Genetic analysis and phenotypic characterization of three novel genes of *Rhizobium tropici* CIAT899 involved in the symbiotic interactions with *Phaseolus vulgaris* plants

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Abstract

Rhizobium tropici CIAT899 is highly tolerant to many environmental stresses and a good competitor for nodule occupancy of *Phaseolus vulgaris*. Random transposon mutagenesis was performed with the aim to identify novel genes of this strain involved in symbiosis and stress tolerance. The analysis of the locus disrupted by the Tn*5* insertion in mutants 899-PV9 and 899-PV4 led the discovery of three novel genes required for and efficient symbiotic interaction with beans plants. The first gene (*syc1*) bears significant similarity to voltage-gated chloride channels. A non-polar deletion in this gene caused serious deficiencies for nodule establishment, nodulation competitiveness and N_2 fixation, probably due to its reduced ability to invade plant cells and to form stable symbiosomes, as judged by electron transmission microscopy. A second gene (*olsC*) found downstream of the former was shown to be homologous with aspartyl/asparaginyl β-hydroxylases and involved in the modification of two species of ornithine-containing lipids, presumably by a hydroxylation. A mutant carrying a non-polar deletion in *olsC* was symbiotically defective, whereas over-expressed OlsC in the complemented strain was related with an acid-sensitive phenotype. The third gene (*sigE*) codes for a putative σ^E factor. Analysis the mutant carrying a deletion in this gene also revealed serious deficiencies for nodule development, nodulation competitiveness and N_2 fixation when inoculated on bean plants. These three different bacterial activities have not been previously reported as required for the symbiotic interaction of rhizobia with its legume host.

Genetische Analyse und phänotypische Charakterisierung dreier neuartiger Gene des Bakteriums *Rhizobium tropici* CIAT899, die eine Rolle während der symbiontischen Wechselwirkungen des Bakteriums mit der Wirtspflanze *Phaseolus vulgaris* spielen

Zusammenfassung

Rhizobium tropici CIAT899 ist ein Bodenbakterium, das eine hohe Toleranz gegenüber einer Vielzahl von Stressbedingungen aufweist. Ferner ist es ein erfolgreicher Besiedler von Wurzelknöllchen der Wirtspflanze *Phaseolus vulgaris* (Bohne). Es wurde eine Insertionsmutagenese mit dem Transposon Tn*5* durchgeführt, um bisher unbekannte Gene zu identifizieren, die eine Rolle im Rahmen von Symbiose und Stresstoleranz spielen. Die Analyse der Transposon-Insertionsstellen in den Mutanten 899-PV9 und 899-PV4, führte zur Identifizierung dreier Gene, die für erfolgreiche symbiontische Interaktionenen mit Bohnenpflanzen notwendig sind. Das Gen *syc1* kodiert für ein Protein, das eine signifikante Homologie zu spannungsabhängigen Chloridkanälen aufweist. Die Deletion des *syc1* Gens führte zu einem ausgeprägten Phänotyp: Knöllchenentwicklung, Effizienz der Knöllchenbesiedlung und Stickstoffixierung waren stark beeinträchtigt. Analyse von Knöllchenquerschnitten mittels Transmission-Elektronen-Mikroskopie zeigte, daß die Bakterien vermutlich nicht in der Lage sind, die Zellen der Wirtspflanze zu invadieren und stabile Symbiosomen zu bilden. Ein weiteres stromabwärts liegendes Gen (*olsC*) kodiert für ein Protein, das Homologie zu Aspartyl-/Asparaginyl β-Hydroxylasen aufweist. Es konnte gezeigt werden, daß OlsC eine Funktion im Rahmen der Modifikation von Ornithinlipiden hat. Bei dieser Modifikation handelt es sich vermutlich um eine Hydroxylierung der Ornithinlipide. Eine *olsC* Deletionsmutante weist einen symbiontischen Phänotyp auf. Die Überexpression von OlsC in *R. tropici* führte zu einer erhöhten Empfindllichkeit der Zellen gegenüber sauren Bedingungen. Das dritte identifizierte Gen (*sigE*) kodiert für einen putativen Sigmafaktor des Typs σ^E. Eine *sigE* Deletionsmutante zeigt Defizienzen in der Knöllchenentwicklung, in der Effizienz der Knöllchenbesiedlung und in der Stickstoffixierung. In dieser Arbeit wird zum ersten Mal berichtet, daß diese drei bakteriellen Gene/Proteine für eine erfolgreiche symbiontische Interaktion zwischen Rhizobien und ihren Wirtspflanzen notwendig sind.

General introduction

Legume-rhizobia interactions and biological nitrogen fixation

Although about 80% of the planet's atmosphere is nitrogen, dinitrogen gas is chemically inert and unavailable to higher organisms, which depend on prokaryotes to convert the N_2 to ammonium, a nitrogenous form that can be assimilated by all other organisms. Since nitrogen is an essential nutrient because it is a major component of proteins and nucleic acids, it is a limiting factor for plant growth. Legumes plants have solved this need by establishing a symbiotic relationship with certain soil bacteria, called "rhizobia", that provide about 65% of the biosphere's available nitrogen and that are of both ecological and agricultural significance (Brewin 2002; Lodwig et al. 2003; Riely et al. 2004).

The symbiotic relationships formed between rhizobia and their legume hosts are the result of a complex signaling network between the host and the symbiont, resulting in the formation of nodules, within are found the nitrogen fixing forms, called bacteroids. Infection of legumes by rhizobia involves chemotaxis of the bacteria toward organic acids, amino acids, sugars and flavonoids excreted by the roots (Fig. 1).

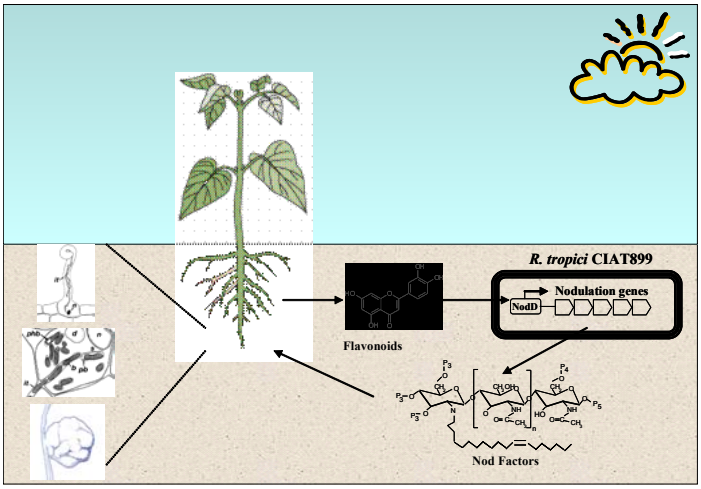

Fig. 1. Symbiotic interactions between rhizobia and legume plants.

Flavonoid compounds trigger expression of rhizobial genes required for nodulation (*nod, nol and noe*), usually regulated by NodD, which is a transcriptional regulator that binds to a conserved region (called Nod box) found in the promoter regions of many nodulation loci. Nodulation genes produce of a second signal in bacteria, which are recognized by the plant host, called Nod factors. Nod factors consist of an acylated chitin oligomeric

backbone with various modifications that determine the host range of the bacterium. They act at nano- to picomolar concentrations and induce the root hair curling, membrane depolarization, changes in ion fluxes, early nodulin gene expression and formation of nodule primordia. The bacteria trapped in the curled root hair induce the formation of an infection thread, a tube of plant origin, which penetrate the outer plant cells while the bacteria proliferate and penetrate individual target cells (Downie and Walker 1999; D'Haeze and Holsters 2002; Gonzalez and Marketon 2003; Riely et al. 2004).

Within the infected cells of the nodules, bacteria are enveloped in a membrane of plant origin, called the peribacteroid membrane (PBM), which divide and differentiate the bacteroids. The organelle-like structure comprised of the PBM and bacteroids is termed the symbiososme, and is the basic nitrogen-fixing unit of the nodule. The peribacteroid and bacteroid membranes govern the nature of the exchanges between plant and bacteroid, which is reduced carbon, probably in the form of dicarboxylic acids, from the plant for fixed nitrogen from the bacteroid. The ammonia produced is released by the bacteroid and is assimilated in the cytoplasm of the infected cell, and then is translocated out of the nodules to other parts of the plant in form of amides or ureides (Udvardi and Day 1997). Many other metabolites are also exchanged, where bacteroids might also be involved in amino acid cycling (Lodwig et al. 2003)).

Bacteroids use the enzyme nitrogenase to catalyze the reduction of N_2 to ammonia, as shown in Equation 1 (Werner 1992):

$$N_2 + 8H+ + 8e- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16Pi$$

The nitrogenase enzyme complex consists of two component proteins. The nitrogenase reductase is the specific electron donor and the dinitrogenase that accumulates electrons and catalyses the reduction of substrates using a highly complex cofactor comprising iron, molybdenum and sulfur. For this reaction are required large amounts of reductants and ATP, which are derived from reduced carbon provided by the plant. In addition, synthesis of the nitrogenase enzyme complex requires the coordinated expression of many genes and a concomitant investment of energy. Therefore, its synthesis and activity is tightly regulated. In rhizobia, nitrogen fixation is regulated by oxygen concentration rather than nitrogen availability requiring concentrations about 1000 times below the ambient. However, oxygen is required for growth and respiration, which is facilitated

through intercellular air spaces and a haemoprotein, called leghaemoglobin, that represents a major component of the cytoplasm in nodules cells (Halbleib and Ludden 2000; Burris 2001; Brewin 2002).

Stress tolerance in rhizobia

In order to accomplish a successful symbiotic interaction with legumes, rhizobia have to cope with different stress conditions they encounter in soil, the rhizosphere and the symbiosome like moisture deficiencies, osmotic and heat stresses, alkalinity and acidity. Soil acidity is one of the main limiting factors since it affects the symbiotic N_2 fixation and crop productivity in many soils of the tropics and subtropics (Aarons and Graham 1991; Zahran 1999; Hungria and Vargas 2000). It causes nitrogen deficiency in legumes as it inhibits rhizobial growth, root infection and bacteroid activity (Munns et al. 1981; Glenn et al. 1999). In the rhizosphere, plants secrete H^+ and organic acids that acidify the soil up to 2 pH units below the surrounding bulk soil (Marschner 1995). In addition, the presence of antibacterial molecules and the strong competence between microorganisms for nutrients constitute further stress factors that could constrain nodulation (Jjemba 2001; Lynch et al. 2002). Finally, rhizobial bacteroids also face an acidic environment in the peribacteroid space, which has been estimated to be up to two pH units more acidic than the plant cell cytosol (Udvardi and Day 1997). Bacteroids also face osmotic and oxidative stresses, as well as microaerobiosis, in the symbiosomes (Day et al. 2001; Nogales et al. 2002). Acid stress is therefore, a common limiting factor all the way from the soil to the symbiosome, which suggests the existence of different mechanisms of adaptation.

It is well known that rhizobial species exhibit different levels of tolerance to acidity (Munns et al. 1979; Graham et al. 1982; Graham et al. 1994). However, the genetic and physiological bases of this acid tolerance are still not clear. Two mechanisms related to the acid tolerance response (ATR) have been identified in rhizobia and enterobacteria (O'Hara and Glenn 1994; Foster 1999; Merrell and Camilli 2002). The first involves the synthesis of outer membrane proteins and changes in the structure of lipopolysaccharides, exopolysaccharides and fatty acids to enhance cell surface stability and to prevent proton permeability (Ballen et al. 1990; Chen et al. 1993a; Reeve et al. 1997). The second mechanism is related to the maintenance of intracellular pH homeostasis (Chen et al.

1993b). Proton influx in low-pH environments is counteracted in the cytoplasm by decarboxylation of amino acids to consume protons and antiporter activity to remove products (Foster 1999; Merrell and Camilli 2002). Export of positively charged substrates could cause hyperpolarization of the inner membrane; however, this process is prevented by chloride channels, which act as electrical shunts (Estevez and Jentsch 2002; Iyer et al. 2002). Furthermore, additional but yet unknown mechanisms for acid tolerance might operate in rhizobia.

Rhizobium tropici CIAT899

Rhizobium tropici CIAT899 is highly tolerant to many environmental stresses and particularly to acidity. It is able to grow on media acidified down to pH 3.5 and it is a good competitor for nodule occupancy of *Phaseolus vulgaris* (common bean) and other hosts under acidic conditions (Martinez-Romero et al. 1991; Graham 1992). Thus, *R. tropici* CIAT899 represents a good model to look for genes involved in symbiosis and acid-stress tolerance (Vinuesa et al. 2003). The current knowledge of pH-regulated genes in rhizobia is still poor, despite their agricultural relevance. Proteome analyses using two-dimensional gel electrophoretic analysis reveal differential protein synthesis after pH shifts (Aarons and Graham 1991; Peick et al. 1999). Mutagenesis with the transposon Tn*5* and selection of mutants on acidified media was used to characterize acid-sensitive mutants in *Rhizobium leguminosarum* (Chen et al. 1993b). It has been shown that the *R. tropici gshB*-like gene and *Sinorhizobium meliloti actA*, *actP, exoH, exoR, actS, actR, phrR* are essential for growth at low pH (Glenn et al. 1999; Riccillo et al. 2000). More recently, Vinuesa et al (2003) reported the isolation of five additional Tn*5*-induced acid sensitive mutants of *R. tropici* CIAT899, all of which displayed symbiotically defective phenotypes in terms of nodulation competitiveness and N_2-fixation on *P. vulgaris*. Two of these mutants (899-PV4 and 899-PV9) were unable to grow in buffered medium at pH 4.5. Strain 899-PV4 was shown to carry a single Tn*5* insertion at the 5' end of *atvA*, an ortholog of the chromosomal virulence gene *acvB* of *Agrobacterium tumefaciens*, which is required by the bacteria for both, a proficient interaction with plants and for acid tolerance (Vinuesa et al. 2003).

In this study, the genetic analysis as well as a phenotypic characterization of the locus disrupted by the Tn*5* insertion in strains 899-PV9 was performed, which bears significant sequence homology with prokaryotic ClC-like chloride channel proteins, and of a gene located downstream, which is involved in membrane lipid modifications, with relevance for acid tolerance and symbiosis. In addition, I analyzed the region downstream *atvA* from strain 899-PV4, which lead to the identification of a putative ECF-σ^E factor from this species also required for an effective interaction with its legume host.

Objectives

This work was done in the project A6 of the Sonderforschungsbereich 395 “Interaction, adaptation and catalytic capability of soil microorganism” using the root nodulating bacterium *Rhizobium tropici* CIAT899 as a model.

The main objective of this work was to identify and characterize novel genes of *Rhizobium tropici* CIAT899 involved in symbiotic interactions with *Phaseolus vulgaris* plants and required for stress tolerance.

The specific objectives include:

1. Perform bioinformatic analyses of sequences of the complementing regions of the previously reported mutants 899-PV4 and 899-PV9 (Vinuesa et al. 2003) to identify and characterize specific open reading frames (ORF) that might be involved in symbiosis or stress tolerance.

2. The construction of non-polar deletions and respective complementing stains

3. The phenotypic characterization of mutants and complemented strains under symbiotic and free-living conditions.

Materials and Methods

Bacterial strains and plasmids

Bacterial strains and plasmids used in the present work are listed in Table 1 and Table 2, respectively. Rhizobial strains were grown in PY, minimal medium or in 20E medium at 28°C (see Media section). Acidic media at pH 4.5 were buffered with 25 mM Homopipes. *Escherichia coli* strains were grown in Luria-Bertani medium at 37°C (Sambrook et al. 1989). When needed, antibiotics were added at the following concentrations: kanamycin (Km) 100 μg/ml, streptomycin (Sm) 150 μg/ml, ampicillin (Ap) 50 μg/ml, tetracycline (Tc) 7.5 μg/ml, gentamicin (Gm) 10 μg/ml, nalidixic acid (Nal) 20 μg/ml.

Table 1. Bacterial strains and plasmids used in this study

Strain	Relevant characteristics	Source or reference
***R. tropici* strains**		
CIAT899	Acid tolerant, Ap^r, Nal^r	Martínez-Romero et al. 1991
899-PV9	CIAT899 derivative (clc::Tn*5*), symbiotically defective, Sm^r, Km^r	Vinuesa et al. 2003
899-PV4	CIAT899 derivative (*atvA*::Tn*5*), symbiotically defective, Sm^r, Km^r	Vinuesa et al. 2003
CIAT899-G1	*gusA*-tagged CIAT899 derivative carrying a single mTn*5gusA30* insertion, used as reporter strain in competition experiments, Sm^r , Sp^r	Vinuesa et al. 2003
899-*syc*Δ1	CIAT899 carrying a 991 bp non-polar deletion in *syc1*	This study
899-*syc*Δ1/ pPV9cos2	899-*syc*Δ1 carrying the complementing cosmid of 899-PV9, Tc^r	This study
899-*olsC*Δ1	CIAT899 carrying a 211 bp non-polar deletion in *olsC*	This study
899-*olsC*Δ1/pBBR-1,6EB	899-*olsC*Δ1 complemented with pBBR-1,6BE, Gm^r	This study
899-*olsC*Δ1/pBBR-MCS5	899-*olsC*Δ1 carrying the vector pBBR-MCS5, Gm^r	This study
899/pBBR-1,6EB	CIAT899 carrying the vector pBBR-1,6EB, Gm^r	This study
899-*olsC*Δ1/pJG21	899-*olsC*Δ1 carrying vector pJG21, Tc^r	This study
899-*olsC*Δ1/pJG21+pBBR-1,6EB	899-*olsC*Δ1 carrying vector pJG21 and pBBR1,6EB, Gm^r, Tc^r	This study
899-Δsig1	CIAT899 carrying a 474 bp non-polar deletion in *sigE*	This study
899-Δsig1/pBBRsigE	899-Δsig1 complemented with pBBRsigE, Gm^r	This study
899-Δsig1/pBBR-MCS5	899-Δsig1 carrying the vector pBBR-MCS5, Gm^r	This study
899/pBBRsigE-GUS	CIAT899 carrying the vector pBBRsigE-GUS, used for transcriptional fusions, Gm^r	This study
899/ pBBR-GUS	CIAT899 carrying the vector pBBR-GUS, used as control of the transcriptional fusions, Gm^r	This study
***E. coli* strains**		
DH5α	*recA*1, Δ*lacU*169, Φ80d*lacZ*ΔM1	Stratagene
S17-1	*thi pro hsdR*$^-$ *hsdM*$^+$ *recA*, RP4 integrated in the chromosome, 2-Tc::Mu-Km::Tn*7*(Tp^r/Sm^r)	Simon et al. 1983

Table 2. Plasmids used in this study

Plasmid	Relevant characteristics	Source or reference
pRK2013	Helper plasmid; Km^r	(Ditta et al. 1980)
pK18*mob*	Conjugative suicide vector with Km^r *lacZ* markers used for positive selection of single recombinants	(Schäfer et al. 1994)
pK18*mobsac*B	Conjugative suicide vector with Km^r *lacZ* and *sacB* markers used for positive selection of double recombinants	Schäfer et al. 1994
pBBR-MCS5	Mobilizable broad host range cloning vector, Gm^r	Kovach et al. 1995
pCR II	PCR cloning vector	Invitrogen
pSUP1011	Mobilizable suicide plasmid for Tn*5* mutagenesis	Simon et al. 1985
pBluescript II SK (pSK)	Standard cloning and sequencing vector, *lac*Z Ap^r	Stratagene
p899PV9ESK	14 kb *Eco*RI fragment from strain 899-PV9, containing the Tn*5*, cloned into pSK. Ap^r, Km^r	This study
p899PV9E-PCR	pSK plasmid containing the flanking region of p899PV9ESK and used for DIG-labeling. Ap^r	This study
pPV9cos2	Cosmid complementing 899-PV9, Tc^r	This study
pPV9PE-SK	6.2 kb *Pst*I-*Eco*RI subclone of pPV9cos2 in pSK, Ap^r	This study
pKRΔ02	Integrative mutagenizing plasmid based on pK18*mobsacB* used to construct strain 899-*syc*Δ1	This study
pKRΔ03	Integrative mutagenizing plasmid based on pK18*mobsacB* used to construct strain 899-*olsC*Δ1	This study
pBBR-1,6BE	1.66 kb *Bam*HI-*Eco*RI fragment cloned into pBBR-MCS5 used for complementing mutation on strain 899-*olsC*Δ1	This study
pJ21	Mobilizable broad host range cloning vector pRK415 containing *olsB* from *Sinorhizobium meliloti*.	Gao et al. 2004
pPV4cos1	Cosmid complementing 899-PV4, Tc^r	
pKRΔsig1	Integrative mutagenizing plasmid based on pK18*mobsacB* used to construct strain 899-Δsig1	This study
pBBRsigE	1.4 kb *Eco*RI-*Hin*dIII fragment cloned into pBBR-MCS5 used for complementing mutation on strain 899-Δsig1	This study
pBBR-GUS	Mobilizable broad host range cloning vector, which contains a promoterless glucuronidase gene (*uidA*) downstream of the polylinker in pBBR-MCS5, Gm^r	This study
pBBRsigE-GUS	713 bp *Xba*I-*Sal*I fragment cloned into pBBR-GUS	This study

Random transposon mutagenesis of *Rhizobium tropici* CIAT899, selection of acid-sensitive mutants and cosmid complementation

Tn*5* mutagenesis of *R. tropici* CIAT899 was carried out using *E. coli* S17-1 carrying pSUP1021 as donor strain (Simon et al. 1983). Transconjugants carrying the transposon were isolated and acid-sensitive mutants 899-PV4 and 899-PV9 were selected on different acidified media, as previously described (Vinuesa et al. 2003). A cosmid library of *R. tropici* CIAT899 made in pVK101 (Vargas et al. 1990) was mobilized *en masse* into these mutants by triparental mating using pRK2013 as helper plasmid (Figurski and

Helinski 1979). Transconjugants that restore symbiotic proficiency on common beans were isolated as previously described (Vinuesa et al. 2003).

Standard DNA manipulations

Genomic DNA from rhizobial strains was isolated using the GenomicPrep Cells & Tissues DNA isolation kit (Amersham) following the manufacturer's instructions. Plasmid DNA from *E. coli* cultures was isolated with the High Pure Plasmid isolation kit (Roche). Restriction endonucleases were purchased from New England Biolabs and used according to standard procedures (Sambrook et al. 1989). PCR amplifications were carried out in a Gene Amp PCR system 2700 (Applied Biosystems) using *Taq* (Roche) or XL polymerase (Applied Biosystems) in a standard 50 µl PCR mix as previously described (Vinuesa et al. 1999).

To map the transposon insertion in mutant 899-PV9, total DNA of this strain was digested with *Eco*RI, transferred to a nylon membrane and hybridized with a digoxygenin-labeled probe generated by the incorporation of DIG-UTP (Roche) into *nptII* marker of Tn*5*. The single hybridizing fragment (~14 kb) was cloned into pBluescript (pSK), yielding p899PV9ESK. This plasmid was used as template for PCR amplification with primers Tn5-77/58EB (Vinuesa et al. 2003) and M13 universal and used for mapping the Tn*5* insertion. The PCR product was cloned into pSK, yielding p899PV9E-PCR, and used for DIG-labeling of the flanking genomic DNA.

Cosmids were isolated and restricted with several enzymes as previously described (Vinuesa et al. 2003). Cosmid pPV9cos2 was hybridized with p899PV9E-PCR and the resulting hybridizing fragment was cloned into pSK, yielding pPV9E-SK. Subclones from pPV9E-SK were sequenced with an ABI Pris 3700 automated sequencer using the universal M13f and M13r primers (Applied Biosystems). PCR primers were subsequently designed to obtain a ~ 8X-coverage of overlapping series of plasmid subclones and PCR products from which a contig was assembled using SeqManII from the DNASTAR package (Lasergene, Madison, WI, USA).

The transposon insertion in the acid sensitive and symbiotic deficient mutant 899-PV4 was mapped to the 5' end of *atvA*, an ortholog of the chromosomal virulence gene *acvB* of *A. tumefaciens* and involved in acid tolerance of *R. tropici* (Vinuesa et al. 2003). In order to obtain additional sequences of genes located downstream *atvA* which might be

involved in symbiotic performance, PCR primers were designed to cover this region, using DNA from the cosmid that complements mutant 899-PV4 as a template.

DNA sequence analyses.

Open reading frames (ORFs) with high coding probability were identified on the contig sequence using FrameD (http://genopole.toulouse.inra.fr/bioinfo.FrameD/FD2) with the *S. meliloti* codon usage table and the pentanucleotide aagga as ribosomal binding site. Homology searching at the nr sequence databases of NCBI was performed with BLASTX and BLASTP programs. Protein sequence analyses to predict secondary structure, cell localization, transmembrane domains, conserved motifs, and hydrophobic profiles were performed using Prosite, ProDom, PsortB, TmPred, PsPred, Pfam and ProteinPredict program servers. A search for putative promoter regions in intergenic regions was performed using the NNPP server (www.fruitfly.org/ seq_tools/promoter.html).

Determination of operon structure by RT-PCR

RNA from *Rhizobium tropici* CIAT899 was isolated using the High Pure RNA isolation kit (Roche) according to the manufacturer's instructions and cDNA was immediately synthesized using the Omniscript RT kit (QIAGEN). This cDNA was used as template for PCR amplification of the intergenic spacer between *syc1* and *olsC* using primers PV9-2000f (5'gcagcggccataccagcatc) and PV9-2985r (5'tcacgccgaaaccgaggag). Positive controls include the amplification of 16S rDNA gene with primers fD1 and rD1 (Weisburg et al. 1991) and the amplification of a 389 bp internal fragment of *olsC* using primers PV9-2571f-H (5'ccaagcttcctcccggaccgcac) and PV9-2960r-B (5'ccggatccagcgggtgtcggtgg). To discard the presence of contamination by *R. tropici* genomic DNA, the master mix used for cDNA synthesis lacking the retrotranscriptase was used as template for PCR amplification of 16S rDNA gene and an internal fragment of *olsC* as a negative control.

Fig. 2. Schematic view of the RT-PCR experiment used to determine if *syc1* and *olsC* were cotranscribed.

Construction of non-polar mutants

To construct a non-polar mutant in *syc1*, a PCR amplification of the fragments located at the opposite ends of the ORF (causing a 991 bp deletion) was performed, using primers with restriction sites underlined, PV9-524f-H (5'gcaagcttgcccgggcggtgtgacg) and PV9-971r-B (5'cggatccggcaacgggcataagaaag) as well as PV9-1962f-B (5'ccggatcctcgctgtcgcgtgctt) and PV9-2272r-E (5'ccgaattcctgccatcggagcgtc) and total DNA of *R. tropici* as template (Fig. 3). The same strategy was used to construct a 211 bp deletion of an internal fragment in *olsC*, using primer pairs PV9-2571f-H (5'ccaagcttcctcccggaccgcac) and PV9-2960r-B (5'ccggatccagcgggtgtcggtgg) and PV9-3171f-B (5'cggatccgcgtcgacaatcacg) and PV9-3622r-E (5'cgcgaattcggtggcggcatgacg). PCR products were digested with *Hin*dIII + *Bam*HI and *Bam*HI + *Eco*RI respectively, and ligated to *Hin*dIII + *Eco*RI restricted pK18*mobsacB*. The resulting plasmids pKRΔ02 and pKRΔ03 were transferred into strain CIAT899 and double recombinants were selected on PY medium at pH 6.8 amended with 12% sucrose as previously reported (Vinuesa et al. 2003). Two non-polar mutants, here after named 899-*syc*Δ1 and 899-*olsC*Δ1, were obtained.

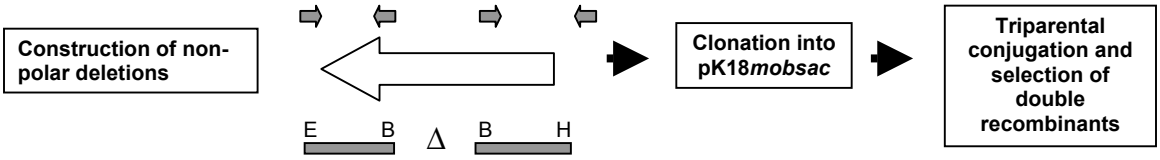

Fig. 3. Process followed to construct non-polar deletion mutants

To construct a non-polar deletion of 474 bp in *sigE*, primers PV4-5419f-E (5'cgaattcatctagtatcgcaggcaac) and PV4-5740r-B (5'cggatcctggaagcggcgcatagtc) as well as PV4-6214f-B (5'cggatccgtgattttggcgaagtc) and PV4-6798r-H (5'gcaagcttcgactacgggcggggag) were used for PCR amplification of fragments located at the opposite ends of the ORF, using total DNA of *R. tropici* as template (Fig. 3). PCR products were digested with *Eco*RI+ *Bam*HI and *Bam*HI+ *Hind*III respectively, and ligated to *Eco*RI + *Hind*III restricted pK18*mobsacB*. The resulting plasmid pKRΔsig1 was transferred into strain CIAT899 and double recombinants were selected as mentioned above. The non-polar mutant, here after named 899-Δsig1, was obtained.

The resulting deletion in each mutant was confirmed by PCR amplification of the ORF in comparison to amplification observed when DNA from parent strain CIAT899 was used as template (Fig. 4).

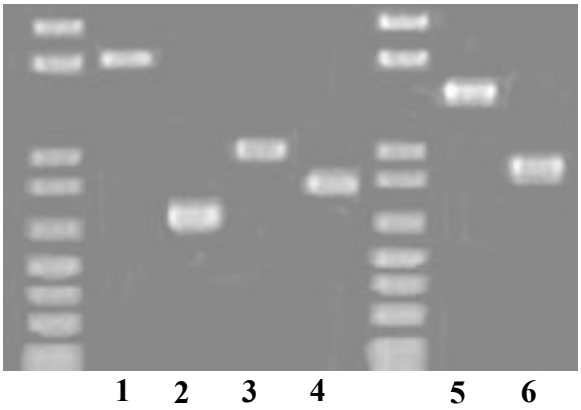

Fig. 4. PCR amplification of *syc1*, *olsC* and *sigE* when using DNA of parent strain CIAT899 (lanes1, 3 and 5) or DNA of mutant 899-*syc*Δ1 (lane 2), 899-*olsC*Δ1 (lane 4) and 899-Δsig1 (lane 6) as templates.

Complementation

A 1660 bp *Eco*RI-*Bam*HI fragment was amplified with primers PV9-1962f-B and PV9-3622r-E and cloned into the pBBR-MCS5 Gm^r vector (Kovach et al. 1995) to obtain pBRR-1,6BE. The cloned fragment contains the whole gene encoded by *olsC* and additional 534 bp upstream of the predicted start codon with the region carrying putative promoter sequence predicted by the NNPP server (Fig.5). This fragment was cloned in opposite direction to the native promoter of the vector in order to avoid vector-derived expression. Plasmid pBRR-1,6BE was transferred into mutant 899-*olsC*Δ1 by triparental mating and transconjugants were selected on PY and MM plates amended with Gm 10, Ap 50 and Nal 20. The empty pBBR-MCS5 vector was transferred into 899-*olsC*Δ1, as a control of the complementation, and transconjugants were selected as mentioned before.

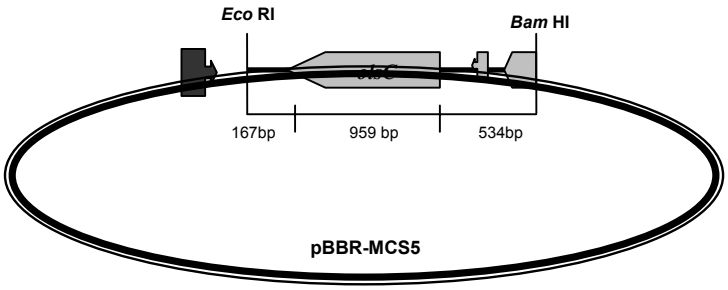

Fig. 5. Map of plasmid pBRR-1,6BE, which contains the *olsC* and its predicted promoter. It was cloned in the opposite direction of the native promoter of vector pBBR-MCS5.

To complement the mutant 899-Δsig1, a 1.4 kb *Eco*RI-*Hind*III fragment was PCR amplified using primers PV4-5419f-E and PV4-6798r-H and cloned into the pBBR-MCS5 Gm^r vector (Kovach et al. 1995) restricted with the same enzymes, the resulting plasmid was named pBBRsigE. The cloned fragment contains the whole gene encoded by *sigE* and 72 bp upstream of its predicted start codon containing one of the promoters predicted by the NNPP server. Plasmid pBBRsigE was transferred into mutant 899-Δsig1 by triparental mating and transconjugants were selected on PY and MM plates amended with Gm 10, Ap 50 and Nal 20. The resulting complementing strain was named 899-Δsig1/pBBRsigE. The vector pBBR-MCS5 was transferred into 899-Δsig1, yielding strain 899-Δsig1/pBBR, and used as a control of the complementation.

Plant tests

Phaseolus vulgaris seeds were surface-sterilized with 1.2% sodium hypochlorite and germinated on 1% agar-water plates for 48h. at 28°C. Seedlings were transferred to 250 ml flasks filled with vermiculite and nitrogen-free nutrient solution (Fahraeus 1957) and inoculated with about 10^5 CFU per plant. Plants were grown in a controlled growth chamber and harvested 21 days post inoculation (dpi). Nitrogenase activity of nodulated roots was determined by acetylene reduction assay (Kuykendall and Elkan 1976). Competition experiments were performed by co-inoculating the mutant strain together with a *gusA*-tagged reported strain, CIAT899-G1, in a 1:10 ratio as previously described (Vinuesa et al. 2003). Plants were harvested 21 dpi and blue nodules were counted after GUS staining (Wilson et al. 1995).

***In vivo* labeling of rhizobial strains with [^{14}C]acetate or [^{14}C]ornithine and analysis of lipid extracts.**

The lipid compositions of *R. tropici* CIAT899, mutant 899-*olsC*Δ1 and complemented mutant 899-*olsC*Δ1/pBBR1,6BE were determined after labeling with [1-^{14}C]acetate (60 mCi/mmol; Amersham) during growth on PY medium for 24 h. The incorporation of ornithine into lipids was followed by labeling *R. tropici* CIAT899 with DL-[1-^{14}C]ornithine (56 mCi/mmol; Amersham) during growth on minimal medium for 48 h. Cultures of 2 ml were inoculated from precultures to an initial OD_{600} of 0.05 in the respective medium. After the addition of 1 μCi [1-^{14}C]acetate or of 0.5 μCi DL-[1-^{14}C]ornithine to the respective cultures, they were incubated at 28^{o}C with appropriate shaking. At the end of the growth period, cells were harvested by centrifugation, resuspended in 100 μl of water and lipid extracts were obtained according to Bligh and Dyer (Bligh and Dyer 1959). Aliquots of the lipid extracts were spotted on high-performance TLC silica gel 60 (Merck) plates, and separated in two dimensions using chloroform-methanol-water (140:60:10, v/v/v) as a mobile phase for the first dimension, and chloroform-methanol-acetic acid (130:50:20, v/v/v) for the second. Primary amino-containing lipids were visualized by spraying the plates with a solution of 0.2% ninhydrin in acetone and subsequent treatment at 100^{o}C for 5 min. To quantify the membrane lipid composition, developed 2D-TLC plates were stained with iodine and the radioactivity of individual spots was quantified in a scintillation counter as previously described (Geiger et al. 1999).

Determination of *sigE* transcriptional regulation

To construct a *sigE*-GUS reporter fusion, a 542 bp *Xba*I-*Sal*I fragment that contains 233 bp of *sigE* and 293 bp upstream its start codon was PCR amplified. The product was cloned upstream the promoterless glucuronidase gene (*uidA*) of the vector pBBR-GUS (Corvera et al. 1999). The resulting plasmid, named pBBRsigE-GUS, was transferred into strain CIAT899 by triparental mating and transconjugants were positively selected by blue staining on PY plates amended with Gm 10, Ap 50, Nal 20 and X-gluc. The resulting strain, 899/pBBRsigE-GUS, was grown to different points of the growth curve and exposed to the above mentioned stresses. The transcriptional activation of *uidA* in this strain was determined by the quantitative β-glucuronidase assay using p-nitrophenyl

glucuronide as substrate (Wilson et al. 1992). Data were normalized to total-cell protein concentration by the Lowry method (Sambrook et al. 1989).

Identification of the *sigE* transcriptional start sites

Total RNA from strain 899/pBBRsigE-GUS, growth on PY medium to early stationary phase, was isolated using the High Pure RNA isolation kit (Roche). The RNA was subject to 5' rapid amplification of cDNA ends using the 5'RACE kit (Invitrogen). Briefly, first-strand cDNA synthesis was performed using the *uidA*-specific primer GUS-LW5 (5'CGATCCAGACTGAATGCCCAC) which is complementary to the region located in the position 96 to 117 from this gene. The resulting cDNA was treated with an RNase mix (mixture of RNase H and RNase T1), to eliminate the original mRNA template, and then purified on a GlassMax DNA column (Gibco). A homopolymeric tail was added to the resulting 3' end using the Terminal deoxynucleotidil transferase (TdT) and dCTP. A PCR amplification of the cDNA was carried out using the 5'RACE anchor primer AAP (5'GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG) and the antisense primer rrsigE-LW (5'GCGTCGACATAGTCTCGTCGGGCAAGGCG). DNA sequencing of the 5'RACE products was performed on an automatic 310 DNA sequencer (Applied Biosystems), using the Big-Dye terminator kit version 3.1 (Applied Biosystems) with primer rrsigE-LW.

Results

Chapter 1

A putative ClC chloride channel from *Rhizobium tropici* is required for symbiosome invasion of *Phaseolus vulgaris* nodules

Introduction

The regulated flow of ions across biological membranes is a process fundamental to all living organisms. It is performed by ion channels, which are integral membrane proteins that form ion-selective, water filled pores across cellular membranes. Chloride channels are a subset of ion channels that are selectively permeable to Cl^- although they generally also transport other anionic species including Br^-, NO_3^- and I^-, sometimes even more efficiently that they transport chloride. However, its name is due to Cl^- is the most abundant and physiologically the most important anion (Maduke et al. 1999; Akabas 2001; Estevez and Jentsch 2002; Mancia and Shapiro 2002).

Chloride channels perform important roles in regulation of cellular excitability, in transepithelial transport, cell volume regulation, signal transduction and acidification of intracellular membranes. Three structural families of chloride channel have been identified based on their differences in the factors that control their gating (process that control opening and closing the pore), ion selectivity (the process of determining the type of ions that can permeate through the channel) and conductance (the rate of ion translocation through the channel). The effect of opening a chloride channel depends on the driving force for the Cl^- movement across the cell membrane. When a chloride channel opens, Cl^- ions may enter or leave the cell depending on the direction of the chloride electrochemical gradient. When the intracellular chloride concentration is higher, the electrical force on the Cl^- ions dominates and Cl^- ions move out the cell. When the intracellular chloride concentration is low, the chemical driving force dominates and Cl^- moves into the cell. The balance between the two forces will determine the direction of chloride movement (Akabas 2001; Estevez and Jentsch 2002).

Three structural classes of chloride channels are well established: the ClC channel family, the cystic fibrosis transmembrane conductance regulator and the γ-aminobutyric acid (GABA)-gated and glycine-gated neurotransmitter receptor. The ClC family is a large

family of proteins found on gram-negative and gram-positive bacteria, cyanobacteria, archaea, yeast, plants and animals that are ubiquitously distributed and with several organisms containing multiple ClC family paralogues. These proteins exhibit 9-12 transmembrane α-helical spanners and appear to be present in the membrane as homodimers, with the N- and C-termini residing in the cytoplasm (Dutzler et al. 2002).

Currently, a small number of prokaryotic ClC channels have been characterized and therefore its function is not completely elucidated. The few characterized proteins include the EriC from *E. coli*, which has been shown to mediate the extreme acid resistance response. In this model, bacteria exposed to acidic conditions decarboxylates amino acids (like glutamate and arginine) to consume protons (Foster 1999; Merrell and Camilli 2002). Positively charged products are then removed by antiporters, generating positive voltages at inside that could activate gating of chloride channels (Dutzler 2004) to prevent hyperpolarization of the inner membrane by Cl^- influx (Iyer et al. 2002). However, further experiments with this protein indicate that EriC may actually not be an ion channel but rather an H^+-Cl^- exchange transporter (Accardi and Miller 2004). A deletion of a *V. cholerae* ClC channel resulted in mild resistance to acidity when pH was adjusted with HCl and enhanced intestinal colonization in infant mice (Ding and Waldor 2003).

Further characterization of other ClC channels in prokaryotes as well as determining the role of each ClC subfamily in symbiosis/pathogenesis and other conditions is therefore required. In this chapter, it is presented the genetic analysis of a putative ClC channel of *R. tropici* which is required for symbiosome invasion of *Phaseolus vulgaris* nodules. This is the first report of a putative ClC channel in rhizobia involved in symbiosis. Additionally, evidence is presented of a second copy of this gene in this species with a not yet determined function.

Results of chapter 1

Complementation of mutant 899-PV9 from *Rhizobium tropici* CIAT899

A symbiotically-defective mutant of *Rhizobium tropici* CIAT899, obtained by random transposon mutagenesis, was designated 899-PV9. When mutant 899-PV9 was inoculated on *Phaseolus vulgaris* plants, nodules were not fully developed, irregular in size, lacked lenticels and had a reduced nitrogenase activity. Southern blot analysis confirmed that the observed phenotype of this strain is due to a single Tn*5* insertion, which was localized between nucleotides C1763 and T1764 after sequence analyses (Fig. 6). Mapping of the insertion junction site was achieved by sequencing plasmid p899PV9-1, which contains the Tn*5* insertion of strain 899-PV9 cloned as an *Eco*RI fragment, using primer Tn5-77/58EB (Vinuesa et al. 2003), which reads outwardly from the IS Tn*5* elements.

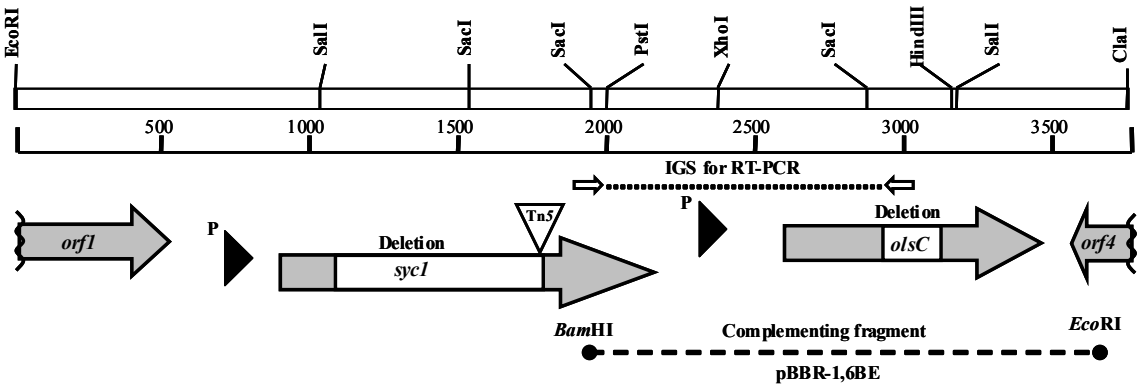

Fig. 6. Genetic and physical maps of the 3761 bp *Eco*RI-*Cla*I region that complements the mutant 899-PV9. Selected restriction sites are shown. Four open reading frames (represented by arrows) were detected. The site of the Tn*5* insertion located in *syc1* between nucleotides C1763 and T1764 is indicated in the triangle. *syc1* and *olsC* were selected for further analyses. Non-polar deletion mutants were generated lacking the regions shown in white. Predicted promoters are shown as black triangles. Dotted line represents the intergenic spacer between *syc1* and *olsC* analyzed by RT-PCR. The 1.66 kb *Bam*HI-*Eco*RI fragment (dashed line) cloned into pBBR-MCS5 and introduced into strain 899-*olsC*Δ1 restored the symbiotic proficiency in this mutant.

Several transconjugants were able to restore symbiotic proficiency of this mutant strain after complementation with a cosmid library. The cosmids isolated from these

transconjugants were identical in their restriction patterns after digestion with different enzymes. One of these cosmids, named pPV9cos2, was digested with *Eco*RI and hybridized against p899PV9-1 and the resulting hybridizing fragment (~6.2 kb*)* was subcloned into pSK, yielding pPV9E-SK, and subjected to DNA sequencing.

Sequence analyses and genetic characterization of *syc1*

Using different computer programs, an analysis of the 3761 bp *Eco*RI-*Cla*I complementing sequence of mutant PV9-899 (accession number AY954450) revealed 4 open reading frames (ORFs) with high coding probability, as predicted by FrameD. ORFs 1 to 3 are transcribed in the same orientation, while ORF4 is transcribed convergently with respect to ORF3. *orf1* and *orf4* were truncated at their 5' ends and code for a putative methyl transferase and for a putative 3-oxo(acyl carrier protein) synthase III, respectively, according to BLASTX searches. The genetic analysis presented herein targeted ORF2-3, which are the ones that could be affected by the Tn*5* insertion in mutant 899-PV9 (Fig. 6).

In frame +2, at position 785 starts a 1368 bp-long ORF (*orf2*) predicted to encode a 48.6 kDa product, which according to homology searches with the Blast program (Fig. 7), showed highly significant sequence similarity to putative ClC chloride channel proteins from diverse β-proteobacteria *Burkholderia cepacia* (E value e^{-104}, 49% identity and 64% similarity), *Ralstonia solanacearum* (E value e^{-103}, 49% identity and 64% similarity), and the γ-proteobacteria *Pseudomonas syringae* (E value e^{-103}, 45% identity and 62% similarity), *Salmonella typhimurium* (E value $2e^{-55}$, 27% identity and 42% similarity) and *Escherichia coli* (E value $4e^{-45}$, 20% identity and 35% similarity). The product of this gene belongs to the voltage-gated chloride channel protein family COG0038 with inner membrane localization.

The rhizobial ClC chloride channel homologue is predicted to have 10 transmembrane helices with both N- and C-terminal domains residing in the cytoplasm, containing the sequence motifs corresponding to the ion-binding site and gating region of the solved *E. coli* and *S. typhimurium* ClC protein structure (Fig. 8). This superfamily of ion channels is found in both, prokaryotic and eukaryotic cells. One of the two ClC paralogs found in *E. coli,* named EriC, has been proposed to act as an electrical shunt for an outwardly

directed proton pump that is linked to amino acid decarboxylation as part of the extreme acid resistance response of this bacterium (Iyer et al. 2002).

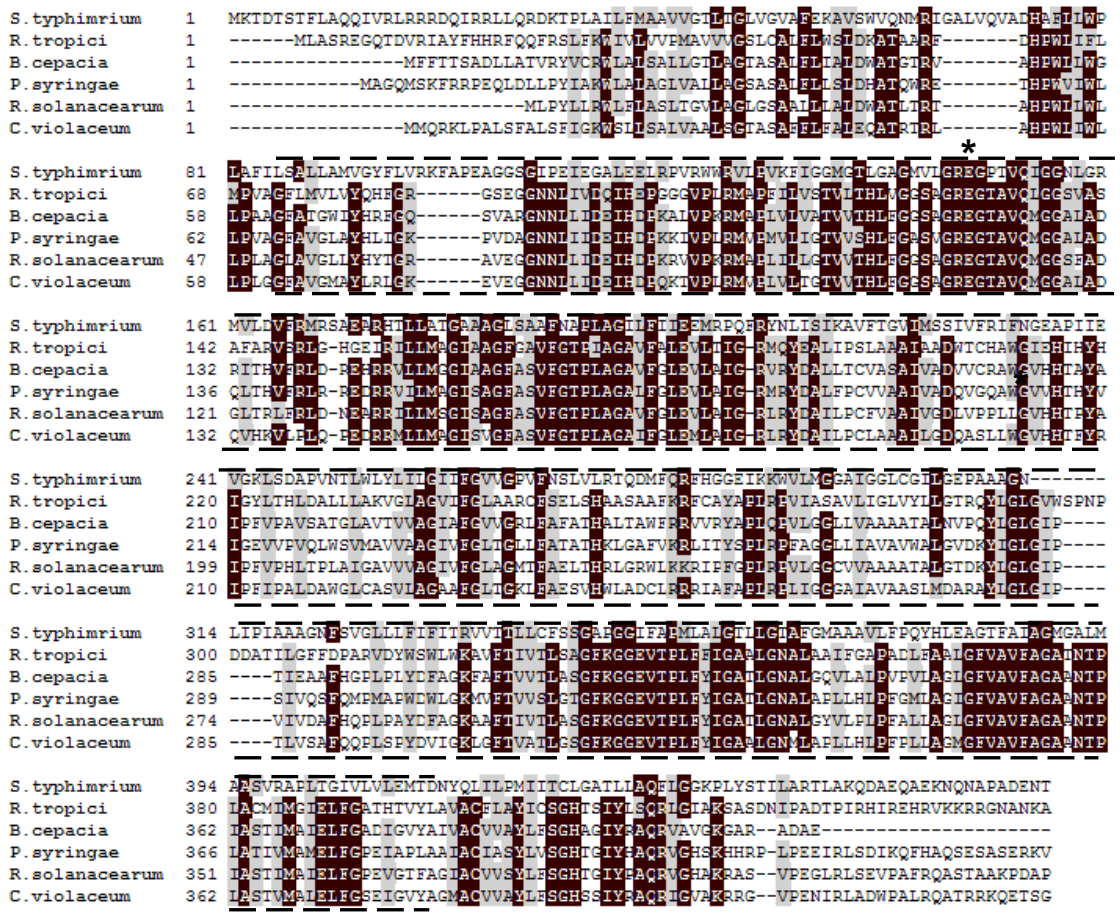

Fig. 7. Amino acid sequence alignment of *Rhizobium tropici, Brucella cepacia*, *Pseudomonas syringae*, *Ralstonia solanacearum, Chromobacterium solanacearum* putative chloride channel sequences against ClC chloride channel from *Salmonella typhimurium.* Glutamate residue E129 (in asterisk) is shown to be highly conserved. It is part of the gating region of this protein (Dutzler et al. 2003).

Protein sequence alignments and secondary structure analyses showed a high conservation (Fig. 8), including the *R. tropici* residue E129 which in the homologous ClC chloride channels from *E. coli* and *S. enterica* is the one responsible for gating according to X-ray crystallographic studies (Dutzler et al. 2002; Dutzler 2004). Therefore, based on the similarity of *orf2* to ClC chloride channels the former was named into *syc1,* for symbiosis-assisting channel. The intergenic spacer (IGS) between *orf1* and *syc1* was

analyzed using the neural network for promoter prediction (NNPP), which with modest resolution located a putative promoter sequence upstream *syc1* start codon (sites 727-772, $r = 0.81$).

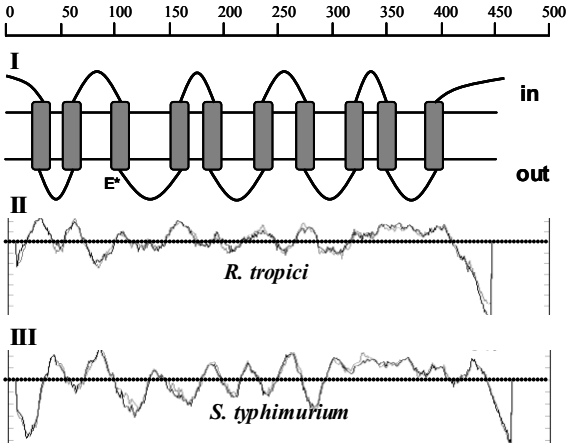

Fig. 8. Predicted secondary structure of *R. tropici* CIAT899 putative chloride channel (panels I and II) in comparison to ClC channel from *Salmonella typhimurium* (panel III). Glutamate residue E129 (with asterisk) is shown to be highly conserved in the gating region of this protein (Dutzler et al. 2003). Transmembrane prediction was obtained through the Predictprotein and TmPred servers.

Construction of a non-polar deletion in *syc1* and phenotypic characterization of the mutant strain

A non-polar mutation in *syc1* was generated by deletion of a 991 bp-long fragment that embraces nearly the whole gene, including the predicted gating region. To construct this mutant, plasmid pKRΔ02 was transferred into CIAT899. Double recombinants were selected for the loss of sensitivity to sucrose and the resulting deletion was confirmed by PCR with primers PV9-524f-H and PV9-2272r-E (Fig. 4). The mutant obtained was designated 899-*syc*Δ1. This strain was able to grow in 20E or PY media acidified to pH 4.5 at similar rates as the parent strain. However, the nodules it formed on bean plants were poorly developed (21dpi), lacked lenticels, were whitish and were decreased in nitrogen fixation to 7% in comparison to the parent strain, as revealed by the acetylene reduction assay (Fig. 9A).

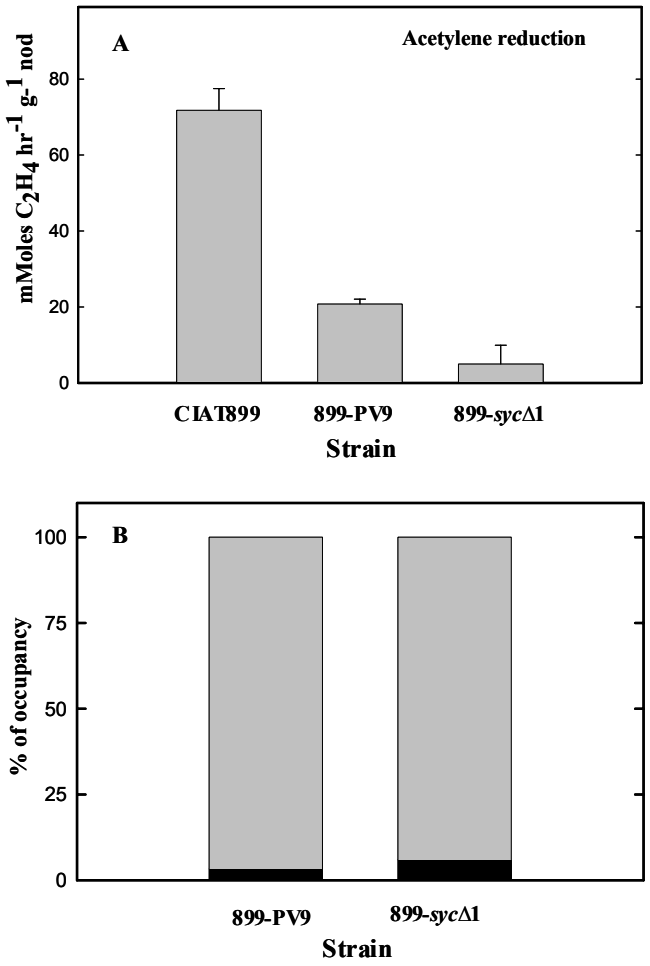

Fig. 9. Phenotype displayed by mutants 899-PV9 and 899-*syc*Δ1 against parent strain CIAT899 on *Phaseolus vulgaris* plants **(A)** Mean acetylene reduction of nodulated roots (21 dpi) of strains 899-PV9 and 899-*syc*Δ1 in comparison to parent strain CIAT899, values are the mean ± SD of three repetitions in two independent experiments. (**B**) Percentage of nodules occupied by mutants against reporter strain CIAT899-G1 in a 10:1 coinoculation experiment.

The nodulation competitiveness of 899-*syc*Δ1 against CIAT899-G1 in a 10:1 co-inoculation experiment showed that only 5.9% of the nodules were occupied by the mutant strain although the mutant strain was 10 times more abundant that the reported strain (Fig. 9B). The phenotype displayed by this strain corresponds to that observed in mutant 899-PV9. Further analysis using light and electron microscopy revealed that both mutants were able to enter the nodules, but unable to form stable symbiosomes.

Micrographs of nodules inoculated with 899-*syc*Δ1 showed poor invasion of plant cells, accumulation of poly-β-hydroxybutyrate (PHB) granules within bacteroids, and presence of amyloplasts, whereareas the parent strain was able to fully invade plant cells (Fig. 10). The symbiotic proficiency of mutant 899-*syc*Δ1 was restored when cosmid pPV9cos2 was provided *in trans*.

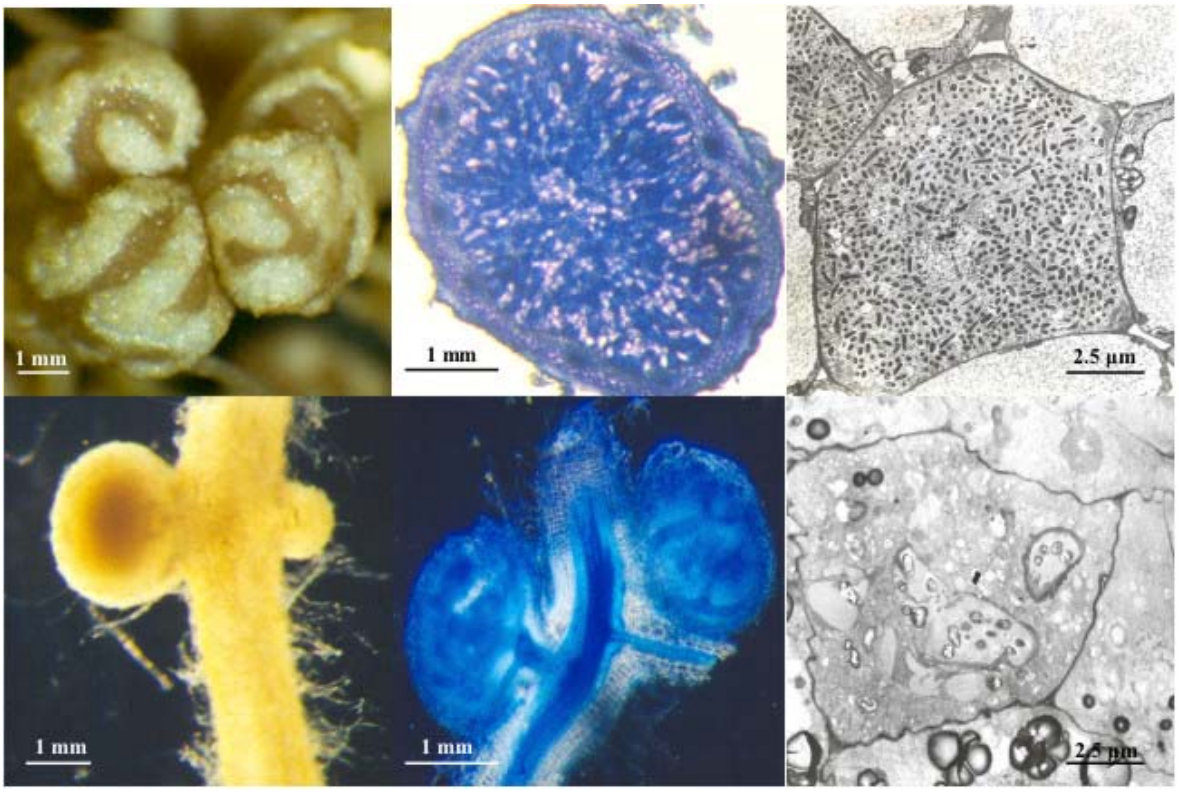

Fig. 10. Root nodules of common bean (*Phaseolus vulgaris*) infected with *Rhizobium tropici* CIAT899 parent strain (upper panel) or with a mutant, defective in a putative chloride channel (lower panel). Intact bean nodules (left panel), cross-sections through bean nodules (middle panel), and electron micrographs (right panel) showing the structure of *R. tropici*-infected bean nodule cells

Discussion of chater 1

The microscopy analyses performed on mutant 899-*syc*Δ1, which carries a deletion in the putative chloride channel encoded by *syc1*, suggests that the observed decrease in nodule development and nitrogen fixation is probably due to its failure to invade plant cells and to form stable symbiosomes. This is the first report showing that a rhizobial homologue of the ClC family of Cl^- channels and Cl^-/H^+ antiporters is essential for the establishment of a fully proficient symbiotic interaction with its legume host, but not for growth under free-living conditions. With the notable exception of *Escherichia coli*, the molecular and physiological functions of dozens of prokaryotic ClC homologues recently uncovered by genome sequencing projects are still unknown. It is worth noting that the ClC-ec1 (or EriC) protein of *E. coli* was recently shown not to be an ion channel, but rather a H^+/Cl^- exchange transporter, demonstrating that the structural boundary separating transporters and channels is not clearcut, as previously thought (Accardi and Miller 2004; Chen 2005). The *E. coli* genome has two ClC homologs. When either one of these genes was individually deleted, no notable phenotype was observed. However, a doble-knock-out strain displayed a dramatic reduction in cell survival and amino acids transport under acid shock (Iyer et al. 2002) Recently a second ClC-like paralog from CIAT899 was cloned (as found in the genomes of many other α-Proteobacteria, including *Agrobacterium tumefaciens* C58, *Brucella suis* 1330 and *B. melitensis* 16M, *Bradyrhizobium japonicum* USDA110 and *Mesorhizobium loti* MAFF303099, but not *Sinorhizobium meliloti* 1021), which suggests that the ClC paralogs might perform different functions in the cell under different physiological conditions, and might be differentially expressed. The complementation results demonstrate that the paralog (*syc1*) mutated in this study is required for a proficient symbiotic interaction with bean plants, but apparently not for acid tolerance or growth under free-living conditions. However, at this point it cannot be defined if the mutations made in *syc1* have a direct or indirect effect on the symbiotic phenotype observed in strains 899-PV9 and 899-*syc*Δ1. At this point, and based on the evidence gained from TEM data, I can not state wether the mutation is affecting bacteroid release from infection threads, symbiosome proliferation or stability. Mutations in the second paralogous gene, the construction of a double mutant and analysis of

transcriptional reporter gene fusions would be very valuable to gain a better understanding of the functions of these genes.

It has recently been shown that *Rhizobium leguminosarum* mutants blocked in amino acid transporters, present poorly developed nodules, reduced N_2 fixation, and the bacteroids are saturated with dicarboxylic acids and polyhydroxybutyrate granules (Lodwig et al. 2003). Since the peribacteroid space is acidic and the mutant 899-*syc*Δ1 displayed a similar phenotype to that observed for the *R. leguminosarum* mutant, it is tempting to speculate that Syc1 might be involved in the adaptation of *R. tropici* bacteroids to the symbiosome's milieu, probably in relation with the electrophysiology of bacteroid membranes, which in turn may affect key aspects of cellular homeostasis like the internal pH of bacteroides, or the transport of metabolites across their cellular membranes. Since ClC channels and antiporters are highly selective for chloride anions (Accardi et al. 2004; Chen 2005), this would imply that Cl^- could play a key role in symbiosome physiology. If so, it remains to be uncovered. Plant voltage-dependent anion channels (VDACs), including ClC homologues, have been recently found to play a broader diversity of functions than previously thought (Barbier-Brygoo et al. 2000; Wandrey et al. 2004). Several plant VDACs and anion transporters have recently been shown to be associated with the symbiosome membrane (Wienkoop et al. 2003; Vincill et al. 2005). Therfore, both plant and bacterial anion channels or antiporters seem to play important, although not yet well understood functions in root nodule symbioses.

Only two previous works describe the phenotypes of microbial cells carrying mutations in ClC homologs in relation to their interaction with eukaryotic hosts. Mutations in *clc-a* from the human pathogenic yeast *Cryptococcus neoformans* resulted in attenuated virulence in a mouse cryptococcosis model. This attenuation resulted from the lack of expression of two important virulence factors, capsule and laccase (Zhu and Williamson, 2003). In contrast, deletion of the single ClC ortholog found in *Vibrio cholerae* enhanced intestinal colonization in infant mice. This gene was found to confer mild resistance to acid when pH was adjusted with HCl, but not with other acids (Ding and Waldor, 2003). Clearly, much research is still needed to provide a basic understanding of the molecular and physiological functions of the diverse ClC homologs found across prokaryotic phyla.

Chapter 2

A putative β-hydroxylase from *Rhizobium tropici* is involved in the modification of two ornithine-containing membrane lipids which are required for symbiosis and acid tolerance

Introduction

Biological membranes consist of many species of lipids that separate interior from exterior and internal cellular compartments. Membrane lipids are amphipathic molecules that form the lipid bilayer by a macromolecular assembly, stabilized by non-covalent interactions. It consists of a wide variety of lipids that are the result of complex metabolic pathways in the cell. Dynamics of lipids in cell membranes influences membrane protein function. The reason of different types of lipids is not clear, although it is assumed that it determines the appropriate membrane environment required for the functioning of membrane-associated proteins that are embedded in the lipid layer (Vance 2001; Yeagle 2001; Cronan 2003).

Membranes of different organisms contain distinct lipid compositions although the most abundant membrane lipids are the phospholipids, present in all organisms from bacteria to humans. The shape of a given phospholipid molecule depends on both its head group and the degree of unsaturation and chain length of the acyl chains. In Rhizobiaceae, like other Gram-negative bacteria, besides the cytoplasmic membrane exist an outer membrane containing membrane-forming lipids, mainly phospholipids, and proteins. The inner membrane is impermeable to solutes unless specific transport systems are present. The outer membrane is rich in structural lipoproteins and proteins and contains pores involved in the transport of high molecular weight compounds. In addition, the outer layer of this membrane contains as additional components the lipopolysaccharides (LPS) consisting of a hydrophobic domain known as lipid A, a nonrepeating "core" oligosaccharide and a distal polysaccharide (or O-antigen). Besides LPS, in this layer are also found the exopolysaccharides (EPS). Both LPS and EPS are known to play important roles in plant-bacterium interactions. Between the inner and outer membrane is an osmotically active compartment called the periplasmic space. Membrane-derived oligosaccharides, peptidoglycan and binding proteins involved in metabolic transport are found in this compartment (Geiger 1998; Heath et al. 2002; Raetz and Whitfield 2002).

Membrane lipids in most bacteria generally consist of glycerophospholipids phosphatidylglycerol (PG), cardiolipin (CL) and phosphatidylethanolamine (PE). In addition to PG, CL and PE, a large diversity of membrane lipids can be found in bacteria, some of them as minor components but some others as major components in the bacterial membrane. They include the methylated derivatives of PE, monomethylphosphatidylethanolamine (MMPE), dimethylphosphatidylethanolamine (DMPE) and phosphatidylcholine (PC). Other phospholipids rarely found are phosphatidylserine (PS) and phosphatidylinositol (PI). Additionally to phosphorus-containing lipids, the membranes of numerous bacteria contain significant amounts of phosphorus-free polar lipids, often derived from amino acids. They have been detected under certain growth conditions and include hopanoids, glycolipids, the sulfur-containing lipid sulfoquinovosyl diacylglycerol (SL), the betaine lipid diacylglyceryl *N,N,N*-trimethylhomoserine (DGTS) and lipids derived from the amino acid ornithine (OL) (Lopez-Lara et al. 2003; Sohlenkamp et al. 2003).

Ornithine-containing lipids are shown to be widespread among bacteria including pathogenic bacteria *Mycobacterium tuberculosis* (Laneelle et al. 1990), *Flavobacterium meningosepticum* (Kato and Goto 1997), *Bordetella pertussis* (Kawai et al. 1982), *Pseudomonas flluorescens* (Minnikin and Abdolrahimzadeh 1974) and *Burhkholderia cepacia* (Taylor et al. 1998). A common characteristic of the ornithine-containing lipids, especially in virulent strains, is the presence of a hydroxyl group in the esterified-linked fatty acid group (Wee and Wilkinson 1988; Laneelle et al. 1990; Inglis et al. 2003). The OL possesses an endotoxin-like structure and have several biological activities including B-cell mitogenicity and macrophage activation. In addition, these lipids are expected to be utilized as nontoxic vaccine adjuvants (Kawai and Akagawa 1989; Kato and Goto 1997; Kawai et al. 1999; Kawai et al. 2002). *Salmonella typhimurium* has the ability to synthesize lipid A that also contains 2-hydroxymyristate. In this species the 2-hydroxylation of myristate in lipid A, catalyzed by LpxO, seems to be responsible for reduced host cell recognition and thought to be important for pathogenesis (Ernst et al. 1999; Ernst et al. 2001; Raetz 2001).

Currently, the functional roles of phospholipids and the other membrane lipids in bacteria, is still not fully elucidated. In the case of rhizobial members, which are able to form nitrogen-fixing root nodules on legume plants, PC is a major component of its membranes and therefore thought to be required for a successful interaction with the eukaryotic host. Examples include *Sinorhizobium meliloti* mutants lacking phosphatidylcholine (PC) that were unable to form nitrogen-fixing nodules on alfalfa (Lopez-Lara et al. 2003; Sohlenkamp et al. 2003) and *pmtA*-deficient mutants of *Bradyrhizobium japonicum* containing reduced levels of PC and produced a decreased number of bacteroids within infected plant cells (Minder et al. 2001).

In the present chapter, a characterization is presented of lipid membranes of *R. tropici* CIAT899 including four ornithine-containing lipids not previously reported. A putative β-hydroxylase from this species was identified, and a non-polar mutation of this gene lead to lack of two presumably hydroxylated forms of ornithine lipids involved in the symbiosis with bean plants and overexpression of this gene resulted in an acid sensitive phenotype.

Results of chapter 2

Genetic analyses of *olsC*

In frame +1, at position 2611 starts a 845-bp-long ORF (*olsC*) predicted to encode a 31.78 kDa product, with highly significant sequence similarity to a putative aspartyl/asparaginyl β-hydroxylase from *Mesorhizobium* sp. (E value $9e^{-90}$, 64% identity and 76% similarity) and from *Azotobacter vinelandii* (E value $4e^{-63}$, 49% identity and 65% similarity), and a lower similarity to lipid A-myristate β-hydroxylase (LpxO) from *Salmonella typhimurium* (E value $5e^{-23}$, 35% identity and 51% similarity). The latter protein has been shown to be responsible for the 2-hydroxylation of myristate in lipid A (Gibbons et al. 2000). Sequence analyses predicted a cytoplasmic localization for the OlsC, with N- and C-terminal hydrophobic domains. The catalytic domain of this aspartyl/asparaginyl β-hydroxylase protein family is well conserved (Fig. 11). In particular, histidine residue H164 is thought to be an iron ligand and therefore essential for the function of the protein (Jia et al. 1994).

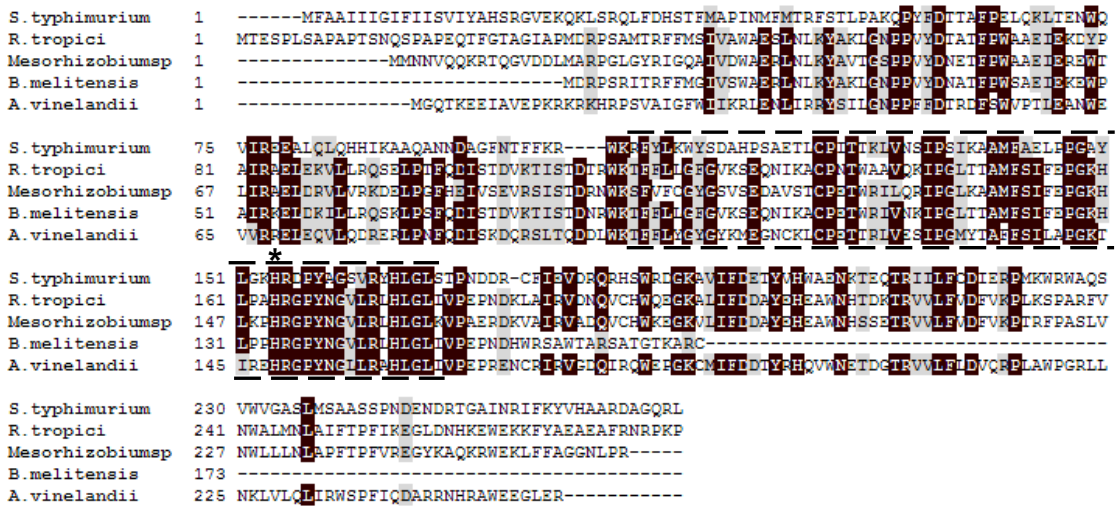

Fig. 11. Amino acid sequence alignment of *Rhizobium tropici, Mesorhizobium sp*, *Brucella melitensis*, *Azotobacter vinelandii* putative aspartyl/asparaginyl beta-hydroxylase sequences against LpxO from *Salmonella typhimurium.* Residue His164 is highly conserved as part of the catalytic domain, which have been demonstrated to be essential for the function of this protein (Jia et al. 1994). Identical residues are underlined in black and similar residues shaded in grey. Dashed lines represent the deleted fragment in the mutant.

A phylogenetic reconstruction based on selected protein sequences of this family found in divergent bacteria (Fig. 12) revealed that the putative aspartyl/asparaginyl β-hydroxylase from *R. tropici* is located in a different clade than the LpxO protein from *S. typhimurium.* This inference suggests that LpxO and OlsC might have slightly different functions or substrate specificities, although they clearly belong to the same protein family (COG3555).

The IGS between *syc1* and *olsC* was analyzed using the NNPP server, which predicted 2 possible promoter sequences upstream *olsC* (sites 2306-2351, r = 0.98; 2419-2464, r = 0.94). This promoter prediction is consistent with a monocistronic organization of the transcript encoded by *syc1*, which is also supported by RT-PCR experiments, complementation analyses, and the phenotype observed by 2D-TLC (discussed below).

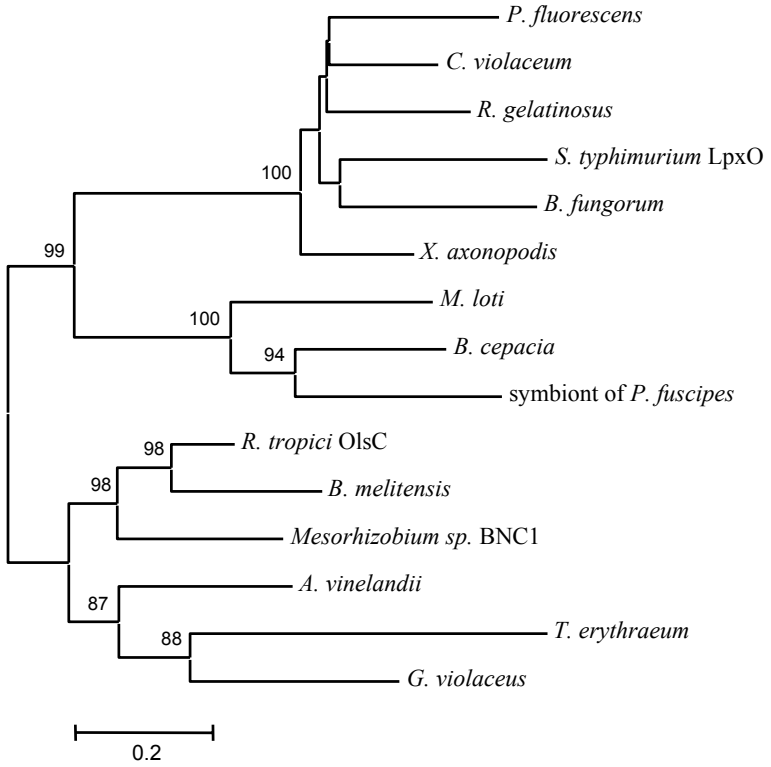

Fig. 12. Phylogenetical analysis *Rhizobium tropici* CIAT899 *olsC* sequence in comparison to homologous sequences from other bacteria. Percentage bootstrap support (1000 replicates) is indicated at branching points.

RT-PCR of the intergenic spacer between *syc*1 and *olsC* confirms that both genes are independently transcribed.

An RT-PCR experiment was designed to determine the transcriptional organization of *syc1* and *olsC* (Fig. 13). For this purpose, RNA was isolated from *Rhizobium tropici* CIAT899 cells grown in PY broth to the early stationary phase, and used to synthesize cDNA with random hexamers as primers. This cDNA was used as template for PCR amplification experiments with primers PV9-2000f and PV9-2985r, which bind up and downstream of the IGS region. No amplification product was detected, which suggests that both ORFs are independently transcribed under these conditions. Therefore, making it unlikely that the Tn*5* insertion in *syc1* has a polar effect on *olsC*. However, IGS region could be amplified with PV9-2000f and PV9-2985r when genomic DNA was used as template. The presence of an internal fragment of *olsC* and 16S rDNA in the cDNA template was confirmed by PCR amplification of these genes, demonstrating a proper cDNA synthesis. In the negative control without reverse transcriptase, no amplification product was observed, which discards the possibility of contamination by *R. tropici* genomic DNA.

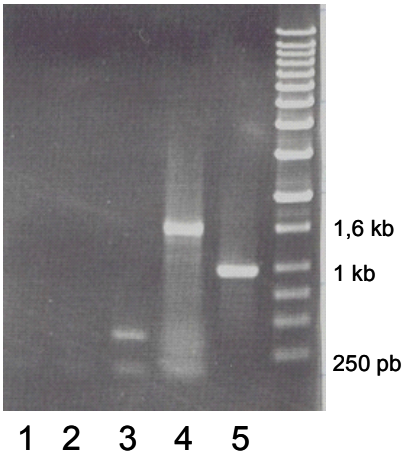

Fig. 13. RT-PCR experiment shows no PCR amplification of the IGS between *syc1* and *olsC* (lane 1), which suggests that both ORFs are independently be transcribed under these conditions. No amplification in the negative control (lane 2) discards the possibility of contamination by *R. tropici* genomic DNA. Positive controls include the PCR amplification of an internal fragment of *olsC* (lane 3) and the 16S rDNA gene (lane 4) when using cDNA as template, or the IGS region when using genomic DNA as template (lane 5).

Construction of a non-polar deletion in *olsC* and phenotypic characterization of mutant strain

A partial deletion (211 bp-long) of the region containing the predicted catalytic domain of OlsC was generated using plasmid pKRΔ03 following the same procedure mentioned for *syc1*. The resulting strain (899-*olsC*Δ1) was confirmed to carry the deletion by PCR with primers PV9-2571f-E and PV9-3622r-H (Fig. 4). Mutant 899-*olsC*Δ1 was able to grow in 20E or PY media acidified to pH 4.5 at a similar rate as the parent strain (Fig. 14).

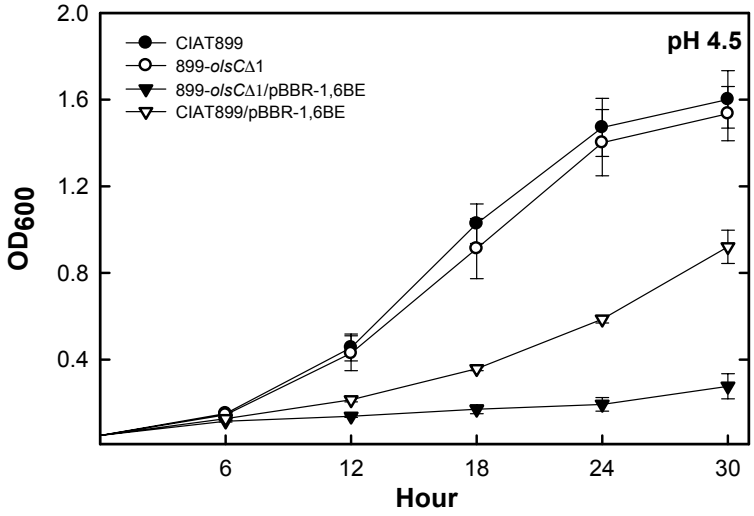

Fig. 14. Growth of *R. tropici* strains CIAT899, 899-*olsC*Δ1, 899-*olsC*Δ1/pBBR-1,6BE and CIAT899/pBBR1,6BE on PY media at pH 4.5. Values are the mean ± SD of 4 independent experiments.

Nodules of bean plants inoculated with this strain (21 dpi) were poorly developed (Ndv$^-$) and lacked lenticels. These nodules also showed reduced levels of nitrogen fixation (about 50%) when compared to the wild type strain CIAT899, as determined by acetylene reduction assays (Fig. 15A). When mutant 899-*olsC*Δ1 was co-inoculated against the *gusA*-tagged reporter strain CIAT899-G1 even in a 10:1 ratio, only 25% of the nodules were occupied by the former, which remained unstained and displayed the same phenotype observed in those nodules induced by 899-*olsC*Δ1. This represents a 3-fold decrease in relative competitiveness of the mutant with respect to the reporter strain (Fig. 15B).

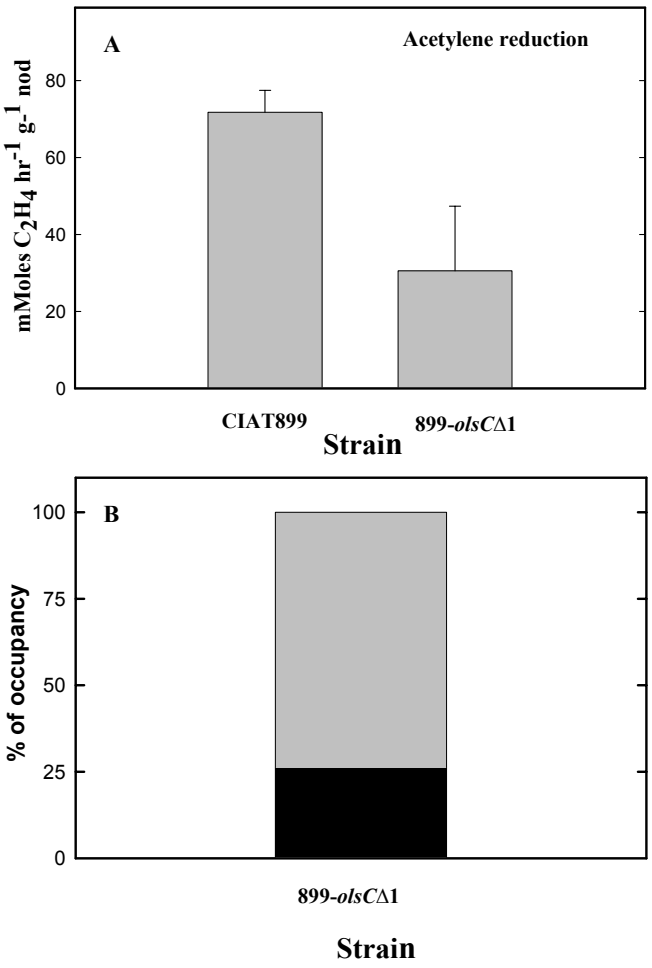

Fig 15. Phenotype displayed by mutants 899-PV9 and 899-*olsC*Δ1 against parent strain CIAT899 on *Phaseolus vulgaris* plants **(A)** Mean acetylene reduction of nodulated roots (21 dpi) of strain 899-*olsC*Δ1 in comparison to parent strain CIAT899, values are the mean ± SD of three repetitions in two independent experiments. (**B**) Percentage of nodules occupied by mutant 899-*olsC*Δ1 against reporter strain CIAT899-G1 in a 10:1 coinoculation experiment.

Light micrographs of nodules inoculated with mutant 899-*olsC*Δ1 revealed lower invasion levels than for the parent strain CIAT899 (Fig. 16), however, not as drastic as observed for mutant 899-*syc*Δ1.

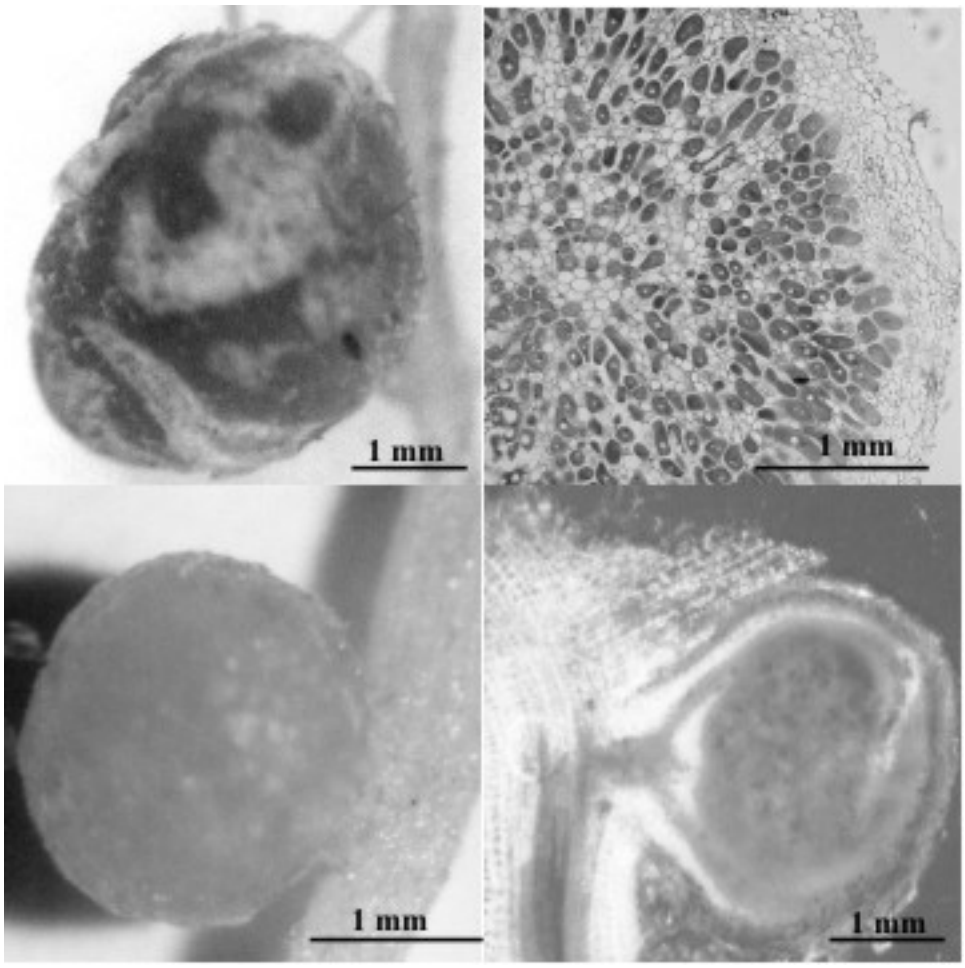

Fig. 16. Root nodules of common bean (*Phaseolus vulgaris*) infected with *Rhizobium tropici* CIAT899 parent strain (upper panel) or with a mutant, defective in a putative β-hydroxylase (lower panel). Intact bean nodules (left panel) and cross-sections through bean nodules (right panel) showing the structure of *R. tropici*-infected bean nodule cells

Complementation of mutant 899-*olsC*Δ1 with pBBR-1,6BE.

In order to complement mutant 899-*olsC*Δ1, plasmid pBBR-1,6BE (Fig. 5) was constructed and provided *in trans*. A 1660 bp *Bam*HI-*Eco*RI fragment containing *olsC* and the region upstream with the predicted promoter region was PCR-amplified and cloned into pBBR-MCS5. To ensure that the protein is expressed from its native promoter, the fragment was cloned in the opposite direction of the vector *lacZ* promoter. Plasmid pBBR-1,6BE was transferred into mutant 899-*olsC*Δ1 and the resulting strain 899-*olsC*Δ1/pBBR-1,6BE was tested on bean plants for symbiotic performance. The

symbiotic proficiency and nitrogen fixation capacity of mutant 899-*olsC*Δ1 was restored to similar levels as exhibited by CIAT899, when complemented with plasmid pBBR-1,6BE. On near neutral media (pH 6.8), the complemented strain 899-*olsC*Δ1/pBBR-1,6BE (generation time g = 2.8 h) grew similarly as CIAT899 (g = 2.4 h), or mutant 899-*olsC*Δ1, with (g = 2.8 h) or without (g = 2.8 h) the empty vector (data not shown). In contrast, when the complemented strain was grown on acidified media at pH 4.5 (Fig. 14), it presented a significantly increased mean generation time (g = 9.2 h) in comparison to CIAT899, or the mutant 899-*olsC*Δ1, with or without the empty vector (g ≈ 2.9 h). These results suggest that the expression or over-expression (due to copy number) of the gene contained in this 1.66 kb fragment is responsible for the reduced growth under acidic conditions displayed by the complemented strain when carrying plasmid pBBR-1,6BE *in trans*. This hypothesis is supported by the fact that strain CIAT899/ pBBR-1,6BE also presented an increased generation time (g ≈ 7.1 h).

Some membrane lipids of *R. tropici* CIAT899 are absent in mutant 899-*olsC*Δ1.

Lipid extracts from *Rhizobium tropici* CIAT899, mutant 899-*olsC*Δ1 carrying the 211 bp deletion in *olsC*, and the complemented mutant 899-*olsC*Δ1/pBBR-1,6BE were separated by two-dimensional thin-layer chromatography (2D-TLC) and individual lipids were quantified (Table 2). Rhizobial membrane phospholipids like phosphatidylethanolamine (PE), dimethylphosphatidylethanolamine (DMPE), cardiolipin (CL), phosphatidylglycerol (PG), sulphoquinovosyl diacylglycerol (SL) and phosphatidylcholine (PC) were identified based on their relative mobilities and in comparison to the well-characterized lipid profile of *Sinorhizobium meliloti* 1021 (Weissenmayer et al. 2002; Gao et al. 2004). As found for *S. meliloti* 1021, PC constitutes also the major membrane lipid of *R. tropici* CIAT899. In addition to the above-mentioned lipids, four additional components can be detected in the chromatogram of *R. tropici* CIAT899, labeled as S1, S2, P1, and P2 (Fig. 17A).

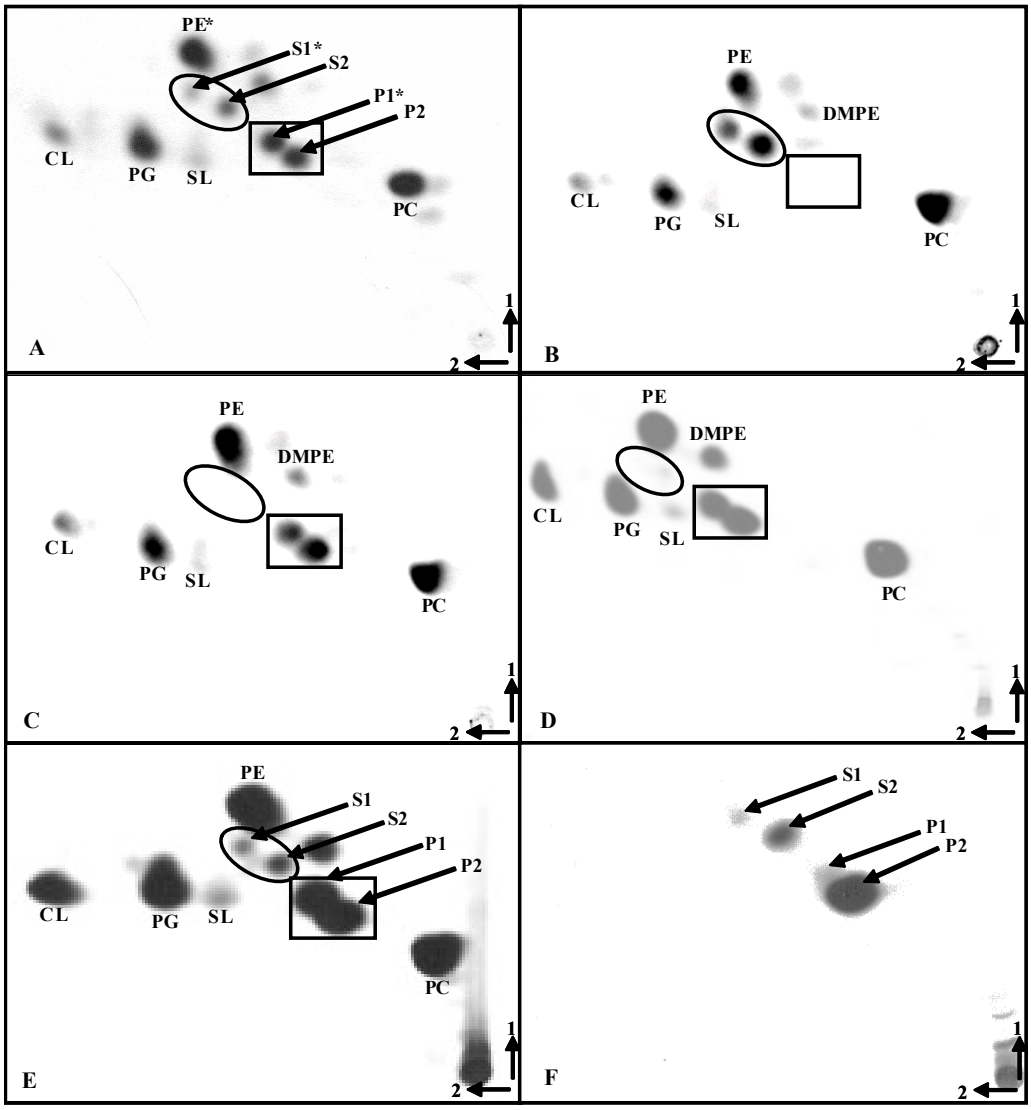

Fig. 17. Membrane lipid analysis of *Rhizobium tropici* strains. Separation of [^{14}C]acetate-labeled lipids from *R. tropici* CIAT899 (**A**), mutant 899-*olsC*Δ1 (**B**), complemented mutant 899-*olsC*Δ1/pBBR-1,6BE (**C**), strain CIAT899/pBBR1,6BE (**D**) and strain 899-*olsC*Δ1/pBBR-1,6BE+pJG21 (**E**) as well as of [^{14}C]ornithine-labeled lipids from *R. tropici* CIAT899 (**F**) using two-dimensional thin-layer chromatography. The lipids cardiolipin (CL), phosphatidylglycerol (PG), sulphoquinovosyl diacylglycerol (SL), phosphatidylethanolamine (PE), dimethylphosphatidylethanolamine (DMPE) and phosphatidylcholine (PC) are indicated. Ovals surround lipid species S1 and S2 which presumably are substrates for the putative *R. tropici* β-hydroxylase to form the lipid products (P1 and P2) enclosed in rectangular boxes. Asterisks indicate ninhydrin-positive lipids.

Staining of developed 2D-TLC chromatograms with ninhydrin demonstrates that PE, S1, and P1 possess primary amino groups. The compound S1 shows the same relative mobility as ornithine-containing lipids (OL) which have been characterized previously in *S. meliloti* (Weissenmayer et al. 2002; Gao et al. 2004). Since OL is also a ninhydrin-positive compound, we suggest that S1 might be the corresponding OL from *R. tropici* CIAT899. The lipid composition of the Tn*5*-generated mutant 899-PV9 and that of the deletion mutant 899-*syc*Δ1, both defective in the predicted chloride channel protein, were indistinguishable from that of the wild type.

Although the lipid composition of mutant 899-*olsC*Δ1 resembles that of the wild type (Fig. 17B), compounds P1 and P2, which together comprised nearly 15% of the wild type membrane lipids, are absent in mutant 899-*olsC*Δ1 (Table 2). In contrast, the wild type has only minor levels of S1 and S2 (7.2% of total membrane lipids) whereas in mutant 899-*olsC*Δ1 these two lipids comprise 22.4% of the total lipid detected. If the mutant is complemented *in trans* with pBBR-1,6BE (Fig. 17C), again P1 and P2 are formed in relatively high amounts (more than 18% of total membrane lipids) whereas S1 and S2 are practically absent from this strain. These data are consistent with a model in which the predicted β-hydroxylase encoded by *olsC* converts the ninhydrin-positive compound S1 to the ninhydrin-positive compound P1 and also the ninhydrin-negative compound S2 to the ninhydrin-negative compound P2, presumably by hydroxylation at a still unknown position in these molecules. As S1 and S2 both function as *in vivo* substrates for the OlsC reaction, one can expect that S1 and S2 would have similar chemical structures and that therefore S2 might be a modified version of the ornithine-containing lipids known to date. The non-reactivity of S2 with ninhydrin might be due to an additional, so far unknown modification at the δ-amino group of its ornithine residue. Similarly, as P1 and P2 are both products of the *in vivo* reaction catalyzed by OlsC, one can expect that P1 and P2 would have similar structures, with P2 having an additional modification at the δ-amino group of its ornithine residue. The nearly complete lack of S1 and S2 in the case of the strain 899-*olsC*Δ1/pBBR-1,6BE can be explained by a more efficient conversion of S1 and S2 to P1 and P2 due to an increased copy number of the gene responsible for the conversion. The latter was concomitant to the 2D-TLC lipid profile observed in strain CIAT899 when plasmid pBBR1,6BE was provided *in trans* (Fig. 17D). Lipids S1 and S2

were restored in strain 899-*olsC*Δ1/pBBR-1,6BE when plasmid pJG21 was provided (Fig.17E). This plasmid contains *olsB* from *S. meliloti* (Gao et al. 2004) and is involved in the biosynthesis of ornithine lipids in this species. The delivery of pJG21 into 899-*olsC*Δ1/pBBR-1,6BE and the subsequent restoration of the 2D-TLC phenotype similar to that observed in parent strain CIAT899 is another indicative the S1, S2, P1 and P2 are different forms of ornithine-containing lipids.

Table 3. Membrane lipid composition of *Rhizobium tropici* CIAT899 wild type, mutant 899-*olsC*Δ1, mutant containing the empty vector 899-*olsC*Δ1/pBBR-MCS5, complemented strain 899-*olsC*Δ1/pBBR-1,6BE, strain CIAT899/pBBR1,6BE and strain 899-*olsC*Δ1/pJG21+pBBR-1,6BE (for lipid designations see Fig. 17). Values are the mean ± standard deviations of three independent experiments.

	Composition (% of total ^{14}C)					
Lipid	**CIAT899**	**899-*olsC*Δ1**	**899-*olsC*Δ1/ pBBR-MCS5**	**899-*olsC*Δ1/ pBBR-1,6BE**	**CIAT899/ pBBR1,6BE**	**899-*olsC*Δ1/pJG21+ pBBR-1,6BE**
PC	40.3± 0.7	47.8 ± 6.0	37.4 ± 5.5	32.5 ± 1.5	31.6 ± 2.2	31.7 ± 4.0
PG	11.9 ±2.1	10.7 ± 1.9	11.2 ± 0.2	12.7 ± 1.3	10.9 ± 0.3	10.0 ± 0.9
CL	2.9 ± 2.0	2.6 ± 0.8	3.2 ± 1.4	2.9 ± 0.3	9.5 ± 1.5	6.4 ± 1.6
PE	23.4 ±3.8	16.3 ± 4.9	26.2 ± 1.7	33.4 ± 0.4	24.0 ± 1.6	18.6 ± 2.2
S1	2.7 ± 2.7	**7.2 ± 1.8**	**10.7 ± 3.0**	n.d.	n.d.	**3.8 ± 0.5**
S2	4.5 ± 1.9	**15.3 ± 1.1**	**11.3 ± 0.8**	0.3 ± 0.2	n.d.	**5.5 ± 0.5**
P1	6.3 ± 3.0	n.d.	n.d.	**6.8 ± 2.9**	**9.7 ± 0.7**	**11.5 ± 2.5**
P2	8.1 ± 4.9	n.d.	n.d.	**11.4 ± 1.4**	**14.3 ± 4.6**	**12.5 ± 2.1**

n.d. not detected

Incorporation experiments with radiolabeled ornithine demonstrated that from all the membrane lipids, in *S. meliloti* only OL become labeled, indicating that ornithine is specifically and exclusively incorporated into OL (Gao et al. 2004). Analysis of the lipid profile from *R. tropici* CIAT899 that had been labeled with [1-^{14}C]ornithine indicates that four compounds have incorporated radiolabeled ornithine (Fig. 17D). The relative mobilities of the radiolabeled compounds coincide with the relative mobilities found for lipids S1, S2, P1, and P2. Therefore, we conclude that all four of these lipids are chemically distinct classes of ornithine-containing lipids (OL) of *R. tropici* CIAT899. As lipid S1 is ninhydrin-positive and migrates in an identical way as the well-characterized OL (Geiger et al. 1999) from *S. meliloti* in 2D-TLCs, we expect that S1 presents a similar

or identical structure as sinorhizobial OL. In contrast, the S2, P1, and P2 classes of ornithine-containing lipids, in this order, migrate more slowly in both dimensions of 2D-TLC systems and therefore must be increasingly more polar due to still unknown modifications by functional groups.

Discussion of chapter 2

It has been speculated that certain membrane lipids might be important in the formation of a successful symbiosis (de Rudder et al. 1997). This idea has been supported by the observations that mutants of *Sinorhizobium meliloti* lacking phosphatidylcholine (PC) are unable to form nitrogen-fixing nodules on alfalfa (Lopez-Lara et al. 2003; Sohlenkamp et al. 2003) and that mutants of *Bradyrhizobium japonicum* with reduced levels of PC presented a reduced number of bacteroids within infected plant cells (Minder et al. 2001). Since PC comprises 50-60% of the lipids in membranes of *S. meliloti* or *B. japonicum* and ornithine-containing lipids about 20% in *R. tropici*, one might expect that major changes in membrane lipid composition, either by mutations or by increased copy number of genes involved in their biosynthesis, might affect the structural properties of cell membranes and as a consequence the proper functioning of membrane-associated proteins.

Rhizobium tropici CIAT899 produces four different classes of ornithine-containing lipids (S1, S2, P1, and P2). Mutant 899-*olsC*Δ1, which forms more S1 and S2 and lacks P1 and P2, is acid-tolerant but symbiotically defective. In contrast, the complemented mutant 899-*olsC*Δ1/pBBR-1,6BE, which showed mainly P1 and P2 and nearly complete lack of S1 and S2, was able to restore the symbiotic proficiency, but was acid-sensitive. The latter indicates that lipids P1 and P2 are necessary for a successful symbiotic interaction of *R. tropici* CIAT899 with plant host whereas lipids S1 and S2 are required for acid tolerance. This hypothesis is consistent with the fact that in the parent strain CIAT899 all four distinct classes of ornithine-containing lipids are present and that this strain is both acid-tolerant and symbiotically proficient.

The analysis performed on mutant 899-*olsC*Δ1 permits us to report for the first time that membrane lipids of *Rhizobium tropici* are involved in symbiosis and that the putative β-hydroxylase encoded by *olsC* is part of a biosynthesis pathway for membrane lipids not previously described for any species. We demonstrate that *R. tropici* lipid species S1, S2, P1 and P2 are indeed ornithine-containing lipids and that the product encoded by *olsC* is necessary to convert lipids S1 and S2 to P1 and P2. Lipids P1 and P2, which presumably carry a hydroxyl group at a still unknown position, are required for an effective symbiotic interaction with bean plants while lack of lipids S1 and S2 was correlated to acidic

sensivity. Hydroxylations at the 2-position of fatty acyl residues of membrane lipids such as PE or OL are known to occur in *Burkholderia cepacia* and other bacteria (Taylor et al. 1998). Therefore, a balanced membrane lipid composition of the *R. tropici* cell membranes is required for both, the symbiotic interaction with plants and for acid tolerance in free-living conditions. This is the first report of a rhizobial membrane lipid other than phospholipids with relevance for symbiosis.

Chapter 3

Rhizobium tropici CIAT899 requires a putative σ^E-factor to establish an effective symbiosis with *Phaseolus vulgaris* plants

Introduction

The initiation of transcription in bacteria depends on the association of the RNA polymerase with small proteins, known as σ-factors, which direct the core enzyme to a specific class of promoter sequences. Prokaryotic species synthesize different σ-factors that recognize different promoter sequences, which allow bacteria to maintain the basal gene expression as well as regulation of gene expression under altered environmental conditions (Wosten 1998; Borukhov and Severinov 2002; Borukhov and Nudler 2003).

Based on sequence similarity, bacterial sigma factors are grouped in two families. The σ^{54} family contains only one group and are not essential for certain growth conditions. They recognize a -12 and -24 promoter element. The σ^{70} family recognize a -10 and -35 promoter element and comprises several groups differentiated by their structure and function. The latter is characterized by the presence of four regions (1-4), where subregions 2.4 and 4.2 are shown to be the most conserved and responsible of the -10 and -35 promoter element recognition, respectively (Burgess and Anthony 2001; Murakami and Darst 2003). Among the σ^{70} family, are the extracytoplasmic function (ECF) σ-factors, also called σ^E or σ^{24} factors. These σ-factors form a subgroup of environmentally responsive transcriptional regulators which respond to events occurring in the periplasm and outer membrane. σ^E controls the transcription of several genes in response to extracellular stresses and that are required for virulence in *E. coli* (Dartigalongue et al. 2001), *H. influenzae* (Craig et al. 2002), *S. typhimurium* (Humphreys et al. 1999; Miticka et al. 2003), *M. tuberculosis* (Manganelli et al. 2001; Manganelli et al. 2004), *P. aeruginosa* (Yu et al. 1996), *V. cholerae* (Kovacikova and Skorupski 2002) and *Y. enterolitica* (Heusipp et al. 2003).

Proteins that negatively regulate transcription by interaction with a σ-factor are known as anti-σ-factors (Hughes and Mathee 1998). Usually, these proteins have inner membrane localization. The N-terminus resides in the cytoplasm and binds reversibly to σ^E, blocking

its association with the RNA polymerase core. The C-terminus of the anti-σ-factor is located in the periplasmic space and is responsible for extracytoplasmic stress sensing. Under stress conditions, proteolytic activity occurs to degrade the anti-σ-factor. This event releases the σ^E-factor, which is then free to bind to RNA polymerase and transcribe the genes in its regulon (Ades 2004; Duguay and Silhavy 2004). The latter enables a quick response to environmental stimuli, since σ-factors are already available with no need to be synthesized *de novo*.

For rhizobia, however, there is no information about the regulation of transcription of genes controlled by ECF σ-factors, particularly in response to environmental stresses or in symbiosis. As part of an attempt to identify novel genes involved in plant-microbe interactions and stress tolerance, we performed random mutagenesis in the bean nodulating bacterium *Rhizobium tropici* CIAT899, which is highly tolerant to many environmental stresses and a good competitor for nodule occupancy. Previously, the genetic analyses of the region disrupted by the Tn*5* insertion in mutant 899-PV4 was reported, that revealed *atvA*, an ortholog of the chromosomal virulence gene *acvB* of *Agrobacterium tumefaciens*, which is required for acid tolerance (Vinuesa et al. 2003). In this work, I present the genetic analysis of the region downstream of *atvA*, which led to the discovery of a gene (*sigE*) that bears high similarity to a σ^E factor and predicted to be responsible for the symbiotic deficiency displayed by 899-PV4. This is the first report of a putative σ^E factor in rhizobia that is required for an effective symbiotic interaction with *Phaseolus vulgaris* plants.

Results of chapter 3

Sequence analyses of *sigE* and *alf1*

The genetic map of the 7102-bp fragment of the complementing sequence of mutant 899-PV4 (Genbank acc. no. AF433669) is shown in Fig. 18. In the region downstream the well characterized *lpiA* and *atvA*, 2 open reading frames (ORFs) with high coding probability were detected, as predicted by FrameD program. These ORFs were are the target of the genetic analysis presented herein, since they could be related to the deficiency observed in mutant 899-PV4 (Vinuesa et al. 2003).

In frame +1, at position 5490 starts a 777 bp-long ORF predicted to encode a 29.41 kDa product, which according to BLASTP searches showed sequence similarity to RNA polymerase σ^E-factors from the pathogenic γ-Proteobacteria *Pseudomonas aeruginosa* AlgU (E value $2e^{-15}$, 35% identity and 52% similarity) and *Escherichia coli* RpoE (E value $1e^{-13}$, 37% identity and 53% similarity). This protein belongs to the sigma factor protein family COG1595 and it is predicted to have a cytoplasmic localization, according to the PsortB program. Analysis of domain conservation with Pfam server revealed significant hits to region 2 (E value $4.8e^{-12}$) which is the most conserved region of this family since it contains both the -10 promoter recognition helix and the primary core RNA polymerase binding determinant and to region 4 (E value $1.8e^{-12}$) which is involved in binding to the -35 promoter element via a helix-turn-helix motif (Campbell et al. 2002). Therefore, based on similarity, this ORF was designated as *sigE,* for sigma-E factor. Analysis of the region upstream of *sigE* using the NNPP server predicted 2 possible promoter sequences (sites 5306-5351, $r = 0.98$; 5419-5464, $r = 0.94$).

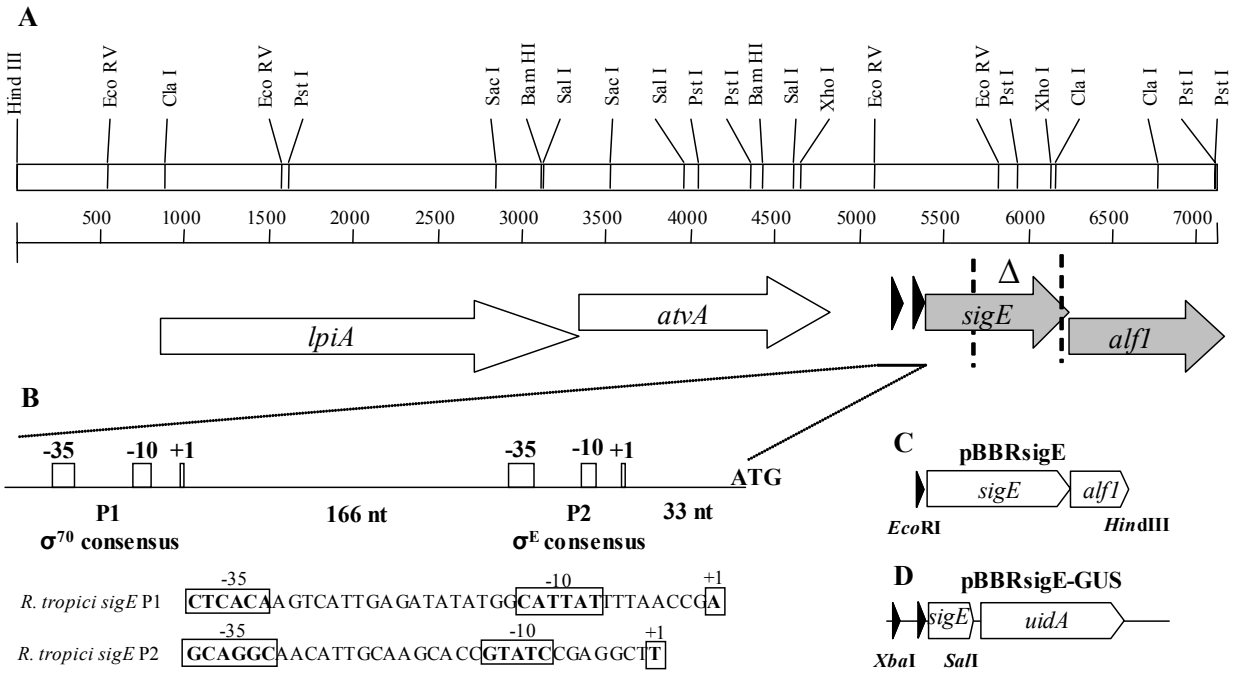

Fig. 18. **A**. Genetic and physical map of the 7102-bp fragment that restores acid tolerance and symbiotic performance in mutant 899-PV4. Selected restriction sites are shown. Four open reading frames (represented by arrows) were detected. A non-polar deletion mutant in *sigE*, lacking the region shown between the dashed lines, was generated. Predicted promoters by NNPP server are shown as black triangles. **B.** Organization of the *R. tropici sigE* P1 and P2 promoters. The distances between the transcriptional start sites and the *sigE* start codon are shown. The nucleotide sequences of the -35 and -10 regions of the promoters enclosed by rectangles. **C.** The region within restriction sites *Eco*RI-*Hind*III, which contains *sigE* and part of *alf1*, represents the fragment used for construction of the complementing plasmid pBBRsigE. **D.** The region within restriction sites *Xba*I-*Sal*I, which contains the region upstream *sigE* and part of this gene, represents the fragment used for the construction of plasmid pBBRsigE-GUS used for transcriptional fusions.

In frame +2, at position 6263 starts a second 804 bp-long ORF predicted to encode a 29.51 kDa product. BLASTP searches revealed homology to trasmembrane transcription regulators (anti-sigma factors) from β-Proteobacterium *Ralstonia metallidurans* (E value

$1e^{-41}$, 38% identity and 56% similarity) and α-Proteobacterium *Mesorhizobium loti* (E value $1e^{-23}$, 29% identity and 43% similarity). This protein belongs to the anti-sigma factor protein family COG5662. Analyses of the secondary structure and transmembrane domains performed with PsiPred and ProteinPredict servers indicate that this protein contains a single transmembrane-spanning segment, with the N-terminus residing in the cytoplasm and the C-terminus in the periplasm. This prediction is in consistent to that observed in other characterized anti-sigma factors and therefore, based on similarity, we designated this ORF as *alf1*, for anti-sigma-like factor.

Construction of a non-polar deletion in *sigE* and symbiotic performance of the mutant strain

A non-polar mutation was generated in *sigE* by deletion of a 474 bp-long internal fragment. To construct this mutant, plasmid pKRΔsig1 was transferred into CIAT899 and double recombinants were selected as mentioned in Materials and Methods. The deletion was confirmed by PCR with primers PV4-5419f-E and PV4-6798r-H (Fig. 4) and the resulting mutant was designated 899-Δsig1.

Nodules formed by *Phaseolus vulgaris* plants inoculated with strain 899-Δsig1 (21 dpi) were not fully developed, whitish, irregular in size and lacked lenticels, which clearly contrast with those induced by parent strain CIAT899 (Figs 19A and 19B). Light micrographs of cross-sections of nodules of bean plants induced by strain 899-Δsig1 revealed that this mutant was able to enter the nodules, but with significant reduced levels of infection (Figs 19C and 19D). Furthermore, the central nodular tissues were not uniformly colonized by the mutant strain presenting rather a patchy pattern when compared with the parent strain (Figs 19E and 19F).

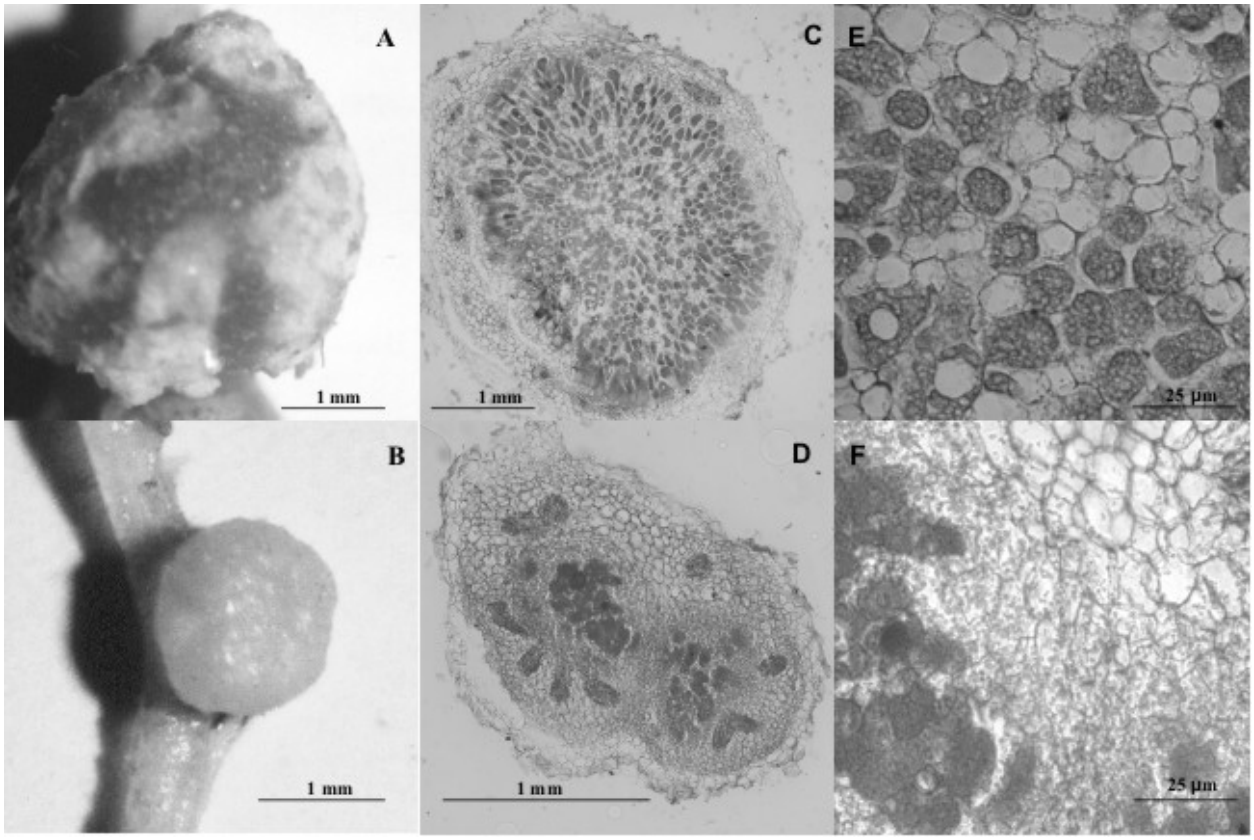

Fig. 19. Root nodules of common bean (*Phaseolus vulgaris*) infected with *Rhizobium tropici* CIAT899 parent strain (upper panel) or with a mutant, defective in a putative σ^E factor (lower panel). Intact bean nodules (**A and B**), cross-sections through bean nodules (**C and D**), and more detailed micrographs (**E and F**) showing the structure of *R. tropici*-infected bean nodule cells.

The nitrogen fixation of strain 899-Δsig1, determined by the acetylene reduction assay, showed a decrease to 25% as compared by the levels exhibited by the parent strain CIAT899 (Fig. 20A), while the nodulation competitiveness of 899-Δsig1 against the *gusA*-tagged reporter strain CIAT899-G1 in a 10:1 co-inoculation experiment showed that only 17% of the nodules were occupied by the former, which indicates that the mutant 899-Δsig1 is a poor competitor for nodule occupancy (Fig. 20B).

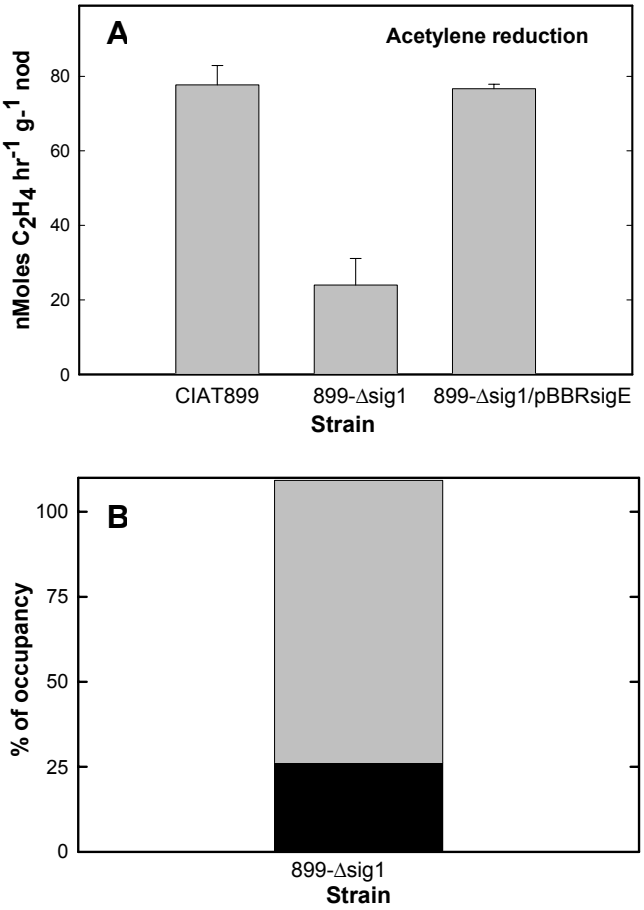

Fig 20. Phenotype displayed by mutant 899-Δsig1 against parent strain CIAT899 on *Phaseolus vulgaris* plants **(A)** Mean acetylene reduction of nodulated roots (21 dpi) of strain 899-Δsig1 in comparison to parent strain CIAT899 or complemented strain 899-Δsig1/pBBRsigE. Values are the mean ± SD of three repetitions in two independent experiments. (**B**) Percentage of nodules occupied by mutant 899-Δsig1 against reporter strain CIAT899-G1 in a 10:1 coinoculation experiment.

Complementation of mutant 899-Δsig1 with pBBRsigE

In order to complement mutant 899-Δsig1, plasmid pBBRsigE was constructed and provided *in trans* (Fig. 18C). To construct pBBRsigE, a 1.4 kb fragment containing *sigE* and the region upstream with the second predicted promoter (with the σ^E consensus) was cloned into pBBR-MCS5, yielding pBBRsigE. This plasmid was transferred into the mutant 899-Δsig1 resulting in strain 899-Δsig1/pBBRsigE. When inoculated on bean

plants the complemented mutant formed again fully developed nodules and with similar levels of nitrogen fixation capacity as exhibited by parent strain CIAT899 (Fig. 20A).

Partial deletion of *sigE* in mutant 899-Δsig1 was not reflect in an increased sensibility to some environmental stresses.

In order to test if mutant 899-Δsig1 was more sensitive than parent strain CIAT899 or complemented strain 899-Δsig1/pBBRsigE to different environmental stresses, bacteria were exposed to specific environmental conditions and percentage of survival was determined. For this purpose, pre-cultures of strains CIAT899, 899-Δsig1 and 899-Δsig1/pBBRsigE were diluted to 10^5 CFU and exposed to osmotic or acid challenge, to increased temperature or to ethanol for 2 hr. The number of CFU determined by plating out serial dilutions of these bacterial samples showed no significant difference in the percentage of survival between strains for the stresses tested (Fig. 21A). Furthermore, the analysis of the growth curves of these strains under the mentioned stresses for a 24 hr. period, neither revealed any significant difference in growth between mutant strain 899-Δsig1 or parent strain CIAT899 (Fig. 21B).

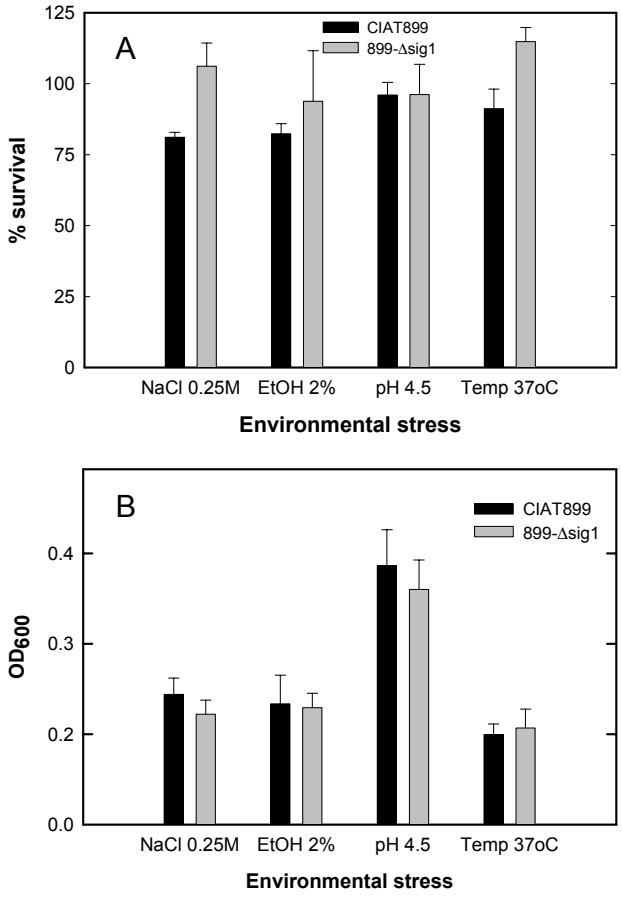

Fig. 21. (**A**) Percentage of survival of strains CIAT899 and 899-Δsig1 after exposition to some environmental stresses for 2 hr. Values are the mean ± SD of three repetitions in two independent experiments. (**B**) Final optical density (600 nm) of strains CIAT899 and 899-Δsig1 after growing for 24hr under some environmental stresses. Values are the mean ± SD of six repetitions.

Transcriptional regulation of the putative σ^E factor in *R. tropici*

In order to monitor the expression of *R. tropici sigE* under different growing conditions, a *sigE*-GUS reporter fusion was constructed as described in materials and methods. The resulting plamid, pBBRsigE-GUS (Fig. 18D), was transferred into CIAT899 yielding strain 899/pBBRsigE-GUS. The empty pBBR-GUS (Corvera et al. 1999) vector was transferred into CIAT899, yielding strain 899-pBBR-GUS, as a negative control. Strains 899/pBBRsigE-GUS and 899/pBBR-GUS were grown to different points of the growth curve or challenged with different stresses and the specific activity of glucuronidase was

determined. Strain 899/pBBRsigE-GUS showed similar levels of specific activity independent of the phase of growth or environmental condition tested (500 $\pm$ 54 $nmol^{-1}$ mg^{-1} protein) while in strain 899/ pBBR-GUS almost no activity was detected (25 $\pm$ 6 $nmol^{-1}$ mg^{-1} protein). This result indicates that *sigE* has a basal expression under free-living conditions, and that at least one of the promoters located in the region 293 bp upstream the gene, is active. This resembles the transcription pattern observed for σ^E-factors in other species including *P. aeruginosa, M. tuberculosis* and *V. cholerae*, which also present basal levels of expression (Schurr et al. 1995; Manganelli et al. 2001; (Kovacikova and Skorupski 2002). To determine the expresion of this gene in symbiotic conditions, we inoculated *Phaseolus vulgaris* seedlings with strain 899/pBBRsigE-GUS or 899/ pBBR-GUS and analyzed the GUS staining of nodules (Wilson et al. 1995). After this treatment, the nodules induced by strain 899/pBBRsigE-GUS presented a positive signal (blue staining) while those induced by strain 899/pBBR-GUS remained unstained (data not shown). These results indicate that expression of the putative *R. tropici* σ^E-factor takes place under both free-living and symbiotic conditions.

Identification of the transcriptional start sites of *sigE*

The 5' rapid amplification of cDNA ends (RACE) was used to identify the transcriptional start sites of *sigE*. Sequence analyses of the PCR products revealed a transcriptional start site (T) 33 nt upstream of the ATG start codon of *sigE* which was consistent with the prediction of the NNPP server (Fig. 18B). This promoter was analogous to the *S. antibioticus phsA* promoter and to other σ^E regulated promoters (Missiakas and Raina 1998). The -35 sequence (GCAGGC) showed only two mismatches when compared to the (GAACGC) motif of *S. antibioticus*, while the -10 sequence (GTATC) showed only one mismatch from the (GTCTC) of the same gene. In addition, a second transcriptional start site (A) was detected 166 nt upstream of the ATG start codon of *sigE*, which was also consistent with the prediction of a second promoter by the NNPP server. This promoter was highly similar to the *E. coli* σ^{70} promoter consensus. The -35 sequence of this *R. tropici sigE* second promoter (CTCACA) showed two mismatches from the TTGACA σ^{70} consensus and the -10 sequence (CATTAT) showed also two mismatches with respect to the (TATAAT) consensus.

Discussion of chapter 3

In this work it is reported a novel gene from *Rhizobium tropici* CIAT899 which was shown to be required for the establishment of a fully developed N_2-fixing symbiosis with bean plants. The analysis performed on mutant 899-Δsig1, which carries a deletion in the putative σ^E factor encoded by *sigE*, suggests that the observed decrease in nodule development and nitrogen fixation might be due to its significantly reduced ability to colonize the central nodular tissue, as revealed by the light microscopy. It has been shown that mutations of σ^E factors in pathogenic bacteria like *S. enterica*, *H. influenzae* and *V. cholerae*, resulted in reduced levels of intracellular survival and consequently were less virulent (Humphreys et al. 1999; Craig et al. 2002; Kovacikova and Skorupski 2002). In this respect strain 899-Δsig1 also displayed reduced "symbiotic infectivity", when co-inoculated with the parental strain. Since mutants of σ^E-factors displayed a similar phenotype in their mammal or legume host cells respectively, it is tempting to suggest that σ^E-factors are regulating the transcription of genes that are important for both pathogenesis and for symbiosis. For *E. coli*, it has been determined that σ^E controls transcription of genes that affect characteristics of the cell envelop, biosynthesis of phospholipids, lipopolysaccharides and lipoproteins, as well as signal transduction pathways (Dartigalongue et al. 2001; Ades 2004). Some of these functions might be also controlled by this putative *R. tropici* σ^E-factor, especially those required for establishing a symbiotic interaction with bean plants. Since strain 899-Δsig1 was not more sensitive than parent strain CIAT899 or complemented mutant 899-Δsig1/pBBRsigE to some environmental stresses, we assume that *sigE* is not essential for growing under free-living conditions, where might exist other σ^E paralogs displaying more relevant roles.

As observed in *E. coli* and *V. cholerae*, the expression of the *R. tropici* putative σ^E-factor seems to be dependent upon two promoters located upstream of *sigE*. P1 appears to be σ^{70} dependent whereas the downstream promoter, P2 is σ^E dependent (Kovacikova and Skorupski 2002). This gene was transcribed constitutively in both free-living and symbiotic conditions, suggesting that the transcriptional regulation is not the principal element of regulation which rather may be posttranscriptional. Usually, this posttranscriptional regulation is mediated by anti-σ factor, which sequester the σ factor in a non functional state, in the absence in the proper stimulus (Hughes and Mathee 1998).

In *R. tropici*, *alf1* is localized immediately downstream of *sigE*, whose product resembles anti-σ factors in primary sequence and secondary structure motifs. This genetic organization is common for other species with this mechanism of regulation, including *E. coli*, *M. tuberculosis*, *H. influenzae* and *S. enterica* (Humphreys et al. 1999; Dartigalongue et al. 2001; Manganelli et al. 2001). Thus, it is tempting to suggest that *alf1* encodes the anti-σ factor that regulates the putative σ^E in this species. Future experiments will address this model.

This bacterial activity has not been previously reported as relevant for the symbiotic process in rhizobia. Further investigations are necessary determine the genes controlled by this putative σ^E factor and elucidate how they influence the symbiotic interaction with its eukaryotic partner.

Further discussion to the 3 chapters is presented in the publications in the following pages.

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Protocols

1. Isolation of genomic DNA (Sambrook et al, 1989)

1. Grow bacteria to late exponential phase. Harvest 5 ml and centrifuge 10 min at 5000 rpm. Discard supernatant.
2. Add 5 ml NaCl 0,88% to the pellet and vortex. Centrifuge and discard supernatant. Repeat this step once.
3. Resuspend pellet in 4.75 ml buffer TE 1X.
4. Add 250µl of 10% SDS (w/v) and 25µl of proteinase K (from 20mg/ml stock). Incubate 1 hour at 37°C.
5. Add 2.5 ml 5M NaCl, mix thoroughly
6. At 750µl CTAB-protein/polysaccharides complexes (10%), mix and incubate 20 minutes at 65°C.
7. Add 2.5 ml chloroform:isoamyl alcohol (24:1, v/v). Centrifuge 10 minutes at 5000 rpm.
8. Transfer the supernatant to a new tube and add 2.5 ml phenol: chloroform (1:1, v/v). Centrifuge as indicated above.
9. Remove the aqueous phase to a new tube and precipitate the DNA by the addition of 2.5 ml of isopropanol. Invert 3-4 times the tube. DNA can be seen as white threads.
10. Transfer the white threads to an epp. and add 500µl ethanol 70%. Centrifuge 5 minutes at 10000 rpm. Discard supernatant and dry pellet in air.
11. To dissolve add 100µl TE 1X. Store at 4°C or -20°C.

CTAB solution: dissolve 10 g of CTAB in 90 ml water. Stir and warm up to 60°C until completely dissolved.
TE 1X: 1 ml 1M Tris-HCL pH 8.0, 0.2 ml 0.5 M EDTA pH 8.0 to 100 ml dH_2O.

2. Extraction of DNA from Gram-negative bacteria (using GenomicPrepCell and Tissue DNA Isolation Kit, Amersham Biosciences)

1. Add 1 ml of cell suspension (overnight culture) to a 1.5 ml tube. Remove supernatant.
2. Add 600µl of cell lysis solution and mix by pipetting
3. Incubate the sample at 80°C for 5 min. Cool down to room temperature.
4. Add 4µl of RNase A and mix by inverting. Incubate 1 hr at 37°C.
5. Add 200µl of protein precipitation solution to the RNase A-treated cell lysate.
6. Vortex vigorously for 20 sec and centrifuge at 13000 rpm for 3 min. The precipitated proteins will form a tight pellet.
7. Transfer supernatant to a 1.5 ml centrifuge tube and at 600µl of 100% isopropanol.
8. Mix the sample by inverting gently 50 times.
9. Centrifuge 1 minute at 13000 rpm. Discard supernatant. DNA will be visible as a white pellet.
10. Add 600µl ethanol 70%. Invert several times and centrifuge as described in last step. Carefully pour off the ethanol.
11. Drain the tube on clean absorbent paper and allow the sample to air dry 10-15 min.
12. Add 100µl of DNA hydration solution and allow DNA to rehydrate overnight at room temperature. Alternatively, heat at 65°C for 1 hr.
13. Store at 2-8°C.

3. DNA amplification by the polymerase chain reaction (PCR)

All PCR amplifications were performed using the following reaction mix and program

Reaction mix

5 µl Taq buffer 10x
2.5 µl DMSO
1.5 µl MgCl2
1.5 µl sense primer (10 pmol)
1.5 µl antisense primer (10 pmol)
1 µl dNTPs (10 mM)
1 µl DNA template (50 ng)
0.3 µl Taq polymerase
37.2 µl dH_2O

Program

1	95 °C	3 min
2	94 °C	1 min
3	60 °C	1 min
4	72 °C	1 min
5	72 °C	5 min
6	4 °C	∞

Repeat steps 2 to 4 for 30 times. Annealing (step 3) vary depending on the primers from 55°C to 60°C

4. DNA isolation from gel (using the QIAquick Gel Extraction Kit, QIAGEN)

1. Excise the DNA fragment from the agarose gel with a clean sharp scalpel
2. Weigh the gel slice in an epp. Add 2 volumes of Buffer QG to 1 volume of gel
3. Incubate at 50°C for 10 min. mix by vortexing every 2-3 min. Gel must be totally dissolved.
4. Add 1 gel volume of isopropanol to the sample and mix
5. Place the column in the collection tube, add the sample and centrifuge for 1 min. at 13000 rpm at RT.
6. To wash, add 500 µl of Buffer PE (containing ethanol 96%). Centrifuge for 1 min.
7. Discard the flow-through and add 200 µl of Buffer PE and centrifuge for 1 min.
8. Place the column in a new 1.5 mil microcentrifuge tube. Add 50 µl of Buffer EB to elute the DNA (previously heated at 65°C). Centrifuge for 1 min. at 13000 rpm at RT.
9. Store DNA at -20°C.

5. Vector preparation for ligation

1. Add 250-500ng vector. Adjust volume the volume to 17µl with dH_2O
2. Add 2µl of buffer and 1µl of the required restriction enzyme, mix thoroughly.
3. Incubate at least for 3 hours at 37°C.
4. Inactivate the restriction enzyme for 15 min at 65°C. Cool the mix down to room temperature. Add 2 µl of the alkaline phosphatase buffer 10X and 1 µl of the phosphatase (CIF, New England Bioloabs). Mix thoroughly.
5. Incubate 60 minutes at 37°C and inactivate phosphatase 15 min at 65 °C.
6. Purify DNA by gel purification, spin-colum purification or phenol extraction.
7. Store al -20°C.

6. Ligation protocol

1. Combine 50ng of vector with a 3-fold molar excess of insert. Adjust the volume to 17µl with dH_2O.
2. Add 2µl of the ligation buffer and 1 µl of the T4 DNA ligase (Roche), mix thoroughly.
3. Incubate at 16°C for at least 16 hours.
4. Store at 4°C

7. Trasformation Protocol

1. Thaw the competent cells (DH5α) on ice
2. Add 10µl from the ligation product to 90µl of the competent cells. Swirl the contents and incubate on ice from 10-20 min.
3. Heat-shock in a water bath/ thermo mixer for 2 min at 42°C. Do not exceed the temperature and time given.
4. Incubate the epp on ice for 2 min.
5. Add 0.9 ml of LB medium and incubate the tubes for 60-90 min at 37°C with shaking.
6. Centrifuge the epp. 1 min at 13000 rpm. Discard supernatant and resuspend pellet in 100µl LB medium by vortexing.
7. Plate 100µl (or less) of the transformation reaction on appropriate antibiotic plates using a sterile spreader
8. Incubate the plates overnight at 37°C. For blue-white color screening, incubate the plates for more than 17 hours. Colonies containing plasmids without inserts will be blue. Colonies containing plasmids with insert will remain white.

8. Generation of competent *E. coli* cells (according to cold $CaCl_2$ method, Sambrook et al, 1989)

1. Grow a preculture of the *E.coli* strain (DH5α) overnight at 37°C.
2. Inoculate a 5 ml LB medium tube with 50µl of the preculture. Incubate it at 37°C with shaking until the culture reaches an OD_{600} between 0.4-0.6
3. Spin down the culture for 1 minute at 9000 rpm at 4°C. Discard the supernatant and wash the pellet in 1.5 ml ice cold 100 mM $MgCl_2$.
4. Centrifuge for 1 min. at 9000 rpm at 4°C. Discard the supernatant and resuspend pellet in 1.5 ml ice cold 0.1 M $CaCl_2$. Incubate on ice for 30-60 minutes.
5. Centrifuge once again with the conditions described above. Resuspend pellet in 1.5 ml ice cold 0.1 M $CaCl_2$, add 15-20% ice cold sterile glycerol, mix thoroughly.
6. Make aliquots of cell suspension in small epp. and freeze them at -70°C. Keep aliquots in -70°C until use.

9. Generation of competent *E. coli* cells (using the RbCl method)

1. Grow a preculture of the *E.coli* strain (DH5α) overnight at 37°C.
2. Inoculate a 100 ml LB medium tube with 1 ml of the preculture. Incubate it at 37°C with shaking until the culture reaches an OD_{600} between 0.4-0.6
3. Cool down the culture on ice for 10 min and centrifuge 5 min at 5000 rpm at 4 °C. Discard the supernatant.
4. Resuspend in 20 ml ice-cold TfbI buffer. Cool down on ice for 30 min
5. Centrifuge as in step 3. Resuspend in 2 ml ice-cold TfbII. Make 200 µl aliquots and store at -70 °C.

TfbI: 30mM KAc, 100 mM RbCl, 10 mM $CaCl_2$, 50 mM MnCl2, 15 % glycerol. Adjust pH to 5.8 with acetic acid 0.2 M. Sterilize with filter.

TfbII: 10 mM MOPS or PIPES, 75 mM $CaCl_2$, 10 mM RbCl, 15 % glycerol. Adjust pH to 6.5 and sterilize with a filter.

10. Toothpick lysis (for quick screening of colonies)

1. Pick a single colony from a freshly streaked selective plate and transfer it in a new plate and incubate for 24 hrs.
2. With a toothpick, pick the grown colony and wipe it on the side of a 0.5 ml epp. Add 30 µl of lysis solution and pipette up and down several times to resuspend the colony. Lysis solution (50mM NaOH, 5% glycerol, 0.5% SDS, 5mM EDTA, 0.1% bromocresol green).
3. Incubate the mixed samples at 65 ^{o}C for 15 minutes or 45 seconds at the microwave. Let cool down.
4. Precipitate proteins with 2.5 µl KCl 4M. Centrifuge 15 sec. at 7000 rpm.
5. Load the supernatant in a dry 1% agarose gel. TAE 1X buffer is added to make contact with the edges of the gel but not high enough to submerge the sample wells.
6. Run the sample at 250 V for 5 min, until the bromocresol green mark is completely into the gel. Add additional TAE buffer to submerge the gel and run at 100 V for 90 min more.
7. Stain with 0.5 µg/ml Etidium Bromide in dH_2O for 15 min with occasional shaking and distain in H_2O for 15 min.
   TAE 10X: 48.4g Tris-HCl, 11.42 ml glacial acetic acid, 0.5M EDTA pH 8.0, in 1L H_2O.

11. PCR over colony (for rapid detection of a PCR products cloned in any vector)

1. Pick a single colony from a freshly streaked selective plate and transfer it in a new plate and incubate for 24 hrs.
2. With a toothpick, pick the grown colony and wipe it on the side of a 0.5 ml epp. Add 20 µl of lysis solution and pipette up and down several times to resuspend the colony.
3. Perform a PCR using the following reaction and an adequate annealing temperature to the primers used. Verify amplification product in an agarose gel.
   Lysis solution: 10 mM $MgSO_4$ and 10 µl Tween 20, in 100 ml H_2O.
   PCR reaction:
   2 µl Taq buffer 10x
   1 µl DMSO
   0.6 µl MgCl2
   0.6 µl sense primer (10 pmol)
   0.6 µl antisense primer (10 pmol)
   0.4 µl dNTPs (10 mM)
   0.2 µl Taq polymerase
   3.5 µl of the colony resuspended in lysis solution
   11.5 µl dH_2O

12. Plasmid Isolation from *E. coli* (according to Sambrook et al. 1989)

1. Grow *E.coli* strain to exponential phase in 3 ml LB tubes containing the appropriate antibiotics.
2. Transfer to 1.5 ml of the culture to an epp and centrifuge 1 min at 13000 rpm. Discard the supernant. Repeat this step two times.
3. Resuspend the pellet to homogeneity in 300 µl buffer 1. Incubate at room temperature for 2 min.
4. Add 300 µl of buffer 2, mix carefully and incubate for 3-4 min for cell lysis.
5. Neutralize by the addition of 300 µl of buffer 3. Mix carefully and incubate 5 min at -20 ^{o}C.
6. Centrifuge at 13000 rpm for 10 min at 4^{o}C.
7. Transfer the clear supernatant to a new epp and extract with 1 vol of phenol:chloroform:isoamyl alcohol (50:49:1) to remove soluble proteins
8. Precipitate solution by adding 1 vol ice-cold isopropanol and 5 µl sodium acetate 3M pH 4.8. Centrifuge 1 min at 13000 rpm at 4 ^{o}C.

9. Wash the DNA pellet with 70% ethanol and centrifuge again. Let air-dry and resuspend in 20-30 µl TE.

Buffer 1: 50 mM Tris-HCl, 10 mM EDTA pH 8.0, add preboiled RNase (100 µg/ml).
Buffer 2: NaOH 100 mM, SDS 1%
Buffer 3: Potasium acetate 2.55 M pH 4.8. Store all solutions at 4 ^{o}C.
TE 1X: 1 ml 1M Tris-HCL pH 8.0, 0.2 ml 0.5 M EDTA pH 8.0 to 100 ml dH_2O

13. Plasmid Isolation (using High pure plasmid isolation kit, Roche)

1. Grow *E.coli* strain to exponential phase in 4 ml LB tubes.
2. Centrifuge 1 minute at 9000 rpm at room temperature. Discard supernatant and resuspend the pellet in 250µl Suspension Buffer + RNase and mix carefully by hand. Do not vortex or pipette.
3. Add 250 µl Lysis Buffer and mix carefully by inverting the tube. Incubate 5 minutes at room temperature. Do not vortex
4. Add 350 µl chilled Binding Buffer. Mix gently by inverting. Incubate 5 minutes on ice.
5. Centrifuge 10 minutes at 13000 rpm
6. Transfer the supernatant to a High Pure filter tube-colector and centrifuge at maximum speed for 1 min.
7. Discard flow-through solution and add 70 µl Wash Buffer II. Centrifuge at maximum speed for 1 min.
8. Discard flow-through solution and centrifuge again to remove residual Wash Buffer.
9. Insert the filter into a clean 1.5 ml epp. and add 100 µl 65^{o}C preheated Elution Buffer. Wait 1 minute and centrifuge 1 minute at maximum speed.
10. Store at -20^{o}C

14. Cosmid isolation (using the very low copy plamids/cosmids QiagenTip100 kit, QIAGEN)

1. Pick a single colony from a freshly streaked selective plate and inoculate a starter culture of 5 ml LB medium containing the appropriate selective antibiotic. Incubate for 8 hours at 37 ^{o}C with shaking.
2. Dilute the starter culture 1/500 into 500 ml of selective LB medium. Grow overnight at 37 ^{o}C with vigorous shaking.
3. Harvest the bacterial cells by centrifugation at 6000 rpm for 15 min at 4^{o}C.
4. Resuspend carefully the bacterial pellet in 20 ml of Buffer P1.
5. Add 20 ml of Buffer P2, mix carefully by interting 4-6 times and incubate at room temperature for 5 min.
6. Add 20 ml of chilled Buffer P3, mix immediately by inverting 4-6 times and incubate on ice for 30 min.
7. Centrifuge at 20000 xg fro 30 min. at 4^{o}C. Remove supernatant containing plasmid/cosmid DNA
8. Re-centrifuge the supernatant at 20000 x g for 15 min at 4^{o}C. Remove supernatant containing plasmid/cosmid DNA
9. Add 42 ml of room-temperature isopropanol to the lysate. Centrifuge at 15000 x g for 30 min at 4^{o}C. Decant carefully the supernatant
10. Redissolve the DNA pellet in 500 µl TE, pH 8,0, and add Buffer QBT to obtain a final volume of 5 ml.
11. Equilibrate a QIAGEN-tip 100 by applying 4 ml Buffer QBt. Allow column to drain completely by gravity flow.
12. Apply the DNA solution to the QIAGEN-tip and allow it to enter the resin by gravity flow.
13. Wash the QIAGEN-tip with 2 x 10 ml of Buffer QC
14. Elute DNA with 5 ml Buffer QF

15. Precipitate DNA by adding 3.5 ml of room-temperature isopropanol to the eluded DNA. Mix and centrifuge immediately at 15000 x g for 30 min at 4°C. Decant carefully the supernant
16. Wash DNA pellet with 2 ml room-temperature ethanol 70% and centrifuge at 15000 x g for 10 min. Carefully decant the supernatant without disturbing the pellet.
17. Air-dry the pellet and redissolve the DNA in a 250 µl elution buffer.
18. Store at -20°C.

15. Triparental conjugation

1. Grow rhizobial recipient strain to exponential phase in 4 ml PY medium with appropriate antibiotics. Grow *E.coli* donor and helper strains to stationary phase in 4 ml LB appropriate antibiotics.
2. Make a new culture of recipient, donor and helper strain without antibiotics and grow to the exponential phase.
3. Incubate recipient strain at 42°C for 15 min to temporarily inactivate its restriction systems. To a 1.5 ml epp, add 0.7 ml recipient stain, 0.2 ml donor strain and 0.1 helper strains.
4. Collect by centrifugation at 13000 rpm for 1 min. Discard the supernatant. Resuspend pellet in 50 µl of PY and spot it in a fresh PY plate. Incubate for 36 hr at 28°C.
5. Scrap off the bacteria and resuspend in 1 ml PY. Make serial dilutions to 10^{-2} and 10^{-3} and plate on selective media. Grow for 2-3 days at 28°C.

16. Transfer of DNA from agarose gels to nylon membranes

1. Set up the vacuum blotting system (Vacu Gene, Pharmacia, Germany)
2. Wet the nylon membrane (Boehringer-Mannheim, Germany) and placed it on the blotter. Cover the remaining area of the blotter with a plastic foil to ensure that vacuum will be generated with the pump.
3. Place the gel (TAE agarose 0.8% w/v gel with the digested DNA or plasmid DNA) on the top of the nylon membrane
4. Add denaturation solution (1.5 M NaCl, O.5 M NaOH) enough to cover the gel surface. Apply 35 mbar vacuum for 20 min.
5. Remove carefully the denaturation solution and add the neutralization solution (1 M ammonium acetate) by covering the gel. Apply 35 mbar vacuum for 20 min.
6. Increase the vacuum to 45 mbar and apply it for additional 30 min to ensure an appropriate transfer.
7. Place the membrane in a paper cover to dry it out. Fix covalently the DNA to the membrane in the UV-crosslinker (Stratagene)
8. Keep membranes dry at RT in a dark and dust-free environment.

17. Dioxigenin-labelled DNA probes (using the PCR DIG Probe Syntesis Kit, Roche)

Hybridization

1. Move the membrane into the hybridization glass tube. Add 20 ml of hybridization solution. Prehybridize it in the hybridization oven at 65-68°C for 30-60 min. Once is over, pour off the solution.

2. Apart, boil for 10 min. the PCR Probe together with 1 ml of hybridization solution in one epp. Cool quickly on ice. Transfer to one 50 ml Falcon tube, add 19 ml hybridization solution and 0.5 µl DIG labeled ladder.
3. Immediately, add the solution to the glass tubes containing the membranes and incubate overnight at 65°C.
4. After hybridization take the solution and pour it back into the falcon tube. It can be used 3 times. Transfer the membrane to a metal container
5. Wash 2x for 5min with 2xSSC+ 0.1% SDS (100 ml from 20x SSC + 10 ml SDS 10% to 1 L)
6. Wash 2x for 15 min with 0.5% SSC + 0.1% SDS at hybridization temperature (25 ml 20xSSC + 10 ml SDS 10% to 1 L)
7. Wash for 1 min. with the washing buffer.
8. Move filters to a metal container and add 30 ml of blocking solution (5 ml of blocking solution stock in 45 ml maleic acid buffer). Incubate for 30-60 min at room temperature with shaking.
9. Pour off the blocking solution, move filters to a plastic bag and add 20 ml antibody solution. Seal the plastic bag and incubate for 30-60 min. with shaking.
10. Pour off antibody solution, put filter into a metal container and wash in washing buffer 2x for 15 min.
11. Pour off washing buffer and add 10-20 ml detection buffer. Incubate 2 min. at room temperature.
12. Move filter to a plastic bag, add 10 ml detection buffer with 45 µl NBT and 35 µl X-Phosphate. Seal the bag and incubate flat in dark for one hour or the time required to see clearly the bands.
13. Pour off substrate and wash with water. Store at 4°C in a sealed plastic bag flooded with 10-15 ml TE 1X buffer.

20X SSC: 3 M NaCl, 300 mM sodium citrate pH 7.0.
Hybridization solution: 5x SSC, 1% w/v blocking reagent (Boehringer,Manheim), 0.1% N-lauroylsarcosine, 0.02% SDS. 20x SSC: 3 M NaCl, sodium citrate, pH 7,0.
Washing buffer: 0.3% Tween 20 in maleic acid buffer
Maleic acid buffer: 150 mM NaCl, 100 mM maleic acid, pH 7.5. Adjust pH with 10 M NaOH.
10X blocking solution stock: 10 % w/v blocking reagent (Boehringer, Mannheim) in maleic acid buffer. Dissolve by stirring on a heat plate. Do not boil.
Antibody solution: Add 2µl antibody (Anti-Dioxygening-AP, Fab fragments, Roche) into 20 ml blocking solution.
Detection buffer: 0.1 M Tris/HCl pH 9.5, 0.1 M NaCl, and 0.05 M $MgCl_2$
NBT: 75 mg/ml nitroblue tetrazolium salt in 70% v/v dimethylformamide (DMF).
X-Phosphate: 50 mg/ml 5-bromo-4-chloro-3-indolyl phosphate toluidinium salt in 100 % DMF.

18. Isolation of total RNA from bacteria (using RNA isolation kit, Roche)

1. Add 2 ml of cell suspension (overnight culture) to a 1.5 ml tube. Collect the sample by centrifugation at 5000 rpm for 5 min. Resuspend the pellet in 200 µl 10 mM Tris, pH 8.0.
2. Add 4 µl lysozyme (50 mg/ml, stored aliquoted at -20 °C). Incubate at 37°C for 10 min .
3. Add 400 µl lysis-binding buffer and mix well.
4. Pipette the sample into the High Pure filter tube with its collection tube.
5. Centrifuge for 15 sec at 10000 rpm. Discard the flowthrough.
6. Pippette 90 µl DNase incubation buffer into a sterile reaction tube, add 10 µl DNase I, mix and pipette the solution in the filter tube. Incubate for 60 min at room temperature. Repeat this step again.
7. Add 500 µl wash bufferII and centrifuge 15 sec at 10000 rpm.
8. Add 200 µl bufferII and centrifuge 2 min at 13000 rpm to remove residual washing buffer.
9. Discard the collection tube and insert the filter tube into a 1.5 ml epp.

10. Add 50-100 µl elution buffer into the filter tube and centrifuge 1 min at 10000 rpm. Repeat this step again.
11. Run the gel in 1X MOPS running buffer for 5 min at a high current. Once samples have entered the gel, submerge the gel in 2-3 mm running buffer. Continue electrophoresis at 80 V for 2-3 hr.

Formaldehyde gel: 0.8-1% agarose gel in 1X MOPS and 2% formaldehyde. Gel should be as fresh and thin as possible. Ethidium bromide staining and distaining should be done to ensure that loaded RNA is intact.

10X MOPS, pH 7.0 (with NaOH): 200 mM MOPS (4-morpholine-propanesulfonic acid), 50 mM Na-Ac, 10 mM EDTA, pH 7.0

Loading buffer: 250 µl formamide, 83 µl formaldehyde 37% (v/v), 50 µl 10X MOPS, 4 µl bromophenolblue 1%, 13 µl PEPC-treated H_2O. Add 3 times of loading buffer per unit of RNA.

19. cDNA synthesis (using the omniscript RT Kit, QIAGEN)

1. Thaw your template RNA solution, the primer solutions, 10X Buffer RT, dNTP Mix, and RNase-free water on ice.
2. Dilute the RNase inhibitor (RNAguard RNase Inhibitor, Amersham) to a final concentration of 10 units/µl in ice-cold 1X Buffer RT (previously diluted with RNase-free water).
3. Prepare the fresh master mix on ice, mix thoroughly and carefully. Add the RNA template to the individual tubes containing the master mix. Mix thoroughly and carefully.
4. Incubate 90 min at 37^{o}C. Inactivate enzyme by heating 5 min at 93 ^{o}C followed by rapid cooling on ice.

   Master mix
   2 µl 10X Buffer RT
   2 µ dNTP Mix (5 mM each dNTP)
   2 µl Oligo-dt primer (10 µM)
   1 µl RNase inhibitor (10 units/ul)
   13 µl RNase-free water
   5 µl RNA template

20. Seed germination

1. Wash seeds thoroughly in water for 3 times
2. Rinse seeds for 1 min in ethanol 70%. Wash 3 times with dH_2O.
3. Rinse seeds for 3 min in NaOCl or for 10 min in H_2O_2 30 % or 30 min in sodium hypochlorite 25 %
4. Wash seeds thoroughly with dH_2O for 7 times.
5. Briefly flame the seeds and plate them out for germination in 1 % agar plates.
6. Incubate on dark at 28^{o}C for 2 days.

21. Isolation of rhizobia from nodules

1. Separate nodules from roots and place them in a epp
2. Wash 3 times with water
3. Rinse nodules 1 min in ethanol 70%
4. Wash nodules 3 times with dH_2O
5. Rinse nodules 3 min in NaOCl 3% or sodium hypochlorite 25%
6. Wash nodules thoroughly with dH_2O for 7 times

7. Squash nodules in 50 µl PY medium and streak a loop of the suspension onto PY plates. Alternatively, Squash nodules directly onto PY or 20E plates.
8. Incubate for 2-3 days at 28°C.

22. Fixation of nodules for light microscopy

1. Put the nodules still attached to a piece of the root in a 1.5 ml epp.
2. Wash 2x with water to remove residual vermiculite.
3. Rinse the nodules for 5 min in phosphate buffer.
4. Rinse the nodules for 4 hr in ice-cold glutaraldehyde solution or until nodules precipitate to the bottom of the recipient.
5. Wash 3x for 20 min with phosphate buffer
6. Place the nodules in the microtome and cut slides of about 40 µm.
7. Rinse slides in sodium hypochloride 10% for 1 hr.
8. Stain samples with 0.1% aqueous methylene blue.
9. Distain for at least 4 hours with dH_2O

Phosphate buffer: 0.2 M KH_2PO_4, 0.2 M Na_2HPO_4, pH 7.0
Glutaraldehyde solution: 4% glutaraldehyde in phosphate buffer

23. Fixation of nodules for electron microscopy

10. Put the nodules still attached to a piece of the root in a 1.5 ml epp.
11. Wash 2x with water to remove residual vermiculite.
12. Rinse the nodules for 5 min in phosphate buffer.
13. Rinse the nodules for 4 hr in ice-cold glutaraldehyde solution or until nodules precipitate to the bottom of the recipient.
14. Wash 3x for 20 min with phosphate buffer
15. Rinse 2 hr in osmiumtetraoxide (2% in phosphate buffer)
16. Wash 2x for 20 min in phosphate buffer
17. Rinse the nodules 30 min in ethanol 30%
18. Rinse the nodules 1 hr in ethanol 50 %
19. Rinse the nodules overnight in ethanol 70%
20. Rinse the nodules 1 hr in ethanol 80%
21. Rinse the nodules in ethanol 90%
22. Rinse the nodules 3x s for 20 min in ethanol 100%
23. Wash the nodules for 30 min in propylenoxide: ethanol 1:1
24. Wash the nodules 2x in propylenoxide
25. Rinse the nodules overnight in Epon:propylenoxide 1:1
26. Add Epon 100% (centrifuge to avoid burbles). Let over night.
27. Capsule the mixture and polymerize at 60 °C for 2 days.

Phosphate buffer: 0.2 M KH_2PO_4, 0.2 M Na_2HPO_4, pH 7.0
Glutaraldehyde solution: 4% glutaraldehyde in phosphate buffer

24. GUS staining

1. Put the nodulated root system in a flask with 100 ml GUS detection buffer
2. Cover the flask with aluminum foil and incubate for 48 hr at 37°C with proper shaking.

3. *gusA*-tagged reporter strain is quantified by counting blue-stained nodules

GUS staining buffer: 4.35 g K_2HPO_4, 3.41g KH_2PO_4, 5 ml SDS 10%, 2.8 ml Sarcosyl 35%, 1 ml Triton X-100, 0.37 g EDTA, 50 mg X-gluc (dissolved in 1 ml DMF) and H_2O to 1000 ml. pH 7.5-8.0.

25. *In vivo* labeling of rhizobial strains with acetate or [1-14C]ornithine and analysis of lipid extracts (modified from Bligh and Dyer, 1959)

1. Grow a preculture of the rhizobia strain overnight at 28°C in 20E, with the antibiotic.
2. *[1-^{14}C] acetate labeling*: Inoculate a 2 ml PY medium tube with the preculture to an OD_{600} between 0.05-0.1, Add 1 µCi [1-^{14}C]acetate to each culture and incubate for 24 h at 28°C with proper shaking. *[1-^{14}C] ornithine labeling*: Inoculate a 2 ml minimal medium tube with the preculture to an OD_{600} between 0.05-0.1, Add 0.5 µCi [1-^{14}C]ornithine to each culture and incubate for 48 h at 28°C with proper shaking.
3. Spin down the culture for 1 min at 13000 rpm. Discard supernatant and resuspend the pellet in 100 µl of H_2O.
4. Add 3.5 vol. methanol- chloroform 2:1, vortex and incubate at room temperature 10 min.
5. Add 1.25 vol chloroform and 1.25 vol H_2O. Vortex briefly, and centrifuge 2 min at 11 000 rpm.
6. Transfer the lower phase (containing dissolved lipids in chloroform) to a new recipient and let air dry.
7. Resuspend in 20 µl methanol-chloroform 1:1. It could be stored at -20°C until run in a thin layer chromatography (TLC) plate.
8. This method could be used to isolate total membrane lipids from cultures of 1 liter or more using the same proportions of the solvents.

Media

1. PY medium (Noel et al. 1984)

Peptone	5 g
Yeast extracts	3 g
$CaCl_2$	1 g
H_2O	to 1000 ml
pH 6.8	

2. Minimal Medium (Kingsley and Bohlool 1992)

Glycerol	5.17 ml	$MnSO_4$	38 mg/250 ml
Na-glutamate	2.085 g	$ZnSO_4$	29 mg/250 ml
Stock I (10X)		$CuCl_2$	43 mg/250 ml
$MgSO_4$	2.46 g/l	CuSO4	118 mg/250 ml
CaCl2	0.68 g/l	Stock V (10^6X)	
KCl	1.12 g/l	$NaMoO_4$	1.21 g/250 ml
Stock II (1000X)		$CoCl_2$	0.16g/250 ml
KH_2PO_4	0.34 g/250 ml	Vitamins (1000X)	
Stock III (100X)		Na pantothenate	0.8 ml/l
FeEDTA	0.55 g/500 ml	Thiamine HCL	0.8 ml/l
Stock IV (100X)		Nicotinamide	0.8 ml/l

3. 20E medium (Werner et al. 1975)

Add 10 ml of stocks I, II, III IV and VI and 2 ml stock V

Stock I

6.8 g/l KH_2PO_4

8.9 g/l K_2HPO_4

Stock II

37.0 g/l $MgSO_4$

Stock III

7.3 g/l $CaCl_2$

Stock IV

50.6 g/l KNO_3

Stock V

0.695 g/100 ml$FeSO_4$

0.93 g/100 ml Titriplex III

Stock VI

0.48 g/l $NaMoO_4$

pH 6.8

4. LB medium (Sambrook et al. 1989)

Peptone	10 g
Yeast extracts	5 g
NaCl	10 g
H_2O	to 1000 ml
pH 7.5	

5. **Fahraeus N-free solution** (Fahraeus 1957)
Add 10 ml of solutions I, II, III, IV and V and 1 ml solution VI to 1000 ml H_2O.

Solution I	
Na_2HPO_4	15 g/l
Solution II	
KH_2PO_4	10 g/l
Solution III	
$CaCl_2$	10 g/l
Solution IV	
$MgSO_4$	12 g/l
Solution V	
Fe Citrate	0.5 g/l
Solution VI	
H_3BO_3	2.86 g/l
$MnSO_4$	2.03 g/l
$ZnSO_4$	220 mg/l
$CuSO_4$	80 mg/l
Na_2MoO_4	80 mg/l

6. YM medium (Somasegaran and Hoben 1994)

K_2HPO_4	0.5 g
$MgSO_4$	0.3 g
NaCl	0.1 g
Yeast extracts	8.5 g
Mannitol	10 g
H_2O	to 1000 ml
pH 6.8	

7. Minimal medium (for *R. tropici* 299)

K_2HPO_4	3.8 g
KH_2PO_4	3 g
Sucrose 20%	5 ml
KNO_3	2.75 g
$MgSO_4$	1 g
Solution VI Fahraeus	1 ml
$CaCl_2$	1 g
Fe citrate	0.025 g
H2O	to 1000 ml

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Erklärung

Ich versiche hiermit, daβ ich meine Dissertation “Genetic analysis and phenotypic characterization of three novel genes of *Rhizobium tropici* CIAT899 involved in symbiotic interactions with *Phaseolus vulgaris”* selbstständig, ohne unerlaubte Hilfe angefertigt und mich dabei keiner anderen als der von mir ausdrücklich bezeichneten Quellen un Hilfen bedient habe.

Die Dissertation wurde in jetzigen oder einer ähnlichen Form noch bei keiner anderen Hochschule eingereicht und hat noch keinen sonstigen Prüfungszwecken gedient.

Marburg, den 01.06 2005 Keilor Rojas Jiménez

Curriculum Vitae

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A ClC chloride channel homologue and ornithine-containing membrane lipids of *Rhizobium tropici* CIAT899 are involved in symbiotic efficiency and acid tolerance

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Running title: ClC channels, ornithine-containing lipids and symbiosis

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Abstract

Rhizobium tropici CIAT899 is highly tolerant to several environmental stresses and a good competitor for nodule occupancy of common bean plants in acid soils. Random transposon mutagenesis was performed with the aim of identifying novel genes of this strain involved in symbiosis and stress tolerance. Here we present a genetic analysis of the locus disrupted by the Tn*5* insertion in mutant 899-PV9, which lead to the discovery of *syc1*, a homologue of the ubiquitous ClC family of voltage-gated chloride channels and Cl^-/H^+ exchange transporters. A non-polar deletion in this gene caused serious deficiencies in nodule development, nodulation competitiveness and N_2 fixation on *Phaseolus vulgaris* plants, probably due to its reduced ability to invade plant cells and to form stable symbiosomes, as judged by electron transmission microscopy. A second gene (*olsC*) found downstream of the former is homologous with aspartyl/asparaginyl β-hydroxylases and modifies two species of ornithine-containing lipids *in vivo*, presumably by a hydroxylation at a still unknown position. A mutant carrying a non-polar deletion in *olsC* is symbiotically defective, whereas over-expressed OlsC in the complemented strain provokes acid-sensitive phenotypes. This is the first report of a ClC homologue being essential for the establishment of a fully developed N_2-fixing root nodule symbiosis and of a putative β-hydroxylase that modifies ornithine-containing membrane lipids of *R. tropici* CIAT899, which in turn are contributing to symbiotic performance and acid tolerance.

Key words: *Rhizobium tropici*, ClC channel family, membrane lipids, ornithine-containing lipids, acid tolerance, symbiosis.

Introduction

In order to accomplish a successful symbiotic interaction with legumes, rhizobia have to cope with different stress conditions they encounter in soil, the rhizosphere and the symbiosome. Soil acidity limits symbiotic N_2 fixation and crop productivity in many soils of the tropics and subtropics (Aarons and Graham 1991; Zahran 1999; Hungria and Vargas 2000). It causes nitrogen deficiency in legumes as it inhibits rhizobial growth, root infection and bacteroid activity (Munns et al. 1981; Glenn et al. 1999). In the rhizosphere, plants secrete H^+ and organic acids that acidify the soil up to 2 pH units below the surrounding bulk soil (Marschner 1995). In addition, the presence of antibacterial molecules and the strong competence between microorganisms for nutrients constitute further stress factors that could constrain nodulation (Jjemba 2001; Lynch et al. 2002). Finally, rhizobial bacteroids also face an acidic environment in the peribacteroid space, which has been estimated to be up to two pH units more acidic than the plant cell cytosol (Udvardi and Day 1997). Bacteroids also face osmotic and oxidative stresses, as well as microaerobiosis, in the symbiosomes (Day et al. 2001; Nogales et al. 2002). Acid stress is therefore, a common limiting factor all the way from the soil to the symbiosome, which suggests the existence of different mechanisms of adaptation.

It is well known that rhizobial species exhibit different levels of tolerance to acidity (Munns et al. 1979; Graham et al. 1982; Graham et al. 1994). However, the genetic and physiological bases of this acid tolerance are still not clear. Two mechanisms related to the acid tolerance response (ATR) have been identified in rhizobia and enterobacteria (O'Hara and Glenn 1994; Foster 1999; Merrell and Camilli 2002). The first involves the synthesis of outer membrane proteins and changes in the structure of lipopolysaccharides,

exopolysaccharides and fatty acids to enhance cell surface stability and to prevent proton permeability (Ballen et al. 1990; Chen et al. 1993a; Reeve et al. 1997). The second mechanism is related to the maintenance of intracellular pH homeostasis (Chen et al. 1993b). Proton influx in low-pH environments such as that faced by *E. coli* during gastric tract infection is counteracted in the cytoplasm by decarboxylation of amino acids to consume protons and antiporter activity to remove products (Foster 1999; Merrell and Camilli 2002). Export of positively charged substrates could cause hyperpolarization of the inner membrane; however, this process is prevented by Cl^-/H^+ exchangers of the ClC family, which act as electrical shunts (Chen 2005; Iyer et al. 2002; Accardi and Miller 2004). Additional but yet unknown mechanisms for acid tolerance might operate in rhizobia.

Rhizobium tropici CIAT899 is highly tolerant to many environmental stresses and particularly to acidity. It is able to grow on media acidified down to pH 4.0 and it is a good competitor for nodule occupancy of *Phaseolus vulgaris* (common bean) and other hosts under acidic conditions (Martínez-Romero et al. 1991; Graham 1992). Thus, *R. tropici* CIAT899 represents a good model to search for genes involved in acid tolerance and to determine their role in symbiosis (Vinuesa et al. 2003). The current knowledge of pH-regulated genes in rhizobia is still poor, despite their agricultural relevance. Proteome analyses using two-dimensional gel electrophoretic analysis reveal differential protein synthesis after pH shifts (Aarons and Graham 1991; Peick et al. 1999). Mutagenesis with the transposon Tn*5* and selection of mutants on acidified media was used to characterize acid-sensitive mutants in *Rhizobium leguminosarum* (Chen et al. 1993b). It has been shown that the *R. tropici gshB*-like gene and *Sinorhizobium meliloti actA*, *actP, exoH,*

exoR, actS, actR, phrR are essential for growth at low pH (Glenn et al. 1999; Riccillo et al. 2000). More recently, Vinuesa et al. (2003) reported the isolation of additional Tn*5*-induced acid sensitive mutants of *R. tropici* CIAT899, all of which displayed symbiotically defective phenotypes in terms of nodulation competitiveness, nodule development and N_2 fixation on *P. vulgaris*. Strain 899-PV4 was shown to carry a single Tn*5* insertion at the 5' end of *atvA*, an ortholog of the chromosomal virulence gene *acvB* of *Agrobacterium tumefaciens*, which is required by the bacteria for both, a proficient interaction with plants and for acid tolerance (Vinuesa et al. 2003).

In this study, we present a genetic analysis as well as a phenotypic characterization of the locus disrupted by the Tn*5* insertion in strain 899-PV9, which bears significant sequence homology with the ubiquitous superfamily of ClC- Cl^- channel proteins and Cl^-/H^+ antiporters (Chen 2005; Accardi and Miller 2004), and of a gene located downstream, which is involved in membrane lipid modifications, with relevance for acid tolerance and symbiosis.

Results

Isolation of cosmids that restore symbiotic proficiency in strain 899-PV9.

A symbiotically-defective mutant of *Rhizobium tropici* CIAT899, obtained by random transposon mutagenesis, was designated 899-PV9. When mutant 899-PV9 was inoculated on *Phaseolus vulgaris* plants, nodules were not fully developed, irregular in size, lacked lenticels and did not express nitrogenase activity. Southern blot analysis (not shown) confirmed that the observed phenotype of this strain is due to a single Tn*5* insertion, which was localized between nucleotides C1763 and T1764 of the sequence (AY954450) reported herein (Fig. 1A). Mapping of the insertion junction site was achieved by sequencing the flanking regions of plasmid p899PV9ESK, which contains the Tn*5* insertion of strain 899-PV9 cloned as an *Eco*RI fragment, using primer Tn5-77/58EB (Vinuesa et al. 2003), which reads outwardly from the Tn*5* IS elements.

Several transconjugants were able to restore symbiotic proficiency of this mutant strain after complementation with a cosmid library. The cosmids isolated from these transconjugants were identical in their restriction patterns after digestion with different enzymes. One of these cosmids, named pPV9cos2, was digested with *Eco*RI, transferred to a nylon membrane and hybridized with Dig-labeled p899PV9-PCR. The resulting hybridizing fragment (~6.2 kb*)* was subcloned into pSK, yielding pPV9E-SK, and subjected to DNA sequencing.

Sequence analyses and characterization of *orf2* and *orf3*.

Using different computer programs, an analysis of the 3761 bp *Eco*RI-*Cla*I complementing sequence of mutant PV9-899 (accession number AY954450) revealed 4

open reading frames (ORFs) with high coding probability, as predicted by FrameD. ORFs 1 to 3 are transcribed in the same orientation, while *orf4* is transcribed convergently with respect to the *orf3*. *orf1* and *orf4* were truncated at their 5' ends. The genetic analysis presented herein targeted *orf2* and *orf3*, which are the ORFs that could be affected by the Tn*5* insertion in mutant 899-PV9 (Fig. 1).

In frame +2, at position 785 starts a 1368 bp-long ORF (*orf2*) predicted to encode a 48.6 kDa product, which according to homology searches with the BLASTP program, showed highly significant sequence similarity to ClC chloride channel proteins from the γ-proteobacteria *Salmonella typhimurium* (E value $2e^{-55}$, 27% identity and 42% similarity) and *Escherichia coli* (E value $4e^{-45}$, 20% identity and 35% similarity). The product encoded by *orf2* belongs to the voltage-gated chloride channel and antiporter protein family (COG0038). They are integral membrane proteins with inner membrane localization in Gram negative bacteria. The rhizobial ClC chloride channel homologue is predicted to have 10 transmembrane helices with both N- and C-terminal domains residing in the cytoplasm, containing the sequence motifs corresponding to the ion-binding site and gating region of the solved *E. coli* and *S. typhimurium* ClC protein structure (Fig. 1B-I). This superfamily of ion channels and antiporters is found in both prokaryotic and eukaryotic cells, including most of the α-proteobacterial genomes sequenced to date. One of the two ClC paralogs found in *E. coli,* named ClC-ec1, has been proposed to act as an electrical shunt for an outwardly directed proton pump that is linked to amino acid decarboxylation as part of the extreme acid resistance response of this bacterium (Iyer et al. 2002). Protein sequence alignments and secondary structure analyses showed a high conservation (Fig. 1B-I-III), including the *R. tropici* residue E129

which in the homologous ClC chloride channels from *E. coli* and *S. enterica* is the one responsible for gating according to X-ray crystallographic studies (Dutzler et al. 2002; Dutzler 2004). Therefore, based on the highly significant homology of *orf2* to ClC chloride channels and antiporters, we named the former locus as *syc1,* for symbiosis-assisting *C*lC-like protein. The intergenic spacer (IGS) between *orf1* and *syc1* was analyzed using the neural network for promoter prediction (NNPP), which located a putative promoter sequence upstream of the *syc1* start codon (sites 727-772, $r = 0.81$).

In frame +1, at position 2611 starts a 845-bp-long ORF (*orf3*) predicted to encode a 31.78 kDa product, with highly significant sequence similarity to the lipid A-myristate β-hydroxylase (LpxO) from *Salmonella typhimurium* (E value $5e^{-23}$, 35% identity and 51% similarity), which has been shown to be responsible for the 2-hydroxylation of myristate in lipid A (Gibbons et al. 2000). Sequence analyses predicted a cytoplasmic localization for the Orf3, with N- and C-terminal hydrophobic domains. The catalytic domain of this aspartyl/asparaginyl β-hydroxylase protein family is well conserved (Fig. 1C). In particular, histidine residue H164 is thought to be an iron ligand and therefore essential for the function of the protein (Jia et al. 1994). A maximum likelihood phylogenetic reconstruction based on selected protein sequences of this family found in divergent bacteria (data not shown) revealed that the putative aspartyl/asparaginyl β-hydroxylase from *R. tropici* is located in a different clade than the LpxO protein from *S. typhimurium.* This inference suggests that LpxO and Orf3 might have slightly different functions or substrate specificities, although they clearly belong to the same protein super-family (COG3555). The IGS between *syc1* and *orf3* was analyzed using the NNPP server, which predicted 2 possible promoter sequences upstream *orf3* (sites 2306-2351, $r = 0.98$; 2419-

2464, $r = 0.94$). This promoter prediction is consistent with a monocistronic organization of the transcript encoded by *syc1*, which is also supported by RT-PCR experiments, complementation analyses, and the phenotype observed by 2D-TLC analyses of radiolabeled lipid extracts (discussed below). Based on the evidence gained from the latter experiments, we named *orf3* as *olsC*, for *o*rnithine *l*ipid *s*ynthesis gene *C*.

RT-PCR of the intergenic spacer between *syc1* and *olsC* confirms that both genes are independently transcribed.

An RT-PCR experiment was designed to determine the transcriptional organization of *syc1* and *olsC* (Fig. 1D). For this purpose, RNA was isolated from *Rhizobium tropici* CIAT899 cells grown in PY broth to the early stationary phase, and used to synthesize cDNA with random hexamers as primers. This cDNA was used as template for PCR amplification experiments with primers PV9-2000f and PV9-2985r, which bind up- and downstream of the IGS region (Fig. 1A). No amplification product was detected (Fig. 1D), which suggests that both ORFs are independently transcribed under these conditions and making it unlikely that the Tn*5* insertion in *syc1* has a polar effect on *olsC*. However, the IGS region could be amplified with PV9-2000f and PV9-2985r when genomic DNA was used as template. The presence of an internal fragment of *olsC* and 16S rDNA in the cDNA template was confirmed by PCR amplification of these genes, demonstrating a proper cDNA synthesis. In the negative control without reverse transcriptase, no amplification product was observed, which discards the possibility of contamination by *R. tropici* genomic DNA.

Construction of non-polar deletions in *syc1* and *olsC* and phenotypic characterization of the mutant strains.

We generated a non-polar mutation in *syc1* by deletion of a 991 bp-long fragment that embraces nearly the whole gene, including the predicted gating region. To construct this mutant, plasmid pKRΔ02 was transferred into CIAT899. Double recombinants were selected for the loss of sensitivity to sucrose and the resulting deletion was confirmed by PCR with primers PV9-524f-H and PV9-2272r-E (data not shown). The mutant obtained was designated 899-*syc*Δ1. This strain was able to grow in 20E or PY media acidified to pH 4.5 at similar rates as the parent strain (Fig. 2A). However, the nodules it formed on bean plants were poorly developed (21dpi), lacked lenticels, were whitish (Figs. 3A and 3B) and presented a 14-fold decrease in nitrogen fixation in comparison to the parent strain, as revealed by the acetylene reduction assay (Fig. 2B). The nodulation competitiveness of 899-*syc*Δ1 against the *gusA*-tagged reporter strain CIAT899-G1 in a 10:1 co-inoculation experiment showed that only 5.9% of the nodules were occupied by the former (Fig. 2C). Coinoculations of CIAT899 and CIAT899-G1 resulted in percentages of GUS-expressing nodules that reflected the proportion of *gusA*-tagged reporter cells in the inoculum mixture, as previously reported (Vinuesa et al., 2003). The symbiotic phenotypes displayed by this strain correspond to those observed in mutant 899-PV9. Further analyses using light and transmission electron microscopy (TEM) revealed that both mutants were able to enter the nodules, but unable to form stable symbiosomes. TEM micrographs of nodules induced by 899-*syc*Δ1 showed poor invasion of plant cells, accumulation of polyhydroxybutyrate (PHB) granules within bacteroids, and presence of amyloplasts in the host cells, whereas the parent strain was able to fully

invade plant cells (Figs. 3D and 3E). The symbiotic proficiency of mutant 899-*syc*Δ1 was restored when cosmid pPV9cos2 was provided in *trans* (data not shown).

A partial deletion (211 bp-long) of the region containing the predicted catalytic domain of OlsC was generated using plasmid pKRΔ03 following the same procedure mentioned for *syc1*. The resulting strain (899-*olsC*Δ1) was confirmed to carry the deletion by PCR with primers PV9-2571f-E and PV9-3622r-H (data not shown). Mutant 899-*olsC*Δ1 was able to grow in 20E or PY media acidified to pH 4.5 at a similar rate as the parent strain (Fig. 2A). Nodules of bean plants inoculated with this strain (21 dpi) were poorly developed and lacked lenticels (Fig. 3C). These nodules also showed reduced levels of nitrogen fixation (about 50%) when compared to the wild type strain CIAT899, as determined by acetylene reduction assays (Fig. 2B). When 899-*olsC*Δ1 was co-inoculated with the *gusA*-tagged reporter strain CIAT899-G1 in a 10:1 ratio, only 25% of the nodules were occupied by the former, which displayed the same Ndv^- phenotype as the nodules induced by 899-*olsC*Δ1 (Fig. 2C). Light micrographs of nodules inoculated with mutant 899-*olsC*Δ1 (Fig. 3F) revealed lower invasion levels than achieved by the parent strain CIAT899, however, not as drastic as observed for mutant 899-*syc*Δ1 (data not shown).

Complementation of mutant 899-*olsC*Δ1 with pBBR-1,6BE.

In order to complement mutant 899-*olsC*Δ1, plasmid pBBR-1,6BE (Fig. 1A) was constructed and provided *in trans*. A 1660 bp *Bam*HI-*Eco*RI fragment containing *olsC* and the upstream region with the predicted promoter region was PCR-amplified and cloned into pBBR-MCS5. To ensure that the gene is expressed from its native promoter,

the fragment was cloned in opposite transcriptional direction to the vector's *lacZ* promoter. Plasmid pBBR-1,6BE was transferred into mutant 899-*olsC*Δ1 and strain 899-*olsC*Δ1/pBBR-1,6BE was tested on bean plants for symbiotic performance. The symbiotic proficiency and nitrogen fixation capacity of strain 899-*olsC*Δ1/pBBR-1,6BE was restored to similar levels as exhibited by CIAT899. On near neutral media (pH 6.8), the complemented strain 899-*olsC*Δ1/pBBR-1,6BE (generation time g = 2.8 h) grew similarly as CIAT899 (g = 2.4 h) or mutant 899-*olsC*Δ1, with (g = 2.8 h) or without (g = 2.8 h) the empty vector (data not shown). In contrast, when the complemented strain was grown on acidified media at pH 4.5 (Fig. 2A), it presented a significantly increased mean generation time (g = 9.2 h) in comparison to CIAT899, or the mutant 899-*olsC*Δ1, with or without the empty vector (g ≈ 2.9 h). These results suggest that the expression or over-expression (due to copy number) of the gene contained in this 1.66 kb fragment is responsible for the reduced growth under acidic conditions displayed by the complemented strain when carrying plasmid pBBR-1,6BE in *trans*.

Some membrane lipids of *R. tropici* CIAT899 are absent in mutant 899-*olsC*Δ1.

Lipid extracts from *Rhizobium tropici* CIAT899, mutant 899-*olsC*Δ1 carrying the 211 bp deletion in *olsC*, and the complemented mutant 899-*olsC*Δ1/pBBR-1,6BE were separated by two-dimensional thin-layer chromatography (2D-TLC) and individual lipids were quantified (Table 1). Rhizobial membrane phospholipids like phosphatidylethanolamine (PE), dimethylphosphatidylethanolamine (DMPE), cardiolipin (CL), phosphatidylglycerol (PG), sulphoquinovosyl diacylglycerol (SL) and phosphatidylcholine (PC) were identified based on their relative mobilities and in

comparison to the well-characterized lipid profile of *Sinorhizobium meliloti* 1021 (Weissenmayer et al. 2002; Gao et al. 2004). As found for *S. meliloti* 1021, PC constitutes also the major membrane lipid of *R. tropici* CIAT899. In addition to the above-mentioned lipids, four additional components can be detected in the chromatogram of *R. tropici* CIAT899, labeled as S1, S2, P1, and P2 (Fig. 4A). Staining of developed 2D-TLC chromatograms with ninhydrin revealed that PE, S1, and P1 possess primary amino groups (data not shown). The compound S1 shows the same relative mobility as ornithine-containing lipids (OL), which have been characterized previously in *S. meliloti* (Weissenmayer et al. 2002; Gao et al. 2004). Since OL is also a ninhydrin-positive compound, we suggest that S1 might be the corresponding OL from *R. tropici* CIAT899. The lipid composition of the Tn*5*-generated mutant 899-PV9 and that of the deletion mutant 899-*syc*Δ1, both defective in the predicted chloride channel protein, were indistinguishable from that of the wild type (data not shown). This is consistent with our conclusion that the Tn*5* insertion in *syc1* does not exert a polar effect on *olsC*.

Although the lipid composition of mutant 899-*olsC*Δ1 resembles that of the wild type (Fig. 4B), compounds P1 and P2, which together comprised nearly 15% of the wild type membrane lipids, are absent in mutant 899-*olsC*Δ1 (Table 1). In contrast, the wild type has only minor levels of S1 and S2 (7.2% of total membrane lipids) whereas in mutant 899-*olsC*Δ1 these two lipids comprise 22.4% of the total lipid detected. If the mutant is complemented in *trans* with pBBR-1,6BE (Fig. 4C), again P1 and P2 are formed in relatively high amounts (more than 18% of total membrane lipids) whereas S1 and S2 are practically absent from this strain. These data are consistent with a model in which the predicted β-hydroxylase encoded by *olsC* converts the ninhydrin-positive compound S1

to the ninhydrin-positive compound P1 and also the ninhydrin-negative compound S2 to the ninhydrin-negative compound P2, presumably by hydroxylation at a still unknown position in these molecules. As S1 and S2 both function as *in vivo* substrates for the OlsC reaction, one can expect that S1 and S2 would have similar chemical structures and that therefore S2 might be a modified version of the ornithine-containing lipids known to date. The non-reactivity of S2 with ninhydrin might be due to an additional, so far unknown modification at the δ-amino group of its ornithine residue. Similarly, as P1 and P2 are both products of the *in vivo* reaction catalyzed by OlsC, again one can expect that P1 and P2 would have similar structures, with P2 having an additional modification at the δ-amino group of its ornithine residue. The nearly complete lack of S1 and S2 in the case of the strain 899-*olsC*Δ1/pBBR-1,6BE can be explained by a more efficient conversion of S1 and S2 to P1 and P2 due to an increased copy number of the gene responsible for the conversion.

Incorporation experiments with radiolabeled ornithine demonstrated that from all the membrane lipids, in *S. meliloti* only OL become labeled, indicating that ornithine is specifically and exclusively incorporated into OL (Gao et al. 2004). Analysis of the lipid profile from *R. tropici* CIAT899 that had been labeled with [1-^{14}C]ornithine indicates that four compounds have incorporated radiolabeled ornithine (Fig. 4D). The relative mobilities of the radiolabeled compounds coincide with the relative mobilities found for lipids S1, S2, P1, and P2. Therefore, we conclude that all four of these lipids are chemically distinct classes of ornithine-containing lipids (OL) of *R. tropici* CIAT899. As lipid S1 is ninhydrin-positive and migrates in an identical way as the well-characterized OL (Geiger et al. 1999) from *S. meliloti* in 2D-TLCs, we expect that S1 presents a similar

or identical structure as sinorhizobial OL. In contrast, the S2, P1, and P2 classes of ornithine-containing lipids, in this order, migrate more slowly in both dimensions of 2D-TLC systems and therefore must be increasingly more polar due to still unknown modifications by functional groups.

Discussion

In this work we report two novel genes of *Rhizobium tropici* CIAT899, which were shown to be required for the establishment of a fully developed N_2-fixing symbiosis with bean plants. The microscopy analyses performed on mutant 899-*syc*Δ1, which carries a deletion in the putative chloride channel encoded by *syc1*, suggests that the observed decrease in nodule development and nitrogen fixation is probably due to its failure to invade plant cells and to form stable symbiosomes. This is the first report showing that a rhizobial homologue of the ClC family of Cl^- channels and Cl^-/H^+ antiporters is essential for the establishment of a fully proficient symbiotic interaction with its legume host, but not for growth under free-living conditions. With the notable exception of *Escherichia coli*, the molecular and physiological functions of dozens of prokaryotic ClC homologues recently uncovered by genome sequencing projects are still unknown. It is worth noting that the ClC-ec1 (or EriC) protein of *E. coli* was recently shown not to be an ion channel, but rather a H^+/Cl^- exchange transporter, demonstrating that the structural boundary separating transporters and channels is not clearcut, as previously thought (Accardi and Miller 2004; Chen 2005). The *E. coli* genome has two ClC homologs. When either one of these genes was individually deleted, no notable phenotype was observed. However, a doble-knock-out strain displayed a dramatic reduction in cell survival and amino acids transport under acid shock (Iyer et al. 2002) We have recently cloned a second ClC-like paralog from CIAT899 (as found in the genomes of many other α-Proteobacteria, including *Agrobacterium tumefaciens* C58, *Brucella suis* 1330 and *B. melitensis* 16M, *Bradyrhizobium japonicum* USDA110 and *Mesorhizobium loti* MAFF303099, but not *Sinorhizobium meliloti* 1021), which suggests that the ClC

paralogs might perform different functions in the cell under different physiological conditions, and might be differentially expressed. Our complementation results demonstrate that the paralog (*syc1*) mutated in this study is required for a proficient symbiotic interaction with bean plants, but apparently not for acid tolerance or growth under free-living conditions. However, at this point we cannot define if the mutations made in *syc1* have a direct or indirect effect on the symbiotic phenotype observed in strains 899-PV9 and 899-*syc*Δ1. At this point, and based on the evidence gained from TEM data, we can not state wether the mutation is affecting bacteroid release from infection threads, symbiosome proliferation or stability. Mutations in the second paralogous gene, the construction of a double mutant and analysis of transcriptional reporter gene fusions would be very valuable to gain a better understanding of the functions of these genes.

It has recently been shown that *Rhizobium leguminosarum* mutants blocked in amino acid transporters, present poorly developed nodules, reduced N_2 fixation, and the bacteroids are saturated with dicarboxylic acids and polyhydroxybutyrate granules (Lodwig et al. 2003). Since the peribacteroid space is acidic and the mutant 899-*syc*Δ1 displayed a similar phenotype to that observed for the *R. leguminosarum* mutant, it is tempting to speculate that Syc1 might be involved in the adaptation of *R. tropici* bacteroids to the symbiosome's milieu, probably in relation with the electrophysiology of bacteroid membranes, which in turn may affect key aspects of cellular homeostasis like the internal pH of bacteroides, or the transport of metabolites across their cellular membranes. Since ClC channels and antiporters are highly selective for chloride anions (Accardi et al. 2004; Chen 2005), this would imply that Cl^- could play a key role in symbiosome physiology. If

so, it remains to be uncovered. Plant voltage-dependent anion channels (VDACs), including ClC homologues, have been recently found to play a broader diversity of functions than previously thought (Barbier-Brygoo et al. 2000; Wandrey et al. 2004). Several plant VDACs and anion transporters have recently been shown to be associated with the symbiosome membrane (Wienkoop et al. 2003; Vincill et al. 2005). Therfore, both plant and bacterial anion channels or antiporters seem to play important, although not yet well understood functions in root nodule symbioses.

Only two previous works describe the phenotypes of microbial cells carrying mutations in ClC homologs in relation to their interaction with eukaryotic hosts. Mutations in *clc-a* from the human pathogenic yeast *Cryptococcus neoformans* resulted in attenuated virulence in a mouse cryptococcosis model. This attenuation resulted from the lack of expression of two important virulence factors, capsule and laccase (Zhu and Williamson, 2003). In contrast, deletion of the single ClC ortholog found in *Vibrio cholerae* enhanced intestinal colonization in infant mice. This gene was found to confer mild resistance to acid when pH was adjusted with HCl, but not with other acids (Ding and Waldor, 2003). Clearly, much research is still needed to provide a basic understanding of the molecular and physiological functions of the diverse ClC homologs found across prokaryotic phyla.

It has been speculated that certain membrane lipids might be important for the establishment of a successful symbiosis (de Rudder et al. 1997). This idea has been supported by the observations that mutants of *Sinorhizobium meliloti* lacking phosphatidylcholine (PC) are unable to form nitrogen-fixing nodules on alfalfa (López-Lara et al. 2003; Sohlenkamp et al. 2003), and that mutants of *Bradyrhizobium japonicum* with diminished levels of PC present a reduced number of bacteroids within infected

plant cells (Minder et al. 2001). Since PC comprises 50-60% of the lipids in membranes of *S. meliloti* or *B. japonicum* and ornithine-containing lipids about 20% in *R. tropici*, one might expect that major changes in membrane lipid composition, either by mutations or by increased copy number of genes involved in their biosynthesis, could affect the structural properties of cell membranes and, as a consequence, the proper functioning of membrane-associated proteins.

Rhizobium tropici CIAT899 produces four different classes of ornithine-containing lipids (S1, S2, P1, and P2). Mutant 899-*olsC*Δ1, which forms more S1 and S2 and lacks P1 and P2, is acid-tolerant but symbiotically defective. In contrast, the complemented mutant 899-*olsC*Δ1/pBBR-1,6BE, which presented mainly P1 and P2 and nearly complete lack of S1 and S2, was able to restore the symbiotic proficiency, but was acid-sensitive. The latter indicates that lipids P1 and P2 are necessary for a successful symbiotic interaction of *R. tropici* CIAT899 with the plant host, whereas lipids S1 and S2 are required for acid tolerance. This hypothesis is consistent with the fact that in the parent strain CIAT899 all four distinct classes of ornithine-containing lipids are present and that this strain is both acid-tolerant and symbiotically proficient.

The analysis performed on mutant 899-*olsC*Δ1 permits us to report for the first time that membrane lipids of *Rhizobium tropici* are involved in symbiosis and that the putative β-hydroxylase encoded by *olsC* is part of a biosynthesis pathway for membrane lipids not previously described for any species. We demonstrate that *R. tropici* lipid species S1, S2, P1 and P2 are indeed ornithine-containing lipids and that the product encoded by *olsC* is necessary to convert lipids S1 and S2 to P1 and P2, presumably by a hydroxylation at a still unknown position. Lipids P1 and P2, which presumably carry a hydroxyl group at a

still unknown position, are required for an effective symbiotic interaction with bean plants while lack of lipids S1 and S2 was correlated with acidic sensivity.. Hydroxylations at the 2-position of fatty acyl residues of membrane lipids such as PE or OL are known to occur in *Burkholderia cepacia* and other bacteria (Taylor et al. 1998).Therefore, a balanced membrane lipid composition of the *R. tropici* cell membranes is required for both, the symbiotic interaction with plants and for acid tolerance under free-living conditions. This is the first report of a rhizobial membrane lipid other than phospholipids with relevance for symbiosis.

Materials and Methods

Bacterial strains and plasmids.

Bacterial strains and plasmids used in the present work are listed in Table 2. Rhizobial strains were grown in PY (Noel et al. 1984), minimal medium (Kingsley and Bohlool 1992) or in 20E medium (Werner et al. 1975) at 28°C. Acidic media at pH 4.5 were buffered with 25 mM Homopipes (Research Organics). *Escherichia coli* strains were grown in Luria-Bertani medium at 37°C (Sambrook et al. 1989). When needed, antibiotics were added at the following concentrations: kanamycin (Km) 100 μg/ml, streptomycin (Sm) 150 μg/ml, ampicillin (Ap) 50 μg/ml, tetracycline (Tc) 7.5 μg/ml, gentamicin (Gm) 10 μg/ml, nalidixic acid (Nal) 20 μg/ml.

Random transposon mutagenesis of *Rhizobium tropici* CIAT899, selection of acid-sensitive mutants and cosmid complementation.

Tn*5* mutagenesis of *R. tropici* CIAT899 was carried out using *E. coli* S17-1 carrying pSUP1021 as donor strain (Simon et al. 1983). Transconjugants carrying the transposon were isolated and acid-sensitive mutants were selected on different acidified media, as previously described (Vinuesa et al. 2003). A cosmid library of *R. tropici* CIAT899 made in pVK101 (Vargas et al. 1990) was mobilized *en masse* into CIAT899-PV9 by triparental mating using pRK2013 as helper plasmid (Figurski and Helinski 1979). Transconjugants that restore symbiotic proficiency on common beans were isolated as previously described (Vinuesa et al. 2003).

Standard DNA manipulations.

Genomic DNA from rhizobial strains was isolated using the GenomicPrep Cells & Tissues DNA isolation kit (Amersham) following the manufacturer's instructions. Plasmid DNA from *E. coli* cultures was isolated with the High Pure Plasmid isolation kit (Roche). Restriction endonucleases were purchased from New England Biolabs and used according to standard procedures (Sambrook et al. 1989). PCR amplifications were carried out in a Gene Amp PCR system 2700 (Applied Biosystems) using *Taq* (Roche) or XL polymerase (Applied Biosystems) in a standard 50 µl PCR mix, as previously described (Vinuesa et al. 1999).

To map the transposon insertion in mutant 899-PV9, total DNA of this strain was digested with *Eco*RI, transferred to a nylon membrane and hybridized with a digoxygenin-labeled probe generated by the incorporation of DIG-UTP (Roche) into *nptII* marker of Tn*5* via PCR, as previously described (Vinuesa et al. 2003). The single hybridizing fragment (~14 kb) was cloned into pBluescript (pSK), yielding p899PV9ESK. This plasmid was used as template for PCR amplification with primers Tn5-77/58EB (Vinuesa et al. 2003) and M13 universal to map the Tn*5* insertion by sequencing the resulting PCR product with the former primer, which reads outwardly from the Tn*5* IS elements. The amplification product was cloned into pSK, yielding p899PV9E-PCR, and used to generate a hybridization probe of the genomic DNA flanking the Tn*5* insertion by DIG-labeling.

Cosmids were isolated and restricted with several enzymes as previously described (Vinuesa et al. 2003). Cosmid pPV9cos2 was hybridized with the probe derived from the insert cloned in p899PV9E-PCR and the resulting hybridizing band was cloned into pSK, yielding pPV9E-SK. Subclones from pPV9E-SK were sequenced with an ABI Pris 3700

automated sequencer using the universal M13f and M13r primers (Applied Biosystems). PCR primers were subsequently designed to obtain an ~ 8X-coverage of overlapping plasmid subclones and PCR products from which a contig was assembled using SeqManII from the DNASTAR package (Lasergene, Madison, WI, USA).

DNA sequence analyses.

Open reading frames (ORFs) with high coding probability were identified on the contig sequence using FrameD (http://genopole.toulouse.inra.fr/bioinfo.FrameD/FD2) with the *S. meliloti* codon usage table and the pentanucleotide aagga as ribosomal binding site. Homology searching at the nr sequence databases of NCBI was performed with the BLASTX and BLASTP programs. Protein sequence analyses to predict secondary structure, cell localization, transmembrane domains, conserved motifs, and hydrophobicity profiles were performed using Prosite, ProDom, PsortB, TmPred, PsPred, Pfam and iProClass program servers. A search for putative promoter regions in intergenic regions was performed using the NNPP server (www.fruitfly.org/ seq_tools/promoter.html).

Determination of operon structure by RT-PCR.

RNA from *Rhizobium tropici* CIAT899 was isolated using the High Pure RNA isolation kit (Roche) according to the manufacturer's instructions and cDNA was immediately synthesized using the Omniscript RT kit (QIAGEN). This cDNA was used as template for PCR amplification of the intergenic spacer between *syc1* and *olsC* using primers PV9-2000f (5'gcagcggccataccagcatc) and PV9-2985r (5'tcacgccgaaaccgaggag). Positive

controls included the amplification of the 16S rDNA gene with primers fD1 and rD1 (Weisburg et al. 1991), and the amplification of a 389 bp internal fragment of *olsC* using primers PV9-2571f-H (5'ccaagcttcctcccggaccgcac) and PV9-2960r-B (5'ccggatccagcgggtgtcggtgg). To discard the presence of contamination by *R. tropici* genomic DNA, the same master mix used for cDNA synthesis, but lacking the retrotranscriptase was used as a negative control for PCR amplification experiments with the primers for the 16S rDNA gene and the internal *olsC* fragment .

Construction of non-polar mutants 899-*syc*Δ1 and 899-*olsC*Δ1.

To construct a non-polar mutant in *syc1*, PCR amplification of two non-overlapping ~400 bp fragments located at the opposite ends of the ORF (resulting in a 991 bp deletion) were performed using primer pairs (restriction sites underlined) PV9-524f-H (5'gcaagcttgcccgggcggtgtgacg) / PV9-971r-B (5'cggatccggcaacgggcataagaaag) and PV9-1962f-B (5'ccggatcctcgctgtcgcgtgctt) / PV9-2272r-E (5'ccgaattcctgccatcggagcgtc), and total DNA of *R. tropici* as template (Fig. 1). The same strategy was used to construct a 211 bp deletion in *olsC*, using primer pairs PV9-2571f-H (5'ccaagcttcctcccggaccgcac) / PV9-2960r-B (5'ccggatccagcgggtgtcggtgg), and PV9-3171f-B (5'cggatccgcgtcgacaatcacg) / PV9-3622r-E (5'cgcgaattcggtggcggcatgacg). The PCR products were digested with *Hin*dIII + *Bam*HI and *Bam*HI + *Eco*RI respectively, and ligated to *Hin*dIII + *Eco*RI restricted pK18*mobsacB*. The resulting plasmids pKRΔ02 and pKRΔ03 were transferred into strain CIAT899 by triparental matings, and double recombinants were selected on PY medium at pH 6.8 amended with 12% sucrose as previously reported (Vinuesa et al. 2003). Two non-polar mutants, here after named 899-*syc*Δ1 and 899-*olsC*Δ1, were obtained.

Complementation of strain 899-*olsC*Δ1 with pBRR-1,6BE.

A 1660 bp *Eco*RI-*Bam*HI fragment was amplified with primers PV9-1962f-B and PV9-3622r-E and cloned into pBBR-MCS5 (Gm^r), a broad host range vector (Kovach et al. 1995) to obtain pBRR-1,6BE. The cloned fragment contains the whole gene encoded by *olsC* and additional 534 bp upstream of the predicted start codon with the region carrying the putative promoter sequence predicted by the NNPP server (see Fig.1). This fragment was cloned in opposite direction to the *lacZ* promoter of the vector in order to avoid vector-derived expression. Plasmid pBRR-1,6BE was transferred into mutant 899-*olsC*Δ1 by triparental mating and transconjugants were selected on PY and MM plates amended with Gm 10, Ap 50 and Nal 20. The empty pBBR-MCS5 vector was transferred into 899-*olsC*Δ1, as a control of the complementation, and transconjugants were selected as mentioned before.

Plant tests.

Phaseolus vulgaris seeds were surface-sterilized with 1.2% sodium hypochlorite and germinated on 1% agar-water plates as described (Vinuesa et al. 1999). Seedlings were transferred to 250 ml flasks filled with vermiculite and nitrogen-free nutrient solution (Fahraeus 1957) and inoculated with about 10^5 CFU per plant. Plants were grown in a controlled growth chamber and harvested 21 days post inoculation (dpi). Nitrogenase activity of nodulated roots was determined by acetylene reduction assay. Competition experiments were performed by co-inoculating the mutant strain together with a *gusA*-tagged reported strain, CIAT899-G1 at a low inoculum titer (~500 cells/plantlet) to

minimize nodule coinfections, as previously described (Vinuesa et al. 2003). Plants were harvested 21 dpi and blue nodules were counted after GUS staining (Wilson et al. 1995).

***In vivo* labeling of rhizobial strains with [^{14}C]acetate or [^{14}C]ornithine and analysis of lipid extracts.**

The lipid compositions of *R. tropici* CIAT899, mutant 899-*olsC*Δ1 and complemented mutant 899-*olsC*Δ1/pBBR1,6BE were determined after labeling with [1-^{14}C]acetate (60 mCi/mmol; Amersham) during growth on PY medium for 24 h. The incorporation of ornithine into lipids was followed by labeling *R. tropici* CIAT899 with DL-[1-^{14}C]ornithine (56 mCi/mmol; Amersham) during growth on minimal medium for 48 h. Two milliliter cultures were inoculated from precultures to an initial OD_{600} of 0.05 in the respective medium. After the addition of 1 μCi [1-^{14}C]acetate or of 0.5 μCi DL-[1-^{14}C]ornithine to the respective cultures, they were incubated at 28^{o}C with appropriate shaking. At the end of the growth period, cells were harvested by centrifugation, resuspended in 100 μl of water and lipid extracts were obtained according to Bligh and Dyer (Bligh and Dyer 1959). Aliquots of the lipid extracts were spotted on high-performance TLC silica gel 60 (Merck) plates, and separated in two dimensions using chloroform-methanol-water (140:60:10, v/v/v) as a mobile phase for the first dimension, and chloroform-methanol-acetic acid (130:50:20, v/v/v) for the second. Primary amine-containing lipids were visualized by spraying the plates with a solution of 0.2% ninhydrin in acetone and subsequent treatment at 100^{o}C for 5 min. To quantify the membrane lipid composition, developed 2D-TLC plates were stained with iodine and the radioactivity of

individual spots was quantified in a scintillation counter as previously described (Geiger et al. 1999).

Nucleotide sequence accession number

The nucleotide sequence (3761 bp) reported in this study has been deposited in GenBank under accession number AY954450.

Acknowledgments

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Figure legends

Fig. 1. A, Genetic and physical maps of the 3761 bp *Eco*RI-*Cla*I region from *Rhizobium tropici* CIAT899 analyzed in this study (acc. no. AY954450). Selected restriction sites are shown. Four open reading frames (represented by arrows) were detected. The site of the Tn*5* insertion located in *syc1* between nucleotides C1763 and T1764 is indicated by an open triangle. Non-polar deletion mutants lacking the regions shown in white were generated in *syc1* and *olsC*. Predicted promoters are shown as thin arrows. The dotted line represents the intergenic spacer between *syc1* and *olsC* subjected to RT-PCR analysis (see panel **D**). The dashed line shows the location of the1.66 kb *Bam*HI-*Eco*RI fragment cloned into pBBR-MCS5 and used to complement strain 899-*olsC*Δ1. **B,** transmembrane topology predicted by Predictprotein for Syc1 (panel B-I); the conserved residue E129 (marked with asterisk) is located in the gating region of EriC (Dutzler et al. 2003); Panels B-II and B-III show hydrophobicity plots generated by TmPred for the *R. tropici* CIAT899 ClC homologue (panel B-II) Syc1 compared with that for the ClC exchange transporter (EriC) from *Salmonella typhimurium* (panel B-III). **C,** Partial sequence alignment of *R. tropici, Mesorhizobium* sp. (ZP 00193099), *B. melitensis* (NP 539381), *A. vinelandii* (ZP 00090437) putative aspartyl/asparaginyl β-hydroxylase sequences and LpxO (Fe^{2+}/alpha-ketoglutarate-dependent dioxygenase) from *S. typhimurium* (AAF87784). Residue His164 is highly conserved as part of the catalytic domain (Jia et al. 1994). Identical residues are underlined in black and similar residues are shaded in grey. **D,** The RT-PCR experiment shows no PCR amplification of the IGS between *syc1* and *olsC* (lane 1), which suggests that both ORFs are independently transcribed under these conditions. No amplification in the negative control (lane 2) discards the possibility

of contamination by *R. tropici* genomic DNA. Positive controls include the PCR amplification of an internal fragment from *olsC* (lane 3) and the 16S rDNA gene (lane 4) when using cDNA as template, or amplification of the IGS region when using genomic DNA as template (lane 5).

Fig. 2. Phenotypes displayed by several *Rhizobium tropici* strains used in this study. **A**, growth of *R. tropici* strains on PY media at pH 4.5. Values are the mean ± SD of 4 independent experiments. **B**, Mean acetylene reduction of nodulated roots inoculated with strains 899-PV9, 899-*syc*Δ1 and 899-*olsC*Δ1 in comparison to the parental strain CIAT899. Values are the mean ± SD of three repetitions in two independent experiments. **C**, Percentage of nodules occupied by mutant strains (black) against reporter strain CIAT899-G1 (gray) in a 10:1 co-inoculation experiment (in favor of mutants) using low inocculum titers (~ 500 cfu/plantlet).

Fig. 3. Macroscopic aspect of nodules induced by strains CIAT899 (**A**), mutant 899-*syc*Δ1 (**B**) and mutant 899-*olsC*Δ1 (**C**) on *Phaseolus vulgaris* plants (21 dpi). Electron micrographs reveal drastic differences in the levels of invasion presented by parent strain (**D**) and mutant 899-*syc*Δ1 (**E**). Notice the accumulation of polyhydroxybutyrate granules in bacteroids of the latter. Mutant 899-*olsC*Δ1 also shows reduced levels of invasion, as revealed by light microscopy (**F**).

Fig. 4. Membrane lipid analysis of *Rhizobium tropici* strains. Separation of [^{14}C]acetate-labeled lipids from *R. tropici* CIAT899 (**A**), mutant 899-*olsC*Δ1 (**B**) and complemented mutant 899-*olsC*Δ1/pBBR-1,6BE (**C**), as well as of [^{14}C]ornithine-labeled lipids from *R.*

tropici CIAT899 (**D**) using two-dimensional thin-layer chromatography. The lipids cardiolipin (CL), phosphatidylglycerol (PG), sulphoquinovosyl diacylglycerol (SL), phosphatidylethanolamine (PE), dimethylphosphatidylethanolamine (DMPE) and phosphatidylcholine (PC) are indicated. Ovals surround lipid species S1 and S2 which presumably are substrates for the putative *R. tropici* β-hydroxylase to form the lipid products (P1 and P2) enclosed in rectangular boxes. Asterisks indicate ninhydrin-positive lipids.

Fig. 1 Rojas-Jiménez et al. MPMI-03-05-0056

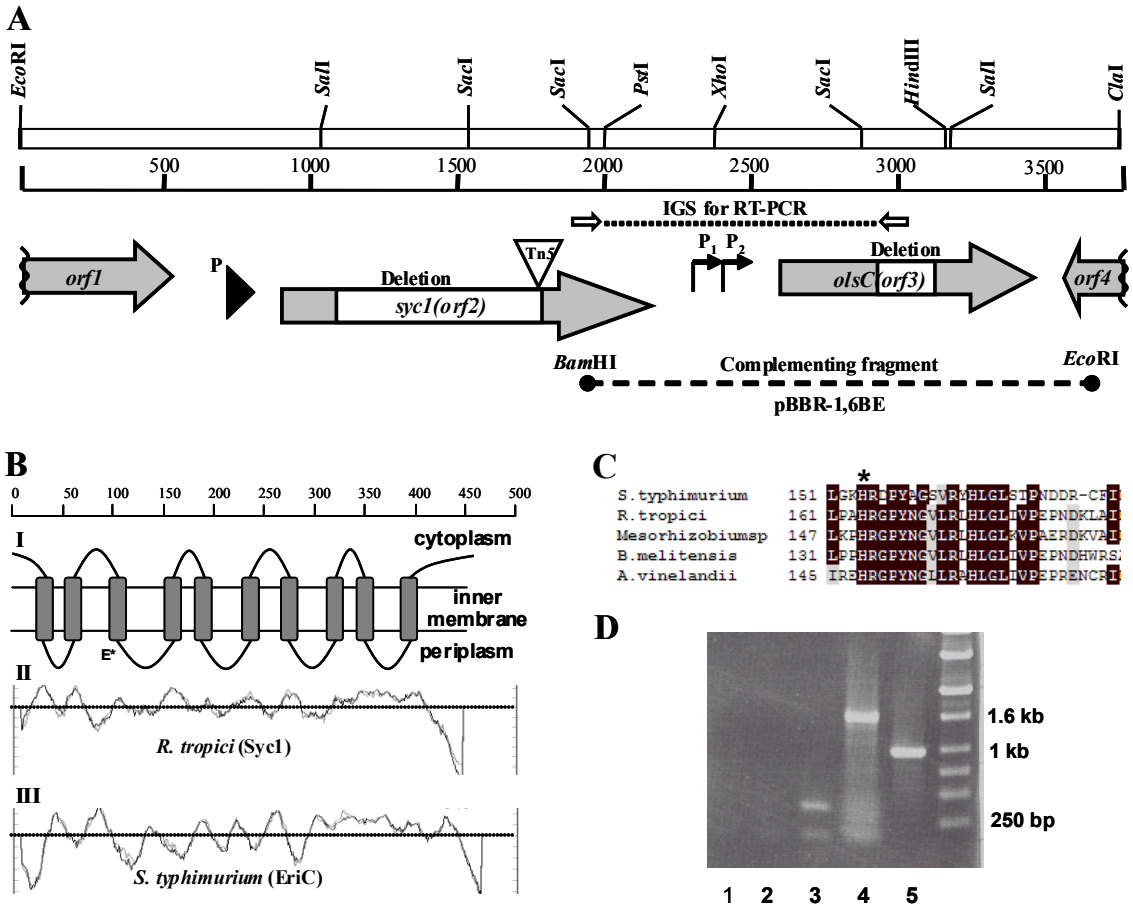


Fig. 2 Rojas-Jiménez et al. MPMI-03-05-0056

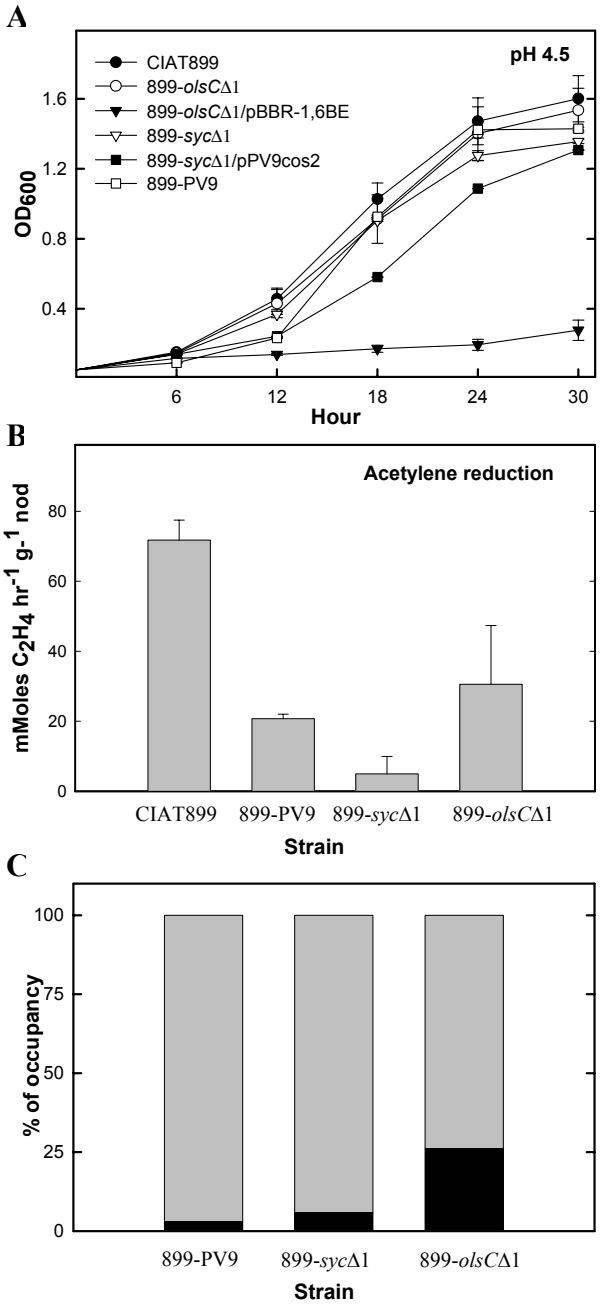
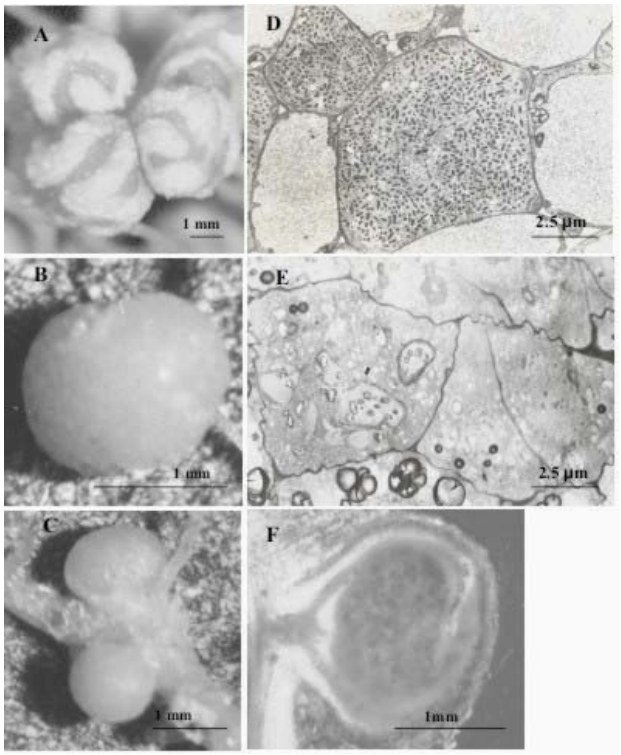


Fig. 3 Rojas-Jiménez et al. MPMI-03-05-0056



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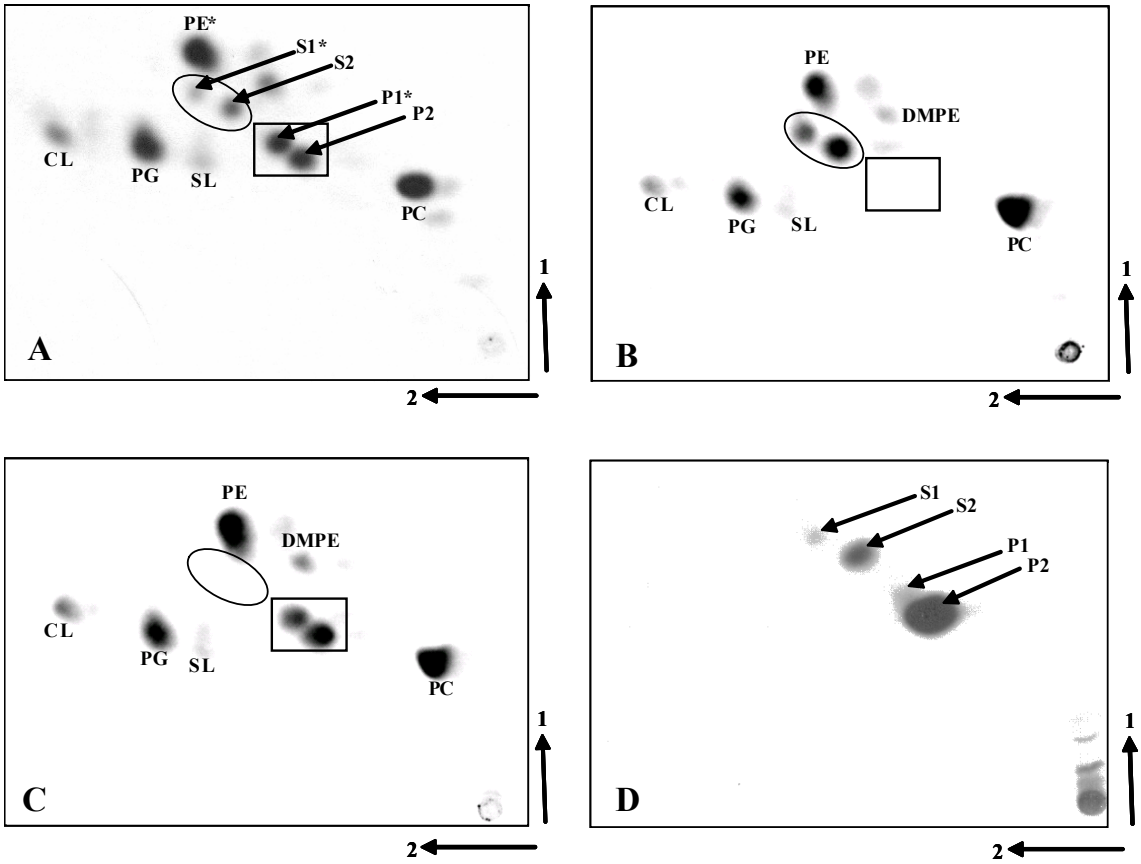

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2 **Table 2**. Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant characteristics	Source or reference
***R. tropici* strains**		
CIAT899	Acid tolerant, Ap^r, Nal^r	Martínez-Romero et al. 1991
899-PV9	CIAT899 derivative (*syc1*::Tn*5*), symbiotically defective, Sm^r, Km^r	Vinuesa et al. 2003
CIAT899-G1	*gusA*-tagged CIAT899 derivative carrying a single mTn*5gusA*30 insertion, used as reporter strain in competition experiments, Sm^r , Sp^r	Vinuesa et al. 2003
899-*syc*Δ1	CIAT899 carrying a 991 bp non-polar deletion in *syc1*	This study
899-*syc*Δ1/ pPV9cos2	899-*syc*Δ1 carrying the complementing cosmid of 899-PV9, Tc^r	This study
899-*olsC*Δ1	CIAT899 carrying a 211 bp non-polar deletion in *olsC*	This study
899-*olsC*Δ1/pBBR-1,6BE	899-*olsC*Δ1 complemented with pBBR-1,6BE	This study
899-*olsC*Δ1/pBBR-MCS5	899-*olsC*Δ1 carrying the vector pBBR-MCS5	This study
***E. coli* strains**		
DH5α	recA1, ΔlacU169, Φ80dlacZΔM1	Stratagene
S17-1	thi pro $hsdR^-$ $hsdM^+$ recA, RP4 integrated in the chromosome, 2-Tc::Mu-Km::Tn7(Tp^r/Sm^r)	Simon et al. 1983
Plasmids		
pRK2013	Helper plasmid; Km^r	Ditta et al. 1980
pK18*mob*	Conjugative suicide vector with Km^r *lacZ* markers used for positive selection of single recombinants	Schäfer et al. 1994
pK18*mobsacB*	Conjugative suicide vector with Km^r *lacZ* and *sacB* markers used for positive selection of double recombinants	Schäfer et al. 1994
pBBR-MCS5	Mobilizable broad host range cloning vector, Gm^r	Kovach et al. 1995
pCR II	PCR cloning vector	Invitrogen

pSUP1011	Mobilizable suicide plasmid for Tn*5* mutagenesis	Simon et al. 1985
pBluescript II SK (pSK)	Standard cloning and sequencing vector, *lacZ* Ap^r	Stratagene
p899PV9ESK	14 kb *Eco*RI fragment from strain 899-PV9, containing the Tn*5* insertion, cloned in pSK. Ap^r, Km^r	This study
p899PV9E-PCR	pSK plasmid containing the flanking region of p899PV9ESK and used for DIG-labeling. Ap^r	This study
pPV9cos2	Cosmid complementing 899-PV9, Tc^r	This study
pKRΔ02	Integrative mutagenizing plasmid based on pK18*mobsacB* used to construct strain 899-*syc*Δ1	This study
pKRΔ03	Integrative mutagenizing plasmid based on pK18*mobsacB* used to construct strain 899-olsCΔ1	This study
pBBR-1,6BE	1.66 kb *Bam*HI-*Eco*RI fragment cloned into pBBR-MCS5 used for complementing mutation on strain 899-*olsC*Δ1	This study

Table 1. Membrane lipid composition of *Rhizobium tropici* CIAT899 wild type, mutant 899-*olsC*Δ1, complemented strain 899-*olsC*Δ1/pBBR-1,6BE and mutant containing the empty vector 899-*olsC*Δ1/pBBR-MCS5 (for lipid designations see Fig. 4). Values are the mean ± standard deviations of three independent experiments.

Lipid	Composition (% of total ^{14}C)			
	CIAT899	**899-*olsC*Δ1**	**899-*olsC*Δ1/ pBBR-1,6BE**	**899-*olsC*Δ1/ pBBR-MCS5**
PC	40.3 ± 4.7	47.8 ± 6.0	32.5 ± 1.5	37.4 ± 5.5
PG	11.9 ±2.1	10.7 ± 1.9	12.7 ± 1.3	11.2 ± 0.2
CL	2.9 ± 2.0	2.6 ± 0.8	2.9 ± 0.3	3.2 ± 1.4
PE	23.4 ± 3.8	16.3 ± 4.9	33.4 ± 0.4	26.2 ± 1.7
S1	2.7 ± 2.7	7.2 ± 1.8	n.d.	10.7 ± 3.0
S2	4.5 ± 1.9	15.3 ± 1.1	0.3 ± 0.2	11.3 ± 0.8
P1	6.3 ± 3.0	n.d.	6.8 ± 2.9	n.d.
P2	8.1 ± 4.9	n.d.	11.4 ± 1.4	n.d.

n.d. not detected

Rhizobium tropici CIAT899 requires a putative σ^E-factor to establish an effective symbiosis with *Phaseolus vulgaris* plants

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Abstract

Random mutagenesis of the root nodulating bacterium *Rhizobium tropici* CIAT899 was performed to identify novel genes involved in symbiosis and stress tolerance. The analysis performed in the non-polar mutant 899-Δsig1, which carries a deletion in the putative σ^E-factor encoded by *sigE*, revealed serious deficiencies for nodule development, nodulation competitiveness and N_2 fixation when inoculated on *Phaseolus vulgaris* plants, indicating that this gene is required to establish a fully proficient symbiotic interaction with its legume host. The expression of the *R. tropici* putative σ^E-factor was constitutive in both free-living and symbiotic conditions and dependent upon two promoters located upstream of *sigE*. Regulation of the expression of this locus seems to be posttranscriptional, possibly through a putative anti-σ factor encoded by *alf1*, which is located downstream of *sigE*. This bacterial activity has not been previously reported as relevant for the symbiotic process in rhizobia.

1. Introduction

The initiation of transcription in bacteria depends on the association of the RNA polymerase with small proteins, known as σ-factors, which direct the core enzyme to a specific class of promoter sequences. Prokaryotic species synthesize different σ-factors that recognize different promoter sequences, which allow bacteria to maintain the basal gene expression as well as regulation of gene expression under altered environmental conditions [1-3].

Based on sequence similarity, bacterial sigma factors are grouped in two families. The σ^{54} family contains only one group and are not essential for certain growth conditions. They recognize a -12 and -24 promoter element. The σ^{70} family recognize a -10 and -35 promoter element and comprises several groups differentiated by their structure and function. The latter is characterized by the presence of four regions (1-4), where subregions 2.4 and 4.2 are shown to be the most conserved and responsible of the -10 and -35 promoter element recognition, respectively [4,5]. Among the σ^{70} family, are the extracytoplasmic function (ECF) σ-factors, also called σ^{E} or σ^{24} factors. These σ-factors form a subgroup of environmentally responsive transcriptional regulators which respond to events occurring in the periplasm and outer membrane. σ^{E} controls the transcription of several genes in response to extracellular stresses and that are required for virulence in *E. coli* [6], *H. influenzae* [7], *S. typhimurium* [8,9], *M. tuberculosis* [10,11], *P. aeruginosa* [12], *V. cholerae* [13] and *Y. enterolitica* [14].

Proteins that negatively regulate transcription by interaction with a σ-factor are known as anti-σ-factors [15]. Usually, these proteins have inner membrane localization. The N-terminus resides in the cytoplasm and binds reversibly to σ^{E}, blocking its association with the RNA polymerase core. The C-terminus of the anti-σ-factor is located in the periplasmic space and is responsible for extracytoplasmic stress sensing. Under stress conditions, proteolytic activity occurs to degrade the anti-σ-factor. This event releases the σ^{E}-factor, which is then free to bind to RNA polymerase and transcribe the genes in its regulon [16,17]. The latter enables a quick response to environmental stimuli, since σ-factors are already available with no need to be synthesized *de novo*.

For rhizobia, however, there is no information about the regulation of transcription of genes controlled by ECF σ-factors, particularly in response to environmental stresses or in symbiosis. As part of an attempt to identify novel genes involved in plant-microbe interactions and stress tolerance, we performed random mutagenesis in the bean nodulating bacterium *Rhizobium tropici* CIAT899, which is highly tolerant to many environmental stresses and a good competitor for nodule occupancy. Previously, the genetic analyses of the region disrupted by the Tn*5* insertion in mutant 899-PV4 was reported, that revealed *atvA*, an ortholog of the chromosomal virulence gene *acvB* of *Agrobacterium tumefaciens*, which is required for acid tolerance [18]. In this work, I present the genetic analysis of the region downstream of *atvA*, which led to the discovery of a gene (*sigE*) that bears high similarity to a σ^E factor and predicted to be responsible for the symbiotic deficiency displayed by 899-PV4. This is the first report of a putative σ^E factor in rhizobia that is required for an effective symbiotic interaction with *Phaseolus vulgaris* plants.

2. Materials and Methods

2.1 Bacterial strains and plasmids

Bacterial strains and plasmids used in the present work are listed in Table 1. Rhizobial strains were grown in PY [19], minimal medium [20] or in 20E medium [21] at 28^{o}C. Acidic media at pH 4.5 were buffered with 25 mM Homopipes. *Escherichia coli* strains were grown in Luria-Bertani medium at 37^{o}C (Sambrook et al. 1989). When needed, antibiotics were added at the following concentrations: kanamycin (Km) 100 µg/ml, ampicillin (Ap) 50 µg/ml, tetracycline (Tc) 7.5 µg/ml, gentamicin (Gm) 10 µg/ml, nalidixic acid (Nal) 20 µg/ml.

2.2 Standard DNA manipulations

Genomic DNA from rhizobial strains was isolated using the GenomicPrep Cells & Tissues DNA isolation kit (Amersham) following the manufacturer's instructions. Plasmid DNA from *E. coli* cultures was isolated with the High Pure Plasmid isolation kit (Roche). PCR amplifications were carried out in a Gene Amp PCR system 2700 (Applied Biosystems) using *Taq* (Roche) or XL polymerase (Applied Biosystems) in a standard 50 µl PCR mix as previously described [22]. PCR primers were designed to cover this region downstream of *atvA* using DNA from the cosmid that complements mutant 899-PV4 as a template.

2.3 DNA sequence analyses

Open reading frames (ORFs) with high coding probability were identified on the contig sequence using FrameD (http://genopole.toulouse.inra.fr/bioinfo.FrameD/FD2) with the *S. meliloti* codon usage table and the pentanucleotide (aagga) as ribosomal binding site. Homology searching at the nr sequence databases of NCBI was performed with BLASTX and BLASTP programs. Protein sequence analyses to predict secondary structure, cell localization, transmembrane domains, conserved motifs, and hydrophobic

profiles were performed using ProDom, PsortB, TmPred, PsiPred, Pfam and ProteinPredict program servers. A search for putative promoter regions in intergenic regions was performed using the NNPP server (www.fruitfly.org/ seq_tools/promoter.html).

2.4 Construction of non-polar mutant 899-Δsig1

To construct a non-polar deletion of 474 bp in *sigE* (Fig. 1A) primers with restriction sites underlined PV4-5419f-E (5'cgaattcatctagtatcgcaggcaac) and PV4-5740r-B (5'cggatcctggaagcggcgcatagtc) as well as PV4-6214f-B (5'cggatccgtgattttggcgaagtc) and PV4-6798r-H (5'gcaagcttcgactacgggcggggag) were used for PCR amplification of fragments located at the opposite ends of the ORF, with total DNA of *R. tropici* as template. PCR products were digested with *Eco*RI+ *Bam*HI and *Bam*HI+ *Hind*III respectively, and ligated to *Eco*RI + *Hind*III restricted pK18*mobsacB*. The resulting plasmid pKRΔsig1 was transferred into strain CIAT899 and double recombinants were selected on PY medium at pH 6.8 amended with 12% saccharose as previously reported [18]. The non-polar mutant, here after named 899-Δsig1, was obtained. The resulting deletion was confirmed by PCR (data not shown).

2.5 Complementation of strain 899-Δsig1 with plasmid pBBRsigE

In order to complement mutant 899-Δsig1, a 1.4 kb *Eco*RI-*Hind*III fragment was PCR amplified using primers PV4-5419f-E and PV4-6798r-H and cloned into the pBBR-MCS5 Gm^r vector [23] restricted with the same enzymes. The resulting plasmid was named pBBRsigE (Fig 1C). The cloned fragment contains the whole gene encoded by *sigE* and 72 bp upstream of its start codon with one of the promoters predicted by the NNPP server. Plasmid pBBRsigE was transferred into mutant 899-Δsig1 by triparental mating and transconjugants were selected on PY and MM plates amended with Gm 10, Ap 50 and Nal 20. The resulting complementing strain was named 899-Δsig1/pBBRsigE. The vector pBBR-MCS5 was transferred into 899-Δsig1, yielding strain 899-Δsig1/pBBR, and used as a control of the complementation.

2.6 Plant tests

Phaseolus vulgaris seeds were surface-sterilized with 1.2% sodium hypochlorite and germinated on 1% agar-water plates for 48h. at 28°C. Seedlings were transferred to 250 ml flasks filled with vermiculite and nitrogen-free nutrient solution [24] and inoculated with about 10^5 CFU per plant. Plants were grown in a controlled growth chamber and harvested 21 days post inoculation (dpi). Nitrogenase activity of nodulated roots was determined by acetylene reduction assay. Competition experiments were performed by co-inoculating the mutant strain together with a *gusA*-tagged reported strain, CIAT899-G1, in a 1:10 ratio as previously described [18]. Plants were harvested 21 dpi and blue nodules were counted after GUS staining [25].

2.7 Analysis of bacterial growth

Strains were grown overnight to exponential phase in the appropriate medium. To analyze the growth curve of the strains, the pre-cultures were inoculated in PY medium to an initial OD_{600} of 0.05 and grown for 24 hr at 28°C. To test the percentage of survival of strains after stress challenge, exponentially growing strains were diluted to 10^5 CFU and exposed to osmotic (0.1-0.25 M NaCl) or acid challenge (pH 4.5), to 37°C temperature or to ethanol (2%) for 2 hr. The number of surviving CFU was determined by plating out serial dilutions of the samples.

2.8 Determination of sigE transcriptional regulation

To construct the *sigE*-GUS reporter fusion, we amplified a 542 bp *Xba*I-*Sal*I fragment that contains 233 bp of *sigE* and 293 bp upstream its start codon and cloned it upstream the promoterless glucuronidase gene (*uidA*) of the vector pBBR-GUS [26]. The resulting plasmid, named pBBRsigE-GUS (Fig 1D), was transferred into strain CIAT899 by triparental mating and transconjugants were positively selected by blue staining on PY plates amended with Gm 10, Ap 50, Nal 20 and X-gluc. The resulting strain, 899/pBBRsigE-GUS, was grown to different points of the growth curve and exposed to

the above mentioned stresses. The transcriptional activation of *uidA* in this strain was determined by the quantitative β-glucuronidase assay using p-nitrophenyl glucuronide as substrate [27]. Data were normalized to total-cell protein concentration by the Lowry method [28].

2.9 Identification of the sigE transcriptional start sites

Total RNA from strain 899/pBBRsigE-GUS, growth on PY medium to early stationary phase, was isolated using the High Pure RNA isolation kit (Roche). The RNA was subject to 5' rapid amplification of cDNA ends using the 5'RACE kit (Invitrogen). Briefly, first-strand cDNA synthesis was performed using the *uidA*-specific primer GUS-LW5 (5'CGATCCAGACTGAATGCCCAC) which is complementary to the region located in the position 96 to 117 from this gene. The resulting cDNA was treated with an RNase mix (mixture of RNase H and RNase T1), to eliminate the original mRNA template, and then purified on a GlassMax DNA column (Gibco). A homopolymeric tail was added to the resulting 3' end using the Terminal deoxynucleotidil transferase (TdT) and dCTP. A PCR amplification of the cDNA was carried out using the 5'RACE anchor primer AAP (5'GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG) and the antisense primer rrsigE-LW (5'GCGTCGACATAGTCTCGTCGGGCAAGGCG). DNA sequencing of the 5'RACE products was performed on an automatic 310 DNA sequencer (Applied Biosystems), using the Big-Dye terminator kit version 3.1 (Applied Biosystems) with primer rrsigE-LW.

3. Results

3.1 Sequence analyses of sigE and alf1

The genetic map of the 7102-bp fragment of the complementing sequence of mutant 899-PV4 (Genbank acc. no. AF433669 and XXXX) is shown in Fig. 1A. Four open reading frames (ORFs) with high coding probability were detected, as predicted by the FrameD program. Previously, we reported that a mutation in *lpiA* caused a decrease in nodulation competitiveness and that *atvA* is required for acid tolerance [18]. Herein, we present the genetic analysis performed in the two ORFs located in the region downstream of *atvA*.

In frame +1, at position 5490 starts a 777 bp-long ORF predicted to encode a 29.41 kDa product, which according to BLASTP searches showed sequence similarity to RNA polymerase σ^E-factors from the pathogenic γ-Proteobacteria *Pseudomonas aeruginosa* AlgU (E value $2e^{-15}$, 35% identity and 52% similarity) and *Escherichia coli* RpoE (E value $1e^{-13}$, 37% identity and 53% similarity). This protein belongs to the sigma factor protein family COG1595 and it is predicted to have a cytoplasmic localization, according to the PsortB program. Analysis of domain conservation with Pfam server revealed significant hits to region 2 (E value $4.8e^{-12}$) which is the most conserved region of this family since it contains both the -10 promoter recognition helix and the primary core RNA polymerase binding determinant and to region 4 (E value $1.8e^{-12}$) which is involved in binding to the -35 promoter element via a helix-turn-helix motif [29]. Therefore, based on similarity, this ORF was designated as *sigE,* for sigma-E factor. Analysis of the region upstream of *sigE* using the NNPP server predicted 2 possible promoter sequences (sites 5306-5351, $r = 0.98$; 5419-5464, $r = 0.94$).

In frame +2, at position 6263 starts a second 804 bp-long ORF predicted to encode a 29.51 kDa product. BLASTP searches revealed homology to trasmembrane transcription regulators (anti-sigma factors) from β-Proteobacterium *Ralstonia metallidurans* (E value $1e^{-41}$, 38% identity and 56% similarity) and α-Proteobacterium *Mesorhizobium loti* (E value $1e^{-23}$, 29% identity and 43% similarity). This protein belongs to the anti-sigma factor protein family COG5662. Analyses of the secondary

structure and transmembrane domains performed with PsiPred and ProteinPredict servers indicate that this protein contains a single transmembrane-spanning segment, with the N-terminus residing in the cytoplasm and the C-terminus in the periplasm. This prediction is consistent with that observed in other characterized anti-sigma factors and therefore, based on similarity, we designated this ORF as *alf1*, for anti-sigma-like factor.

3.2 Construction of a non-polar deletion in sigE and symbiotic performance of the mutant strain

We generated a non-polar mutation in *sigE* by deletion of a 474 bp-long internal fragment. To construct this mutant, plasmid pKRΔsig1 was transferred into CIAT899 and double recombinants were selected as mentioned in Materials and Methods. The deletion was confirmed by PCR with primers PV4-5419f-E and PV4-6798r-H (data not shown) and the resulting mutant was designated 899-Δsig1.

Nodules formed by *Phaseolus vulgaris* plants inoculated with strain 899-Δsig1 (21 dpi) were not fully developed, whitish, irregular in size and lacked lenticels, which clearly contrast with those induced by parent strain CIAT899 (Figs 2A and 2B). Light micrographs of cross-sections of nodules of bean plants induced by strain 899-Δsig1 revealed that this mutant was able to enter the nodules, but with significant reduced levels of infection (Figs 2C and 2D). Furthermore, the central nodular tissues were not uniformly colonized by the mutant strain presenting rather a patchy pattern when compared with the parent strain (Figs 2E and 2F).

The nitrogen fixation of strain 899-Δsig1, determined by the acetylene reduction assay, showed a 4-fold decrease as compared by the levels exhibited by the parent strain CIAT899 (Fig. 3), while the nodulation competitiveness of 899-Δsig1 against the *gusA*-tagged reporter strain CIAT899-G1 in a 10:1 co-inoculation experiment showed that only 17% of the nodules were occupied by the former, which indicates that the mutant 899-Δsig1 is a poor competitor for nodule occupancy (data not shown).

3.3 Complementation of mutant 899-Δsig1 with pBBRsigE

In order to complement mutant 899-Δsig1, plasmid pBBRsigE was constructed and provided *in trans* (Fig. 1C). To construct pBBRsigE, a 1.4 kb fragment containing *sigE* and the region upstream with the second predicted promoter (with the σ^E consensus) was cloned into pBBR-MCS5, yielding pBBRsigE. This plasmid was transferred into the mutant 899-Δsig1 resulting in strain 899-Δsig1/pBBRsigE. When inoculated on bean plants the complemented mutant again formed fully developed nodules and with similar levels of nitrogen fixation capacity as exhibited by parent strain CIAT899 (Fig. 3).

3.4 Partial deletion of sigE in mutant 899-Δsig1 was not reflected in an increased sensibility to some environmental stresses

In order to test if mutant 899-Δsig1 was more sensitive than parent strain CIAT899 or complemented strain 899-Δsig1/pBBRsigE to different environmental stresses, bacteria were exposed to specific environmental conditions and percentage of survival was determined. For this purpose, pre-cultures of strains CIAT899, 899-Δsig1 and 899-Δsig1/pBBRsigE were diluted to 10^5 CFU and exposed to osmotic or acid challenge, to increased temperature or to ethanol for 2 hr. The number of CFU determined by plating out serial dilutions of these bacterial samples showed no significant difference in the percentage of survival between strains for the stresses tested (Fig. 4A). Furthermore, the analysis of the growth curves of these strains under the mentioned stresses for a 24 hr. period, neither revealed any significant difference in growth between mutant strain 899-Δsig1 or parent strain CIAT899 (Fig. 4B).

3.5 Transcriptional regulation of the putative σ^E-factor in R. tropici

In order to monitor the expression of *R. tropici sigE* under different growing conditions, a *sigE*-GUS reporter fusion was constructed as described in materials and methods. The resulting plamid, pBBRsigE-GUS (Fig. 1D), was transferred into CIAT899 yielding strain 899/pBBRsigE-GUS. The empty pBBR-GUS [26] vector was transferred into CIAT899, yielding strain 899-pBBR-GUS, as a negative control. Strains 899/pBBRsigE-GUS and 899/pBBR-GUS were grown to different points of the growth

curve or challenged with different stresses and the specific activity of glucuronidase was determined. Strain 899/pBBRsigE-GUS showed similar levels of specific activity independent of the phase of growth or environmental condition tested (500 $\pm$ 54 $nmol^{-1}$ mg^{-1} protein) while in strain 899/ pBBR-GUS almost no activity was detected (25 $\pm$ 6 $nmol^{-1}$ mg^{-1} protein). This result indicates that *sigE* has a basal expression under free-living conditions, and that at least one of the promoters located in the region 293 bp upstream the gene, is active. This resembles the transcription pattern observed for σ^E-factors in other species including *P. aeruginosa, M. tuberculosis* and *V. cholerae*, which also present basal levels of expression (Schurr et al. 1995; Manganelli et al. 2001; [13]. To determine the expresion of this gene in symbiotic conditions, we inoculated *Phaseolus vulgaris* seedlings with strain 899/pBBRsigE-GUS or 899/ pBBR-GUS and analyzed the GUS staining of nodules [25]. After this treatment, the nodules induced by strain 899/pBBRsigE-GUS presented a positive signal (blue staining) while those induced by strain 899/pBBR-GUS remained unstained (data not shown). These results indicate that expression of the putative *R. tropici* σ^E-factor takes place under both free-living and symbiotic conditions.

3.6 Identification of the transcriptional start sites of sigE

The 5' rapid amplification of cDNA ends (RACE) was used to identify the transcriptional start sites of *sigE*. Sequence analyses of the PCR products revealed a transcriptional start site (T) 33 nt upstream of the ATG start codon of *sigE* which was consistent with the prediction of the NNPP server (Fig. 1B). This promoter was analogous to the *S. antibioticus phsA* promoter and to other σ^E regulated promoters [30]. The -35 sequence (GCAGGC) showed only two mismatches when compared with respect the (GAACGC) motif of *S. antibioticus*, while the -10 sequence (GTATC) showed only one mismatch from the (GTCTC) of the same gene. In addition, a second transcriptional start site (A) was detected 166 nt upstream of the ATG start codon of *sigE* (Fig. 1B), which was also consistent with the prediction of a second promoter by the NNPP server. This promoter was highly similar to the *E. coli* σ^{70} promoter consensus. The -35 sequence of this *R. tropici sigE* second promoter (CTCACA) showed two mismatches from the

TTGACA σ^{70} consensus and the -10 sequence (CATTAT) showed also two mismatches with respect to the (TATAAT) consensus.

4. Discussion

In this work it is reported a novel gene from *Rhizobium tropici* CIAT899 which was shown to be required for the establishment of a fully developed N_2-fixing symbiosis with bean plants. The analysis performed on mutant 899-Δsig1, which carries a deletion in the putative σ^E factor encoded by *sigE*, showed a decrease in nodule development and nitrogen fixation that might be due to a significantly reduced ability to colonize the central nodular tissue, as revealed by the light microscopy. It has been shown that mutations of σ^E factors in pathogenic bacteria like *S. enterica*, *H. influenzae* and *V. cholerae*, resulted in reduced levels of intracellular survival and consequently were less virulent [7,8,13]. In this respect strain 899-Δsig1 also displayed reduced "symbiotic infectivity", when co-inoculated with the parental strain. Since mutants of σ^E-factors displayed a similar phenotype in their mammal or legume host cells respectively, it is tempting to suggest that σ^E-factors are regulating the transcription of genes that are important for both pathogenesis and for symbiosis. For *E. coli*, it has been determined that σ^E controls transcription of genes that affect characteristics of the cell envelop, biosynthesis of phospholipids, lipopolysaccharides and lipoproteins, as well as signal transduction pathways [6,17]. Some of these functions might be also controlled by this putative *R. tropici* σ^E-factor, especially those required for establishing a symbiotic interaction with bean plants. Since strain 899-Δsig1 was not more sensitive than parent strain CIAT899 or complemented mutant 899-Δsig1/pBBRsigE to some environmental stresses, we assume that *sigE* is not essential for growing under free-living conditions, where might exist other σ^E paralogs displaying more relevant roles.

As observed in *E. coli* and *V. cholerae*, the expression of the *R. tropici* putative σ^E-factor seems to be dependent upon two promoters located upstream of *sigE*. P1 appears to be σ^{70} dependent whereas the downstream promoter, P2 is σ^E dependent [13]. This gene was transcribed constitutively in both free-living and symbiotic conditions, suggesting that the transcriptional regulation is not the principal element of regulation which rather may be posttranscriptional. Usually, this posttranscriptional regulation is mediated by anti-σ factor, which sequester the σ factor in a non functional state, in the absence in the proper stimulus [15]. In *R. tropici*, *alf1* is localized immediately downstream of *sigE*,

whose product resembles anti-σ factors in primary sequence and secondary structure motifs. This genetic organization is common for other species with this mechanism of regulation, including *E. coli*, *M. tuberculosis*, *H. influenzae* and *S. enterica* [6,8,10]. Thus, it is tempting to suggest that *alf1* encodes the anti-σ factor that regulates the putative σ^E in this species. Future experiments will address this model.

This bacterial activity has not been previously reported as relevant for the symbiotic process in rhizobia. Further investigations are necessary determine the genes controlled by this putative σ^E factor and elucidate how they influence the symbiotic interaction with its eukaryotic partner.

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Escherichia coli plasmids pK18 and pK19: Selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. Gene. 145, 69-73.

Table 1. Bacterial strains and plasmids used in this study

Strain	Relevant characteristics	Source or reference
***R. tropici* strains**		
CIAT899	Acid tolerant, Ap^r, Nal^r	Martínez-Romero et al. 1991
899-PV4	CIAT899 derivative (*atvA*::Tn*5*), symbiotically defective, Sm^r, Km^r	Vinuesa et al. 2003
CIAT899-G1	*gusA*-tagged CIAT899 derivative carrying a single mTn*5gusA30* insertion, used as reporter strain in competition experiments, Sm^r , Sp^r	Vinuesa et al. 2003
899-Δsig1	CIAT899 carrying a 474 bp non-polar deletion in *sigE*	This study
899-Δsig1/pBBRsigE	899-Δsig1 complemented with pBBRsigE, Gm^r	This study
899-Δsig1/pBBR-MCS5	899-Δsig1 carrying the vector pBBR-MCS5, Gm^r	This study
899/pBBRsigE-GUS	CIAT899 carrying the vector pBBRsigE-GUS, used for transcriptional fusions, Gm^r	This study
899/ pBBR-GUS	CIAT899 carrying the vector pBBR-GUS, used as control of the transcriptional fusions, Gm^r	This study
***E. coli* strains**		
DH5α	*recA*1, Δ*lacU*169, Φ80d*lacZ*ΔM1	Stratagene
S17-1	*thi pro hsdR*$^-$ *hsdM*$^+$ *recA*, RP4 integrated in the chromosome, 2-Tc::Mu-Km::Tn*7*(Tp^r/Sm^r)	Simon et al. 1983
Plasmids		
pRK2013	Helper plasmid; Km^r	Ditta et al. 1980
pK18*mobsac*B	Conjugative suicide vector with Km^r *lacZ* and *sacB* markers used for positive selection of double recombinants	Schäfer et al. 1994
pBBR-MCS5	Mobilizable broad host range cloning vector, Gm^r	Kovach et al. 1995
pCR II	PCR cloning vector	Invitrogen
pBluescript II SK (pSK)	Standard cloning and sequencing vector, *lacZ* Ap^r	Stratagene
pPV4cos1	Cosmid complementing 899-PV4, Tc^r	
pKRΔsig1	Integrative mutagenizing plasmid based on pK18*mobsacB* used to construct strain 899-Δsig1	This study
pBBRsigE	1.4 kb *Eco*RI-*Hin*dIII fragment cloned into pBBR-MCS5 used for complementing mutation on strain 899-Δsig1	This study
pBBR-GUS	Mobilizable broad host range cloning vector, which contains a promoterless glucuronidase gene (*uidA*) downstream of the polylinker in pBBR-MCS5, Gm^r	Corvera et al. 1999
pBBRsigE-GUS	713 bp *Xba*I-*Sal*I fragment cloned into pBBR-GUS	This study

Figure Legends

Fig. 1A. Genetic and physical map of the 7102-bp fragment that restores acid tolerance and symbiotic performance in mutant 899-PV4. Selected restriction sites are shown. Four open reading frames (represented by arrows) were detected. A non-polar deletion mutant in *sigE*, lacking the region shown between the dashed lines, was generated. Predicted promoters by NNPP server are shown as black triangles. The region within restriction sites *Eco*RI-*Hind*III, which contains *sigE* and part of *alf1*, represents the fragment used for construction of the complementing plasmid pBBRsigE. The region within restriction sites *Xba*I-*Sal*I, which contains the region upstream *sigE* and part of this gene, represents the fragment used for the construction of plasmid pBBRsigE-GUS used for transcriptional fusions. **1B.** Organization of the *R. tropici sige* P1 and P2 promoters. The distances between the transcriptional start sites and the *sige* start codon are shown. The nucleotide sequences of the -35 and -10 regions of the promoters enclosed by rectangles.

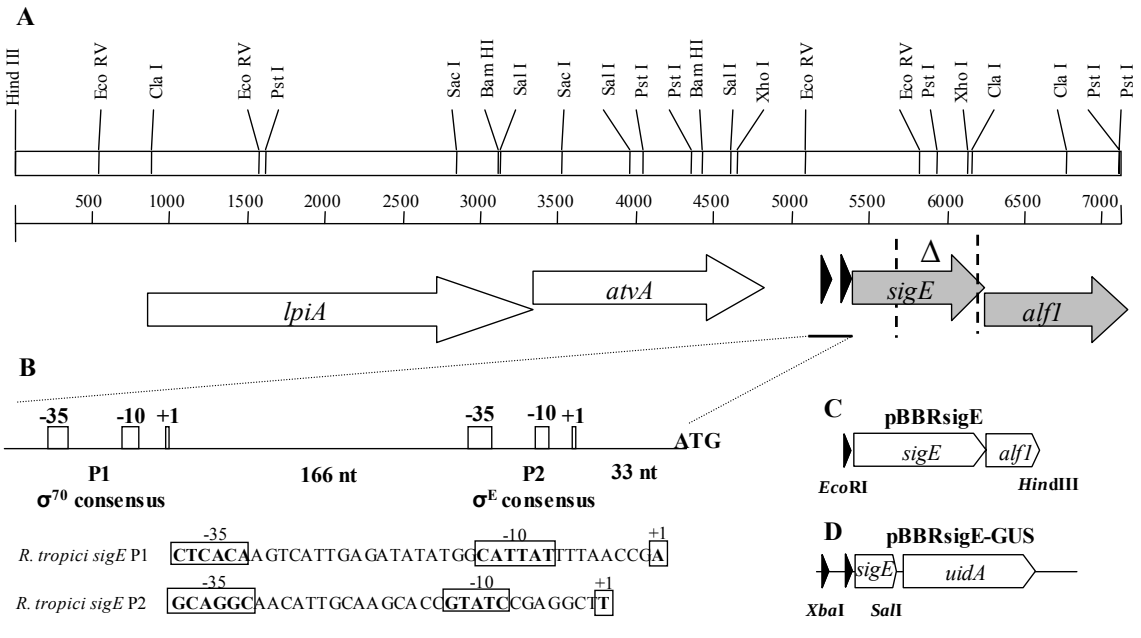
Fig. 2. Root nodules of common bean (*Phaseolus vulgaris*) infected with *Rhizobium tropici* CIAT899 parent strain (upper panel) or with a mutant, defective in a putative σ^E factor (lower panel). Intact bean nodules (A and B), cross-sections through bean nodules (C and D), and more detailed micrographs (E and F) showing the structure of *R. tropici*-infected bean nodule cells.

Fig 3. Phenotype displayed by mutant 899-Δsig1 against parent strain CIAT899 on *Phaseolus vulgaris* plants **(A)** Mean acetylene reduction of nodulated roots (21 dpi) of strain 899-Δsig1 in comparison to parent strain CIAT899, values are the mean $\pm$ SD of three repetitions in two independent experiments. (**B**) Percentage of nodules occupied by mutant 899-Δsig1 against reporter strain CIAT899-G1 in a 10:1 co-inoculation experiment.

Fig. 4. (**A**) Percentage of survival of strains CIAT899 and 899-Δsig1 after exposition to some environmental stresses for 2 hr. Values are the mean $\pm$ SD of three repetitions in two independent experiments. (**B**) Final optical density (600 nm) of strains CIAT899 and

899-Δsig1 after growing for 24hr under some environmental stresses. Values are the mean ± SD of six repetitions.

Fig. 1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σE consensus
R. tropici sigE P1 CTCACAAGTCATTGAGATATATGCCATTATTTAACCCA
R. tropici sigE P2 GCAGGCAACATTGCAAGCACCGTATCCGAGGCTT
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XbaI
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Fig. 2

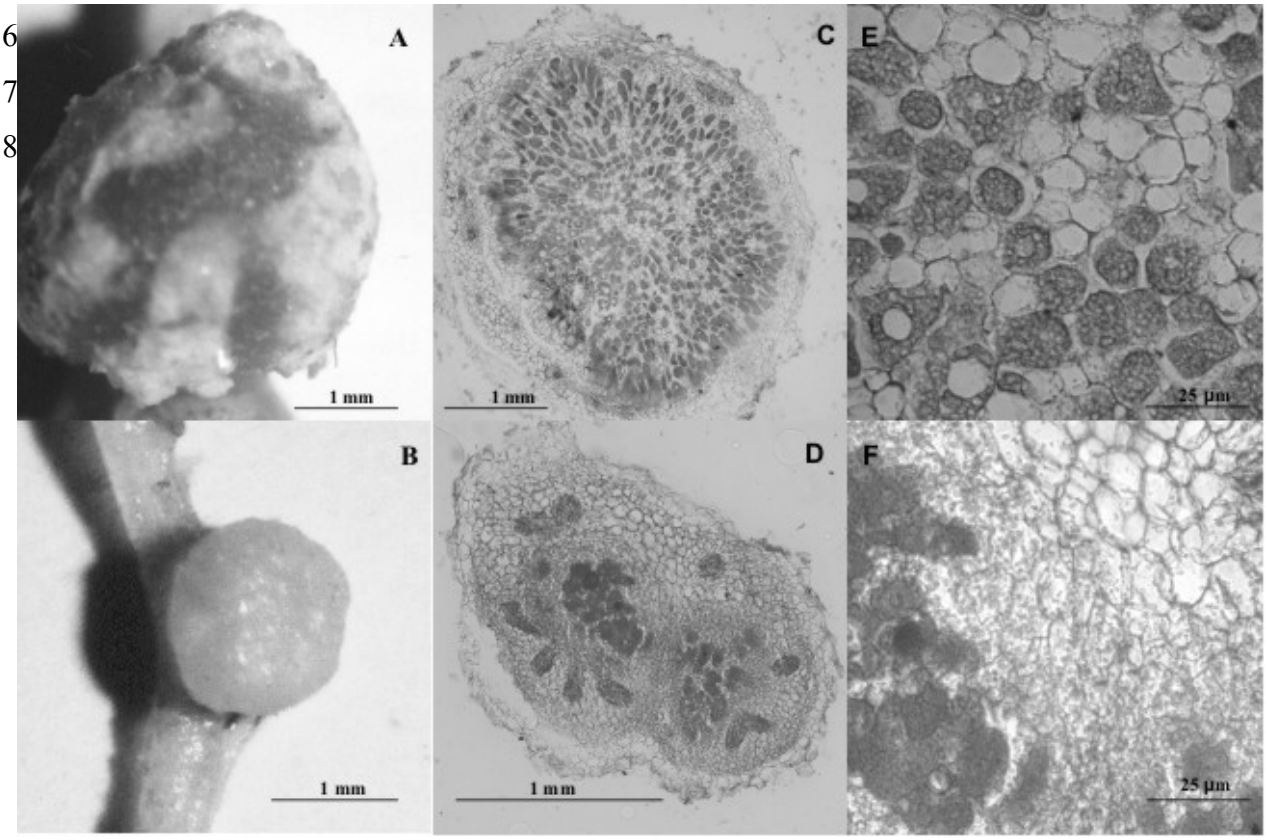
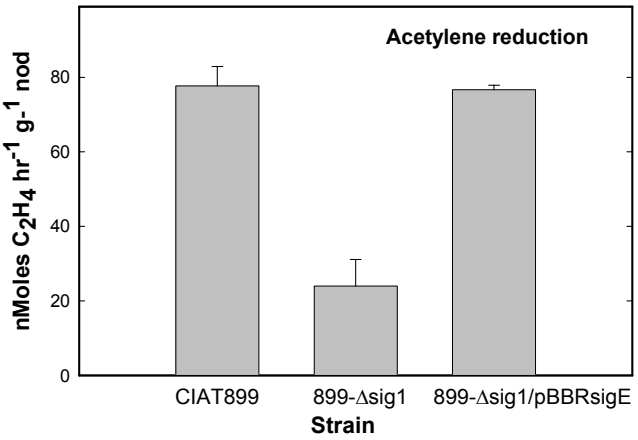


Fig. 3


Acetylene reduction
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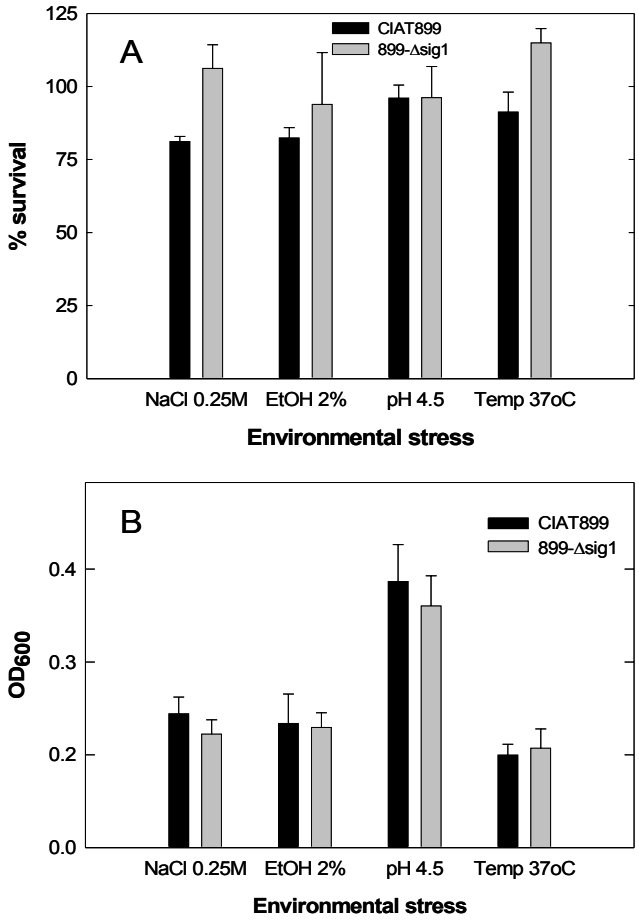
CIAT899
899-Δsig1
899-Δsig1/pBBRsigE
Strain

Fig. 4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EtOH 2%
pH 4.5
Temp 37oC
Environmental stress

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Phylogenetic Relationships of Rhizobia Based on Citrate Synthase Gene Sequences

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Summary

Partial nucleotide sequences of the citrate synthase (*gltA*) gene from different rhizobia genera were determined. Tree topologies based on this housekeeping gene were similar to that obtained using 16S rRNA sequences. However *gltA* appeared to be more reliable at determining phylogenetic relationships of closely related taxa. We propose *gltA* sequences as an additional tool to be used in molecular phylogenetic studies.

Key words: Phylogeny – *gltA* – 16S rRNA

Introduction

The members of the genus *Rhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Azorhizobium*, and *Sinorhizobium*, collectively named rhizobia, have been classified on the basis of polyphasic taxonomy [17]. This approach uses phenotypic and genotypic characteristics and includes 16S rRNA gene sequence determination. Hitherto, the analysis of 16S rRNA genes represented the most powerful method for investigating the phylogenetic relationships among microorganisms. However, this gene has limitations as a taxonomic tool such as slow evolution, genetic crossing-over and, because it is a highly conserved molecule, may not be useful for discriminating closely related bacterial species. Furthermore, the presence of a 16S rRNA gene as part of a complete ribosomal operon has been found on a plasmid in *Bacillus megaterium* [14] and allele differences have been detected in 16S rRNA genes, making these genes a not infallible guide to define evolutionary relationships [31]. Studies of additional genes are required to know more about the evolution of the genome and improve the knowledge of the phylogenetic relationships of rhizobia.

The citrate synthase gene (*gltA*) is present in almost all living cells. It contains conserved and variable regions and its product is the first enzyme in the tricarboxylic acid cycle, and is thus considered a key regulator of intracellular ATP production in both prokaryotic and eukaryotic cells. Based on these characteristics *gltA* gene sequences have been used for estimating phylogenies among some bacterial groups [1, 2, 10]. In the present work we study the usefulness of *gltA* gene sequences for determining phylogenetics relationships among rhizobia and compare it to phylogenies obtained with 16S rRNA gene sequences.

Rhizobia strains were grown in PY medium and *Escherichia coli* strains were cultivated in LB medium. Plasmid purification, genomic DNA extraction and Southern blotting were performed according to published protocols [23]. For sequencing, double stranded DNA was purified and sequencing was performed in an automatic ALF DNA sequencer (Pharmacia Biotech Uppsala) or in an automatic Perkin Elmer/Applied Biosystems 377-18 sequencer. *gltA* genes from *Agrobacterium* sp. Ch-Ag-4 (**AY094145**), *Agrobacterium* sp. K-Ag-3 (**AY094144**), *Rhizobium etli* CFN42 (**AY094142**), *Rhizobium galegae* HAMBI540 (**AY094149**), *Rhizobium* sp. CFN234 (**AY094151**), *Sinorhizobium terangae* USDA4102 (**AY094150**), *Mesorhizobium mediterraneum* USDA3392 (**AY094148**), *Mesorhizobium huakuii* CCBAU2609

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*(***AY094147**), and *Azorhizobium caulinodans* ORS571 (**AY094143**) were amplified by PCR with forward primers **P231-101** (5′AGAAGAAGGACTTCGACTA3′) or **512–MAP** (5′TACAAGTACCATATCGGCCAGCCC T3′) and reverse primer **OP85-1R** (5′CATGGTCGTGG GGAAGCC3′). They correspond respectively to base positions 299–317, 529–553, and 1123–1140 of the chromosomal *Rhizobium tropici ccsA* gene [7]. Primer pair **512-MAP** and **OP85-1R** was used to amplify 590 bp from *Azorhizobium caulinodans* ORS571 and *Mesorhizobium huakuii* USDA 4779. Primer pair **P231-101** and **OP85-1R** was used to amplify 805 bp for the remaining strains used in this study. PCR fragments were obtained using the following temperature program: initial denaturation at 93 °C for 3 min, then 34 cycles of annealing at 48 °C for 2 min, extension at 72 °C for 2 min and denaturation at 93 °C for 2 min. PCR products were purified from agarose gels with the Geneclean kit (BIO101). PCR products were cloned into the sequencing vector pMos-Blue T (Amersham Life Sciences). In this work we also included previously reported *gltA* sequences of *Rhizobium tropici* CFN299 (L41815, Z34516; [7, 20]) *Sinorhizobium fredii* (AY157738), *Sinorhizobium. meliloti* 1021 (SMc02087; [4]), *Brucella melitensis* 16M (BMEI0836; [3]), *Brucella suis* 1330 (AE014415; [21]), *Agrobacterium tumefaciens* C58 (Atu1392; [27]), *Mesorhizobium loti* MAFF303099 (m1r0629; [11]), *Bradyrhizobium japonicum* (blr4839; [12]), and *Rhodopseudomonas palustris* (BX572602; [15]). Gene accession numbers are shown in parenthesis, sequences in bold were determined in this study.

Phylogenetic analysis of *gltA* genes was performed. Partial nucleotide sequences (568 bp) of *gltA* genes were first translated into amino acid sequences, aligned using CLUSTAL W [24] and the nucleotide sequence were aligned against aligned amino acid sequences by using DAMBE [29] and optimised by hand with BIOEDIT [6]. Different analyses to assay data robustness of *gltA* sequences corroborate that our dataset showed a strong phylogenetic signal and there is no evidence of saturation. Nucleotide substitution rate over site analysis confirmed a homogeneous distribution of the variation along the sequence using all three codon positions, 1 and 2 positions and 3rd positions only. Substitution saturation test [28] showed no significant saturation ($Iss < Iss.c$, $p < 0.01$) when all three codon positions, 1 and 2 positions and 3rd positions were analysed. A plot of the number of transitions and transversion versus genetic distances confirmed this result (data not shown). Molecular phylogenies were constructed using the neighbour joining (NJ) method [22] with MEGA 2 software [13]. Statistical tests of nucleotide frequencies and transition/transversion substitution rates confirmed Tamura-Nei (TN93) as an accurate distance-correction model for our datasets. Maximum-parsimony (MP) analysis was also performed. Statistical support of branches in the phylogeny was calculated with 1000 bootstrap replications. Very similar topologies between neighbour joining and MP phylogenies were observed. Identical groups were identified particularly for those clades with a strong bootstrap support.

Analysis of GltA protein. A multiple aligment of partial GltA protein sequences (189 AA) was constructed using t_coffee [18]. We then carried out a Bayesian estimation of the phylogeny for this region with the program mrBayes 3.0b4 [9] using 250000 generations, sampling and printing frequencies of 100 and 4 chains for the

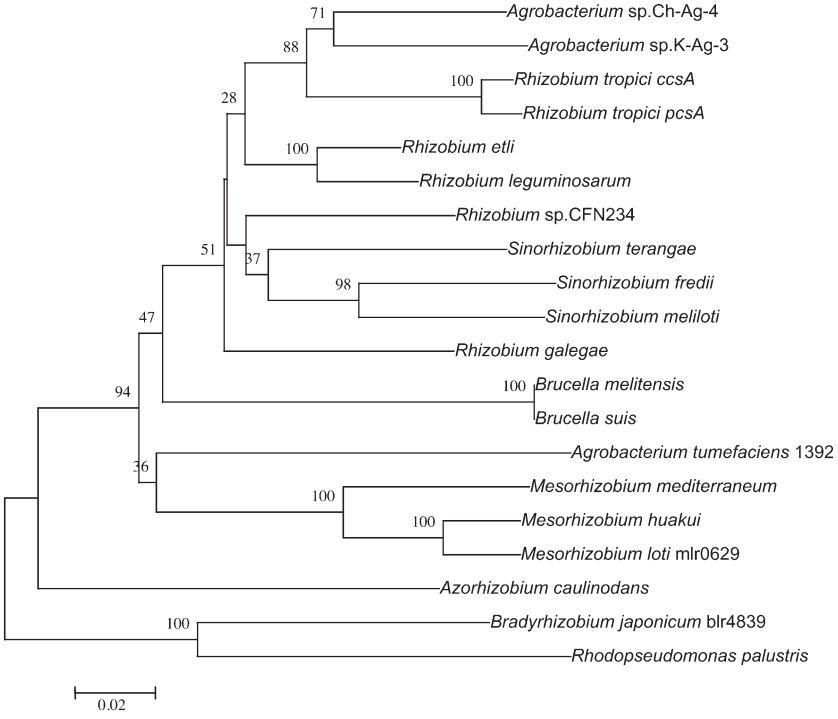

Fig. 1. Phylogenetical relationships of *Rhizobium*, *Sinorhizobium, Mesorhizobium*, *Bradyrhizobium*, *Azorhizobium*, *Agrobacterium, Brucella* and related species based on *gltA* gene sequences. Phylogenetic trees were obtained by neighbour-joining analysis. TN93 distance correction was applied. Percentage bootstrap support (1000 replicates) is indicated at branching points.

Markov Chain Monte Carlo analysis thus generating 2500 trees. As stationarity was reached at around 500 cycles, we used a burnin of 1500 trees so that only 1000 tree samples were used for the determination of a consensus tree and the determination of posterior probabilities for the trees. The consensus tree was displayed with TREEVIEW [19]. We found that topologies generated with DNA or protein showed very similar phylogenetic relationships of rhizobia.

The analysis of 590 bp of *gltA* genes from rhizobia, shows that the sequence identity of this fragment ranged from 75% to 95%, thus the sequence of *gltA* is much variable among *Rhizobium* species than are 16S rRNA genes (87% to 99%). The 590 bp analysed also showed 261 variable sites and 195 parsimony informative sites, as well as conservation of 16 NADH binding residues and 5 active site residues. Phylogenies of rhizobia based on *gltA* genes sequences (Fig. 1) showed distinct phylogenetic linages. *R. tropici* was clearly separated from *Agrobacterium* sp K-Ag-3 and Ch-Ag-4 with the identity among those strains being 90%. Previously, Willems et al showed that by 16S rRNA sequences, *R. tropici* and *Agrobacterium* spp were highly related, with an identity of 99%. In this, case *gltA* sequences appeared to be more reliable at determining relationships among *Rhizobium* and *Agrobacterium* spp than sequences of 16S rRNA. Furthermore total DNA hibridization experiments showed that *R. tropici* and *Agrobacterium* sp Ch-Ag-4 were not highly related [16], these data support the relationships based on *gltA* genes sequences. Previously we reported [8] that *R.* sp CFN234 belonged to *R. etli* linage (based on 16S rRNA gene sequences), however by phylogentic analysis of *gltA* genes, *R.* sp CFN234 is separated from *R. etli*. Our results with *gltA* support data generated with total DNA hybridisation experiments, which show a low level of homology between *R. etli* and *R.* sp CFN234 [16]. Phylogenetic trees generated with *gltA* showed a close relationships among *M. mediterraneum, M. huakui* and *M. loti*. In this cluster the *gltA* gene of *M. huakuii* is closely related to the *gltA* (mlr0629) of *M. loti* MAFF303099. This observation confirms previous data which suggest that *M. loti* MAFF303099 might belong to *M. huakuii* [25]. *R. galegae* is normally found clustered to *Agrobacterium* rather than *Rhizobium* in the 16S rRNA phylogeny, however phylogentic relationships with *gltA* genes clearly separate *R. galegae* from *A. tumefaciens*. This finding is in agreement with phylogenies obtained with *atpD* or *recA*, [5] and so the use of three molecular markers support *R. galegae* being as distant from *Agrobacterium* as it is from rhizobia. Phylogenetic relationships of *R. etli*, *R. leguminosarum*, *S. terangae*, *S. fredii*, *S. meliloti*, *B. melitensis*, *B. suis*, *B. japonicum*, *A. caulinodans* and *R. palustis* derived from *gltA* sequences are congruent to those obtained with 16S rRNA genes sequences.

In previous studies we reported the presence of two *gltA* genes in *Rhizobium tropici* [7]. To determine if the presence of multiples copies is common in rhizobia, hybridisation experiments were performed. Total DNA of the *Rhizobium* strain analysed were purified and digested with two different restriction enzymes. Our results showed that only *Rhizobium tropici* contain two *gltA* genes, therefore the presence of multiples *gltA* genes is not general in rhizobia. *gltA* gene sequences have been used in several bacterial groups for taxonomy. In *Bartonella,* phylogenetic trees reflect a much higher sequence diversity in *gltA* genes than that observed for the 16S rRNA genes [2]. In *Ehrlichia* the architecture of the trees constructed with *gltA* was similar to that derived from the 16S rRNA gene sequences but showed more significant bootstrap values [10]. In the case of rhizobia, we found that *gltA* is useful for estimating evolutionary relationships of closely related taxa, We propose *gltA* sequences as an additional tool for molecular phylogenetic studies.

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