in root hair formation, and hence in potential infection sites (reported in cowpea; Kopittke *et al.,* 2007). Figure 2 shows the EDXMA results for Cu and Fe for nodular tissues of plants exposed to 1.6 or 192 µM Cu. Data are expressed as percentages of the total signal. No significant differences were seen in terms of the cytoplasmic and vacuolar localisation of Cu and Fe inside the nodule cells, although a decreasing Cu gradient was in the cell walls from the outer to the inner nodule cortex. Cu was not detected in the control nodules. No significant differences were seen between the control and 192 µM Cu-treated plants in terms of the Fe signal in the cell walls of cortex cells (zones 1 and 2). In contrast, in zone 3 and in the infected zone, Fe values higher than those for controls were observed in the nodules of plants grown with 192 µM Cu.

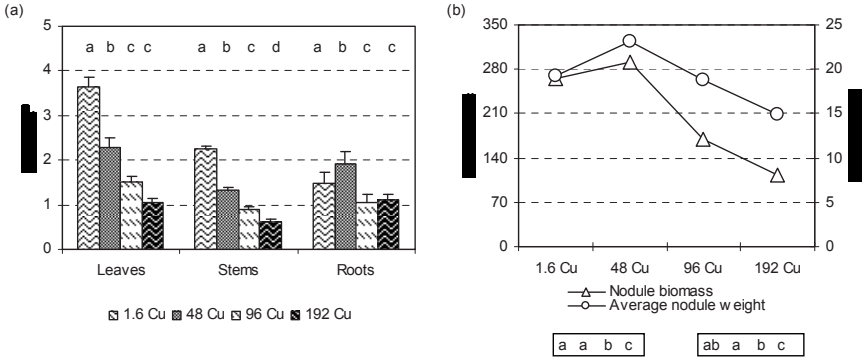

Figure 1. Fresh weights of leaves, stems and roots (a) and nodule biomass (b).

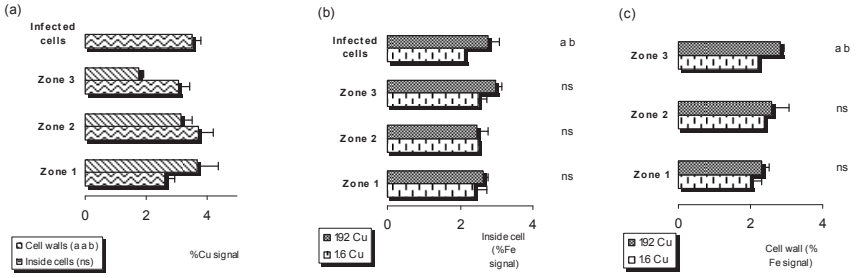

Figure 2. Cu localisation in nodules treated with 192 µM Cu (a); Fe localisation in citoplasm-vacuole (b) and cell walls (c) treated with 1.6 and 192 µM Cu. Values are means ± SE of four replicates

Acknowledgments

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Response of biserrula to inoculation with selected rhizobial strains

Albareda M[1], Rodríguez-Navarro DN[2], Temprano FJ[2*]

[1]*Centro de Biotecnología y Genómica de Plantas (CBGP), Universidad Politécnica de Madrid, Campus de Montegancedo - Carretera M40 km 38, 28223 Pozuelo de Alarcón, Madrid, Spain;* [2]*IFAPA-Centro Las Torres, Carretera Sevilla-Alcalá del Río, km 12,2, 41200 Alcalá del Río (Sevilla), Spain.*
**francisco.temprano@juntadeanadlucia.es*

Summary
Biserrula [*Astragalus pelecinus* (L.) Barneby] is an annual forage legume useful for the improvement of pastures in areas of Mediterranean climate. It establishes a symbiotic association with specific rizobia of the *Mesorhizobium* genus and can fix atmospheric N_2 which is incorporated into pastures. Thirty two soils from pasture areas in Southwest Spain were analysed for the presence of specific *Mesorhizobium* (*Biserrula*) native populations: 23 % of those soils showed a low density of *Mesorhizobium* populations whereas 60 % showed populations higher than 10^3 bacteria/g. The nitrogen fixing capacity of the isolates from these soils was variable and, in general, they showed to be effective when compared with the uninoculated control. Greenhouse tests showed a significative interaction strain x cultivar for nitrogen fixation when 3 biserrula cultivars where inoculated with 6 *Mesorhizobium* strains. A field experiment carried out in an alluvial soil in South Spain, with low density *Mesorhizobium* native populations, showed a positive yield response when biserrula seeds were inoculated with some selected strains. An increase of 65% of forage yield was achieved with the best *Mesorhizobium* strain when compared with the non-fertilized control.

Introducción
La biserrula es una leguminosa anual originaria de la cuenca mediterránea que se utiliza para la mejora de pastos en áreas con veranos secos y cálidos como ocurre en el suroeste de España (Loi *et al.,* 2005). Las leguminosas deben proveer al agrosistema del nitrógeno que necesita para su mantenimiento y productividad, dado que es desaconsejable el uso de fertilizantes nitrogenados en los pastizales extensivos. La biserrula establece una simbiosis muy específica con bacterias del género *Mesorhizobium* y en algunos suelos, donde esas bacterias se encuentran ausentes o en muy baja densidad, se hace necesario inocular las semillas de esta leguminosa antes de su introducción en el pastizal (Nansadena *et al.,* 2004).

Materiales y Métodos
Se tomaron muestras de 32 suelos de pastos del suroeste de España (Extremadura y Andalucía) y se analizó la presencia y densidad de las poblaciones de *Mesorhizobium* (*Biserrula*) por el método del NMP utilizando plantas de biserrula [*Astragalus pelecinus* (L.) Barneby cv. Cashba] (Brockwell, 1985). De nódulos de plantas cultivadas en esos suelos se aislaron 65 estirpes de *Mesorhizobium* (*Biserrula*) y se evaluó, bajo condiciones controladas de invernadero, su capacidad de nodulación y fijación de nitrógeno con biserrula (cv, Cashba) en tubos de 20 x 200 mm con un medio sin nitrógeno reactivo. Posteriormente, en las mismas condiciones, en jarros de Leonard, se analizó la capacidad simbiótica de 8 aislamientos seleccionados de los anteriores con 3 cultivares de biserrula (Casba, PVPA y 6110).

El ensayo de campo se llevó a cabo en un suelo aluvial (Xerofluvent, franco-arenoso, pH 8.0, 21% de carbonatos totales, 1,1 % M.O., 0,10% de N total, 5 ppm de P, 194 ppm de K), con un bajo contenido (< 10 bacterias/g) de poblacione nativas de *Mesorhizobium* (*Biserrula*). Se emplearon parcelas elementales de 1,5 x 1 m^2 en diseño de bloques al azar con 4 repeticiones. Las semillas de biserrula cv. 6110 se inocularon con las estirpes de *Mesorhizobium* (*Biserrula*) B1-6, B12-1 y BIS24, utilizando inoculantes con turba como sustrato (Somasegaran y Hoben, 1994) alcanzando una dosis de 2-4 x 10^4 bacterias/semilla. A los 7 meses de cultivo (Noviembre-Mayo), se evaluó la nodulación de las plantas y el rendimiento del forraje (materia seca) y su contenido en N (Kjeldahl).

Resultados y Discusión

Un 23% de los suelos analizados mostraron ausencia o baja densidad (<10^2 bacterias/g) de *Mesorhizobium* específico de biserrula, mientras que el 60% de los suelos poseían densidades superiores a 10^3 bacterias/g. Los aislamientos de *Mesorhizobium* (*Biserrula*) de los suelos mostraron, en los ensayos de invernadero, una efectividad fijadora de nitrógeno variable,

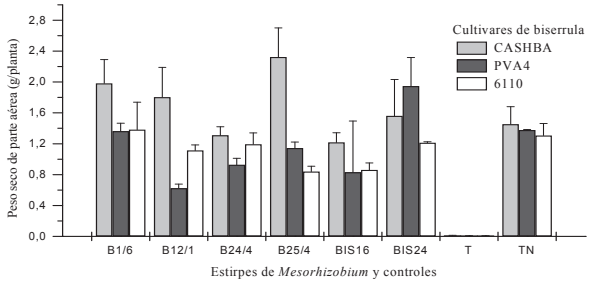

Figura. Efectividad fijadora de nitrógeno (peso seco) de estirpes de *Mesorhizobium (Biserrula*) en tres cultivares de biserrula en condiciones controladas de invernadero.

dando lugar, en general, a pesos de las plantas significativamente superiores a los del control no inoculado. Sin embargo, sólo un 10% de los aislamientos se mostró más efectivo que la estirpe de referencia (WSM1497) seleccionada por su alta efectividad. Las estirpes inoculadas en 3 cultivares de biserrula mostraron cierta interacción con el cultivar para la nodulación y la efectividad en la fijación de nitrógeno (19% de la varianza total, p<0,005) (Figura). El ensayo de campo mostró un respuesta positiva de los rendimientos de biserrula (materia seca y nitrógeno total) a la inoculación (Tabla). La estirpe B1-6 dio lugar a rendimientos semejantes a los del control fertilizado con 100 UF/ha y aumentó los rendimientos del control sin fertilizar en más del 65%. Los controles sin inocular tuvieron una nodulación escasa debido a a la baja densidad de las poblaciones nativas de *Mesorhizobium* (*Biserrula*), viéndose notablemente incrementada en los tratamientos inoculados.

Inoculantes y controles	Nodulos. planta^{-1}		Rendimiento del forraje (Kg.ha^{-1})	
	Número	Peso seco (mg)	Materia seca	N total
No inoculado	1.00 c	21.9 c	3276 c	91.6 c
No inoculado + fertilizante N	0.70 c	10.7 c	5675 a	158.9 a
B1-6	6.89 a	138.6 b	5508 a	151.7 ab
B12-1	3.31 b	86.9 b	5041 ab	146.5 ab
BIS24	3.60 b	143.2 a	4225 bc	122.8 bc
M.D.S. (p>0.05)	1.85	45.1	1041	33.3

Tabla. Nodulación y rendimientos de biserrula cv. 6110 en un suelo aluvial del sur de España en respuesta a la inoculación con 3 estirpes de *Mesorhizobium*(*Biserrula*).

Agradecimientos

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Impact of linear alkylbenzene sulfonate on the structure of the *Pseudomonas* spp. community in an agricultural soil microcosm

Sánchez-Peinado M[1,2], Molina-Muñoz M[1,2], Martínez-Toledo MV[1,2], González-López J[1,2], Pozo C[1,2], Rodelas B[1,2*]

*[1]Departamento de Microbiología, Universidad de Granada, 18070 Granada, Spain; [2]Instituto del Agua, Universidad de Granada, Spain. *mrodelas@ugr.es*

Summary

Linear Alkylbenzene Sulfonate (LAS) is the most used anionic surfactant in a world-wide scale and is considered a high-priority pollutant. LAS is readily biodegradable under aerobic conditions and mostly removed in wastewater treatment plants, but an important fraction (20–25%) is immobilized in sewage sludge and persists under anoxic conditions. Due to the application of the sludge as a fertilizer, LAS reaches agricultural soil. Molecular-based community level analyses have been seldom applied in studies regarding the effects of LAS on the soil microbial communities. We developed and used a microcosm soil system to evaluate the effects of a commercial mixture of LAS on the structure of the *Pseudomonas* spp. community in an agricultural soil, by means of a cultivation-independent approach, based on specific amplification of *Pseudomonas* spp. 16S-rRNAs by PCR and their further separation by temperature-gradient gel electrophoresis (TGGE). Cluster analysis of TGGE profiles in either water (control) or LAS-amended soil microcosms revealed significant differences in the composition of the communities analysed. 42 TGGE bands were identified by reamplification and sequencing. Although the primers used for PCR amplification were previously described as specific for *Pseudomonas* spp., 32% of identified band sequences were distantly related to the genus and found phylogenetically close to members of the *Xanthomonadaceae*. 50% of the identified populations displayed close identities only to environmental sequences on the DNA databases.

Introducción

El sulfonato de alquilbenceno lineal (LAS) es el surfactante aniónico más utilizado a escala mundial, y está considerado un contaminante de alta prioridad (HERA, 2007; Jansen *et al.,* 2007). Aunque el LAS está descrito como biodegradable en medio acuoso en condiciones aerobicas, una fracción importante (20–25%) es retenida en los lodos de las depuradoras de agua residual y persiste bajo condiciones anaerobias. El LAS alcanza el suelo mediante la práctica común de emplear los lodos de depuradora como abono agrícola. Aunque se han realizado numerosos estudios acerca de la toxicidad del LAS para los microorganismos del suelo, muy pocas veces se han aplicado métodos independientes de cultivo con estos fines (Sánchez-Peinado *et al.,* 2010). En este estudio, se ha utilizado un sistema de microcosmos edáfico con el fin de evaluar el efecto de dos concentraciones diferentes de LAS sobre la estructura de la comunidad de *Pseudomonas* spp. en un suelo agrícola, empleando para ello la técnica independiente de cultivo PCR-TGGE (electroforesis en gel con gradiente de temperatura).

Materiales y Métodos

Los microcosmos empleados y la metodología utilizada para el estudio mediante PCR-TGGE han sido en general los descritos con anterioridad por Sánchez-Peinado *et al.* (2010). Se utilizaron los cebadores específicos del género *Pseudomonas* previamente descritos por Milling *et al.* (2004). Los perfiles de TGGE obtenidos se analizaron mediante el programa Gel Compar II v. 5.10 (Applied Maths, Bélgica). Las poblaciones relevantes detectadas en los perfiles de TGGE se identificaron mediante la reamplificación por PCR del material de las correspondientes bandas, la secuenciación de los amplicones de ADN, y la comparación de las secuencias con las depositadas en las bases de datos públicas.

Resultados y Discusión

El análisis de clusters de los perfiles de la comunidad de *Pseudomonas* spp. obtenidos mediante TGGE indicó diferencias significativas entre los microcosmos sometidos a la presencia de LAS y los controles adicionados de agua estéril. Mediante la reamplificación de las bandas de TGGE y la secuenciación del ADN, se identificaron un total de 42 poblaciones entre las detectadas por TGGE. A pesar de que los cebadores utilizados en las reacciones de PCR están descritos como específicos de *Pseudomonas* spp., un 32% de las bandas identificadas se revelaron filogenéticamente distantes de este género, siendo evolutivamente más próximas a miembros de la familia *Xanthomonadaceae*. El 50% de las poblaciones identificadas en este estudio sólo mostró un alto porcentaje de identidad con secuencias ambientales depositadas en las bases de datos de ADN.

Agradecimientos

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Effect of alfalfa on the yield of *Solanum andigenum*/*Vicia faba* cultivated in rotation in farms

Arone GJ[1], Calderon C[2]

[1]Facultad de Ciencias Agrarias, Universidad Nacional de Huancavelica, Perú; [2]Departamento de Suelos, Facultad de Agronomía, Universidad Nacional Agraria La Molina, Perú.

Summary

The performance of two farms located in the Andean region of Oyolo (Ayacucho, Peru) has been evaluated. One farm had been without cultivation for 9 years, and the other was cultivated with alfalfa (*Medicago sativa*) for 15 years. The farms were sowed in rotation, first with natives potatoes (*Solanum andigenum*) and later with faba bean (*Vicia faba*), following the traditional Andean system. Native populations of *Azotobacter* and of *Azospirillum* in the rizosphere, and endorizosphere, respectively, of each crop, as well as the number of nodules formed in beans were evaluated. Whereas the resting farm produced 25.85 Tm/ha of potato and 2.53 Tm/ha of dry grain of beans, the performance of the farm cultivated with alfalfa was of 41.29 Tm/ha of potato and 3.48 Tm/ha of dry grain of bean. In general, the number of colony forming units of *Azotobacter* and *Azospirillum* from each rizosphere and endorizosphere of the plants from the resting farm was greater to that detected in the plants from the farm cultivated with alfalfa. The highest performance of the farm with alfalfa could be due to the beneficial effect of the legume on the properties of the soil.

Introducción

En la Zona Andina del Perú, en particular en la región ocupada por una de las etnias "ayllus" de Oyolo (Ayacucho, Perú), se practica con éxito un tipo de agricultura basada en eventos propios de su cosmovisión y prácticas de producción ancestrales (pastoreo, descanso de chacras, rotación de cultivos, etc.). Los ayllus siembran papa nativa (*Solanum andigenum*) como el primer cultivo de la rotación, seguido por el cultivo de haba (*Vicia faba*), sea en suelo descansado, esto es, sin cultivar durante más de dos años, o en suelo cultivado con alfalfa (*Medicago sativa*) durante más de 5 años de producción. Los agroquímicos no se emplean en la agricultura tradicional andina. Este sistema de producción se practica en los Andes peruanos desde hace más de 8.000 años y ha sido la base de la alimentación de los ayllus durante todo este tiempo (Valladolid, 1994).

¿En qué se basa la sostenibilidad del sistema de agricultura tradicional andina? En este trabajo se ha estudiado, por una parte, el rendimiento de papa y haba cultivadas en dos chacras, una descansada (CHD) y la otra cultivada con alfalfa (CHCA). Por otra parte, se ha evaluado la presencia de *Azotobacter* en suelo y rizosfera y la de *Azospirillum* en la endorizosfera y rizosfera. También se ha cuantificado la nodulación de las plantas de haba.

Materiales y Métodos

Se han utilizado dos chacras de los ayllus de Oyolo (Ayacucho, Perú) ubicadas en las coordenadas 15º 10' 45'' latitud sur y 73º 11' 28'' longitud oeste. Las chacras se sitúan a una altitud de 3600 msnm, donde la temperatura media anual es de 10-11ºC, y la precipitación de 460-500 mm/año. Las campañas se llevó a cabo durante los años 1995-1996 y 1996-1997. Las propiedades físico-químicas del suelo de las chacras se determinaron mediante su análisis de caracterización en el Laboratorio de Análisis de Suelos de la Universidad Nacional Agraria La Molina. En la primera campaña, ambas chacras se sembraron con papa nativa, empleando de una a tres semillas/golpe. En la segunda campaña, después de la cosecha de papa, en el mismo suelo, sin previa labranza (labranza cero), se sembró haba con trinche, dejando de 2 a 3 semillas/golpe. El riego en ambas chacras fue complementario a la lluvia. La presencia de *Azotobacter* se determinó empleando la metodología propuesta por Zapater (1975), y para *Azospirillum* se empleó la descrita por J. Dobereiner referido por Julca (1991). El rendimiento del cultivo de papa se evaluó en 100 plantas extraídas al azar, y el de haba en las plantas recogidas en 60 m^2. El recuento de nódulos se realizó en 16 plantas de haba extraídas al azar cuando se encontraban al 50% de floración.

Resultados y Discusión

En términos generales, en la primera campaña, independientemente de las fechas de muestreo, la población de *Azotobacter* fue mayor en la rizosfera (R) de las plantas de papa de la CHD respecto a las plantas de la CHCA. Resultados similares se obtuvieron en el suelo no rizosférico (SNR). Por otro lado, la población de *Azospirillum* en la endorizosfera (E) de las plantas de papa de la CHCA fue mayor que de la CHD. En la segunda campaña, para las tres fechas de muestreo, la población R de *Azotobacter* de las plantas de haba presentó un comportamiento similar al observado en la primera, y el número de UFCs de *Azotobacter* del SNR varió con las fechas de muestreo. Por otra parte, la población de *Azospirillum* de R y de E de la plantas de haba de la CHD fue superior a la de la CHCA. Conviene resaltar que el aporque que se realizó antes del segundo muestreo afectó temporalmente la población de *Azotobacter* en el suelo R, y no a la de *Azospirillum*. Esta observación no se manifestó en la segunda campaña, dado que no se aporca el cultivo de haba (Yang y Crowley, 2000). En todas las evaluaciones de cada una de las campañas se observó que el número de UFCs de *Azotobacter* en la R de las dos plantas fue mayor que el detectado en el SNR. Igualmente, la población E de *Azospirillum* fue superior a la de R. La población de *Azotobacter* en la primera campaña tanto en la R de papa como en el SNR fue mayor que en la R de haba y SNR de la segunda campaña; no se observaron diferencias en el número de UFCs de la población E y R de *Azospirillum*. Es posible que la mayor abundancia de *Azotobacter* y *Azospirillum* en la CHD respecto a la CHCA se deba al contenido y calidad de los rastrojos (Primavesi, 1984).

El número de nódulos/planta de haba en la CHD fue de 263, y de 223 en la CHCA. El color rojizo de los mismos después del corte de nódulos indicaba que se trataba de nódulos activos. El rendimiento de papa fue de 25,85 Tm/ha en la CHD y de 41,29 Tm/ha en la CHCA. En la segunda campaña, el rendimiento del cultivo de haba fue de 2,53 Tm/ha en la CHD y de 3,48 Tm/ha en la CHCA. Para ambos cultivos, los rendimientos obtenidos fueron superiores al promedio nacional de papa (9,7 Tm/ha) y de haba (1 Tm/ha), y similares a los rendimientos logrados en agricultura convencional (Igúzquiza, 2000; Camarena y Huaringa,1991). Nuestros estudios sugieren que el mayor rendimiento obtenidos en la CHCA puede atribuirse a que la alfalfa promueve el crecimiento de bacterias (PGPRs), que favorecen el crecimiento vegetal, así como el de hongos formadores de micorrizas, etc. Además, la alfalfa incrementa la materia orgánica, la profundidad efectiva del suelo, mejora la porosidad, estabilidad de los agregados e incorpora al suelo de 127 a 900 kg de N/ha/año, los que favorecen al cultivo de rotación (Altieri y Nicholls, 2000). Los suelos de ambas chacras son de textura franco-arenosa y, entre ellos, no se detectaron diferencias para pH, contenido de carbonatos; carbono orgánico y nitrógeno total, sin embargo, los cultivos varían en rendimiento. Los análisis de suelos no contemplan la fertilidad biológica, por lo que no revelan la fertilidad total de los suelos

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