A ClC Chloride Channel Homolog and Ornithine-Containing Membrane Lipids of *Rhizobium tropici* CIAT899 Are Involved in Symbiotic Efficiency and Acid Tolerance

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Rhizobium tropici* CIAT899 is highly tolerant to several environmental stresses and is a good competitor for nodule occupancy of common bean plants in acid soils. Random transposon mutagenesis was performed to identify 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 *sycA*, a homolog of the ClC family of chloride channels and Cl⁻/H⁺ exchange transporters. A nonpolar deletion in this gene caused serious deficiencies in nodule development, nodulation competitiveness, and N₂ 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 *sycA,* is homologous to aspartyl/asparaginyl β-hydroxylases and modifies two species of ornithine-containing lipids in vivo, presumably by hydroxylation at a still-unknown position. A mutant carrying a nonpolar deletion in *olsC* is symbiotically defective, whereas overexpressed OlsC in the complemented strain provokes an acid-sensitive phenotype. This is the first report of a ClC homolog being essential for the establishment of a fully developed N₂-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.

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; Hungria and Vargas 2000; Zahran 1999). It causes nitrogen deficiency in legumes as it inhibits rhizobial growth, root infection, and bacteroid activity (Glenn et al. 1999; Munns et al. 1981). In the rhizosphere, plants secrete H^+ and organic acids that acidify the soil up to

two 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; Walker et al. 2003). 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, 1994). However, the genetic and physiological bases of this acid tolerance are still not clear. Two mechanisms related to the acid-tolerance response have been identified in rhizobia and enterobacteria (Foster 1999; Merrell and Camilli 2002; O'Hara and Glenn 1994;). 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 *Escherichia 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 (Accardi and Miller 2004; Chen 2005; Iyer et al. 2002;). 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 (Graham 1992; Martínez-Romero et al. 1991). Thus, *R. tropici* CIAT899 represents a good model to search for genes involved in acid toler-

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ance 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 Tn5 and selection of mutants on acidified media was used to characterize acid-sensitive mutants in *R. 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*, and *phrR* are essential for growth at low pH (Glenn et al. 1999; Riccillo et al. 2000). More recently, Vinuesa and associates (2003) reported the isolation of additional Tn5-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 Tn5 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 Tn5 insertion in strain 899-PV9, which bears significant sequence homology with the ubiquitous superfamily of ClC-Cl$^-$ channel proteins and Cl$^-$/H$^+$ antiporters (Accardi and Miller 2004; Chen 2005), and of a gene located downstream that 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 *R. tropici* CIAT899, obtained by random transposon mutagenesis, was designated 899-PV9. When mutant 899-PV9 was inoculated on *P. vulgaris* plants, nodules were not fully developed, were 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 Tn5 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 Tn5 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 Tn5 insertion sequence elements.

Several symbiotically proficient transconjugants were recovered after complementation of the mutant strain 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, was transferred to a nylon membrane, and was hybridized with digoxigenin (DIG)-labeled p899PV9-PCR. The resulting hybridizing fragment (approximately 6.2 kb) was subcloned into pSK, yielding pPV9EG-SK, and was subjected to DNA sequencing.

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

Using different computer programs, an analysis of a 3,761-bp *Eco*RI-*Cla*I sequence complementing mutant PV9-899 (accession number AY954450) revealed four open reading frames (ORF) with high coding probability, as predicted by FrameD (Fig. 1A). ORF 1 through 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. The genetic analysis presented herein targeted *orf2* and *orf3*, which are the ORFs that could be affected by the Tn5 insertion in mutant 899-PV9 (Fig. 1).

In frame +2, at position 785, a 1,368-bp ORF (*orf2*) begins that is predicted to encode a 48.6-kDa product that, according to homology searches with the BLASTP program, showed highly significant sequence similarity to ClC chloride channel proteins from diverse β-proteobacteria *Burkholderia cepacia* (E value e^{-104}, 49% identity, 64% similarity), *Ralstonia solanacearum* (E value e^{-103}, 49% identity, 64% similarity), and the γ-proteobacteria *Pseudomonas syringae* (E value e^{-103}, 45% identity, 62% similarity), *Salmonella typhimurium* (E value 2e^{-55}, 27% identity, 42% similarity), and *E. coli* (E value 4e^{-45}, 20% identity, 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 homolog is predicted to have 10 transmembrane helices, with both N- and C-terminal domains residing in the cytoplasm and containing the sequence motifs corresponding to the ion-binding site and gating region of the solved *E. coli* and *Salmonella typhimurium* ClC protein structures (Fig. 1B, part 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, parts I through III), including the *R. tropici* residue E129, which, in the homologous ClC chloride channels from *E. coli* and *S. typhymurium*, is the one responsible for gating, according to X-ray crystallographic studies (Dutzler 2004; Dutzler et al. 2002). Therefore, based on the highly significant sequence similarity of *orf2* to ClC chloride channels and antiporters, we named the former locus *sycA*, for symbiosis-assisting ClC homolog. The intergenic spacer (IGS) between *orf1* and *orf2* was analyzed using the neural network for promoter prediction (NNPP) server, which located a putative promoter sequence upstream of the sycA start codon (sites 727 to 772, r = 0.81).

A 845-bp-long ORF (*orf3*) starts in frame +1 at position 2,611 and is predicted to encode a 31.78-kDa product, with highly significant sequence similarity to a putative aspartyl/asparaginyl β-hydroxylase from a *Mesorhizobium* sp. (E value 9e^{-90}, 64% identity, 76% similarity) and *Azotobacter vinelandii* (E value 4e^{-63}, 49% identity, 65% similarity) and a lower similarity to lipid A-myristate β-hydroxylase (LpxO) from *Salmonella typhimurium* (E value 5e^{-23}, 35% identity, 51% similarity). LpxO 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 *Salmonella typhimurium*. This inference suggests that LpxO and Orf3 might have slightly different functions or substrate specificities,

although they clearly belong to the same protein superfamily (COG3555). The IGS between *sycA* and *orf3* was analyzed using the NNPP server, which predicted two possible promoter sequences upstream of *orf3* (sites 2,306 to 2,351, $r = 0.98$; 2,419 to 2,464, $r = 0.94$). This promoter prediction is consistent with a monocistronic organization of the transcript encoded by *sycA*, which is also supported by reverse transcription-polymerase chain reaction (RT-PCR) experiments, complementation analyses, and the phenotype observed by two-dimensional thin-layer chromatography (2D-TLC) analyses of radiolabeled lipid extracts (discussed below). Based on the evidence gained from the latter experiments, we named *orf3 olsC*, for ornithine lipid synthesis gene C.

RT-PCR of the IGS between *sycA* and *olsC* confirms that both genes are independently transcribed.

An RT-PCR experiment was designed to determine the transcriptional organization of *sycA* and *olsC* (Fig. 1D). For this purpose, RNA was isolated from *R. tropici* CIAT899 cells grown in PY broth (Noel et al. 1984) to the early stationary phase and was 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 ORF are independently transcribed under these conditions and making it unlikely that the Tn*5* insertion
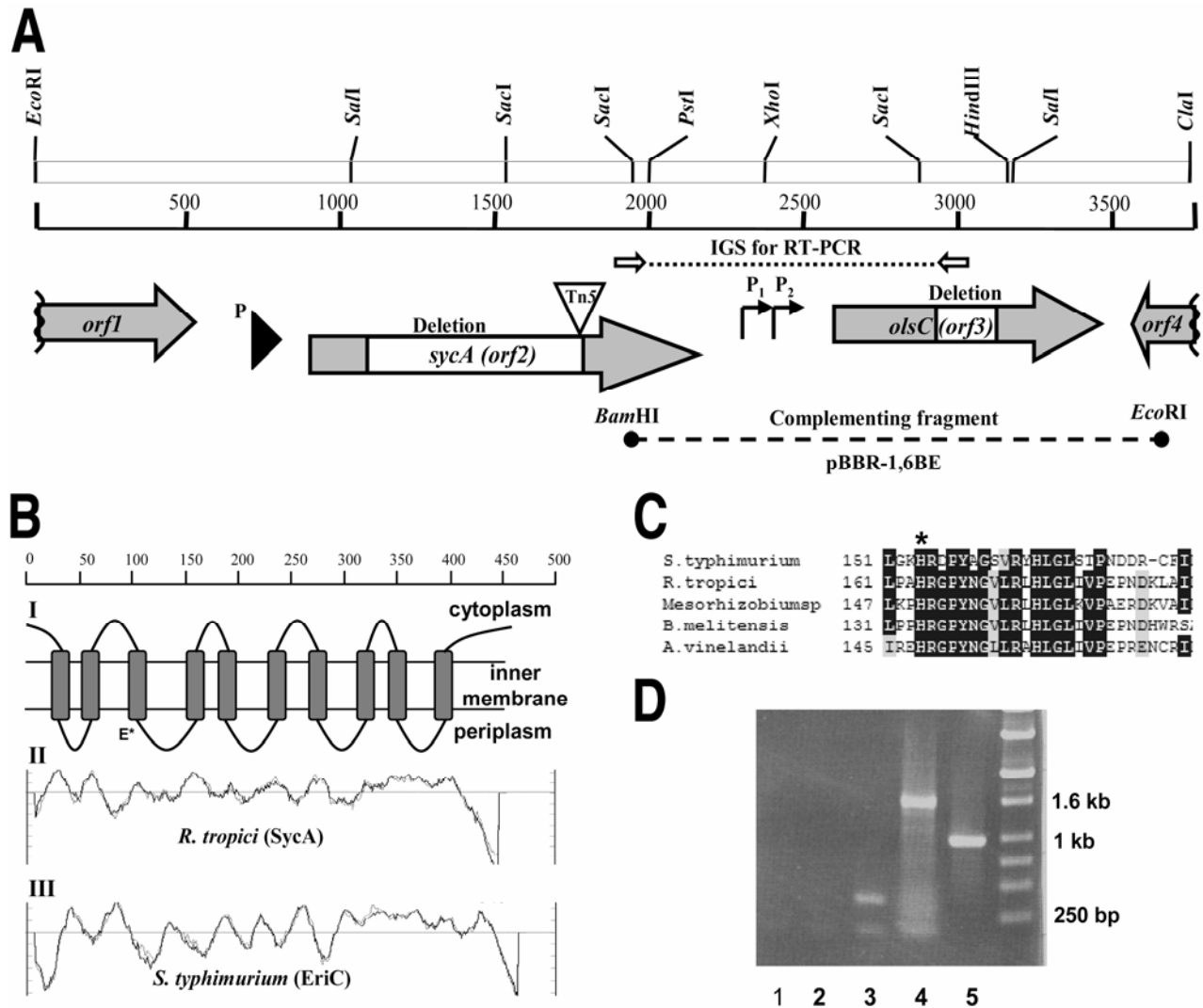

Fig. 1. A, Genetic and physical maps of the 3,761-bp *Eco*RI-*Cla*I region from *Rhizobium tropici* CIAT899 analyzed in this study (accession number AY954450). **A,** Selected restriction sites. Four open reading frames (ORF) (represented by arrows) were detected. The site of the Tn*5* insertion located in *sycA* between nucleotides C1763 and T1764 is indicated by an open triangle. Nonpolar deletion mutants lacking the regions shown in white were generated in *sycA* and *olsC*. Predicted promoters are shown as thin arrows. The dotted line represents the intergenic spacer between *sycA* and *olsC* subjected to reverse transcriptase-polymerase chain reaction (RT-PCR) analysis (shown in D). The dashed line shows the location of the 1.66-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 SycA (panel I); the conserved residue E129 (marked with asterisk) is located in the gating region of EriC (Dutzler et al. 2003). Panels II and III show hydrophobicity plots generated by TmPred for the *R. tropici* CIAT899 ClC homolog *sycA* (II) compared with that for the ClC exchange transporter (EriC) from *Salmonella typhimurium* (III). **C,** Partial sequence alignment of *R. tropici, Mesorhizobium* sp. (ZP 00193099), *Brucella melitensis* (NP 539381), *Azotobacter vinelandii* (ZP 00090437) putative aspartyl/asparaginyl β-hydroxylase sequences, and LpxO (Fe^{2+}/α-ketoglutarate-dependent dioxygenase) from *Salmonella 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 gray. **D,** The RT-PCR experiment shows no PCR amplification of the intergenic spacer (IGS) between *sycA* and *olsC* (lane 1), which suggests that both ORF are independently transcribed under these conditions. No amplification in the negative control (lane 2). 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).

in *sycA* 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 excludes the possibility of contamination by *R. tropici* genomic DNA.
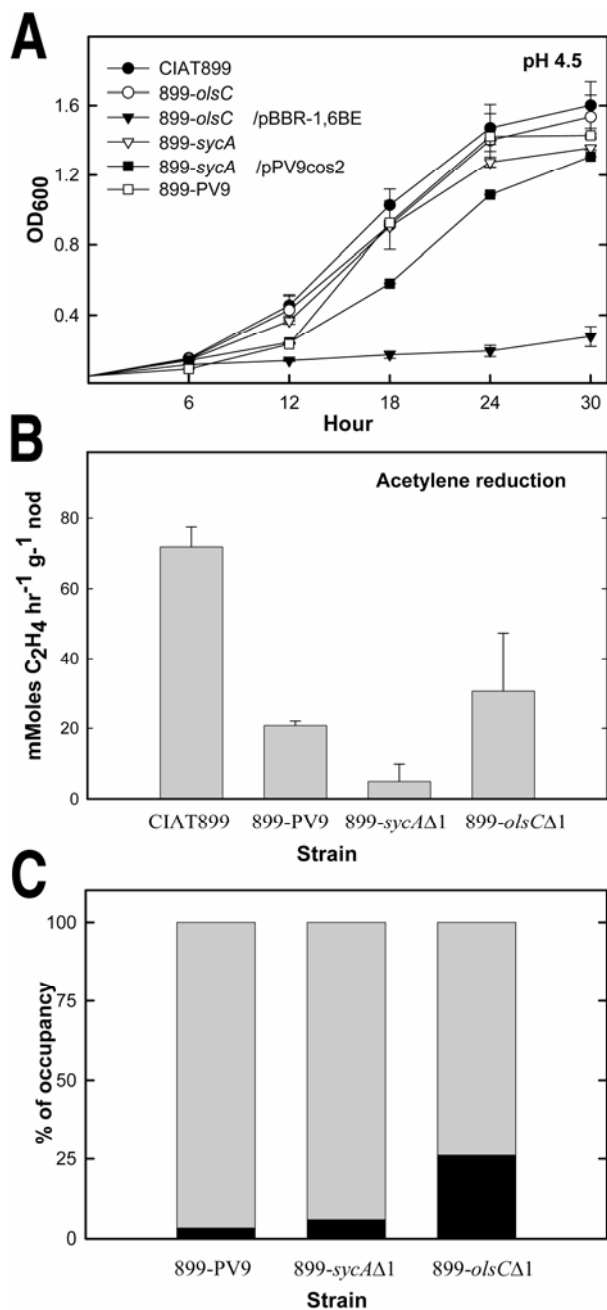

Fig. 2. Phenotypes displayed by several *Rhizobium tropici* strains used in this study. **A**, growth of *R. tropici* strains on PY media at pH 4.5. Values are the mean ± SD of four independent experiments. **B**, Mean acetylene reduction of nodulated roots inoculated with strains 899-PV9, 899-*sycA*Δ1, 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 coinoculation experiment (in excess of mutants), using low-inoculum titers (approximately 500 CFU per plantlet).

Construction of nonpolar deletions in *sycA* and *olsC* and phenotypic characterization of the mutant strains.

We generated a nonpolar mutation in *sycA* by deletion of a 991-bp 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-*sycA*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 (21 days postinoculation [dpi]), lacked lenticels, were whitish (Fig. 3A and B) and presented a 14-fold decrease in nitrogen fixation in comparison with the parent strain, as revealed by the acetylene reduction assay (Fig. 2B). The nodulation competitiveness of 899-*sycA*Δ1 against the *gusA*-tagged reporter strain CIAT899-G1 in a 10:1 coinoculation experiment (in favor of the mutant) 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 were unable to form stable symbiosomes. TEM micrographs of nodules induced by 899-*sycA*Δ1 showed poor invasion of plant cells, accumulation of polyhydroxybutyrate granules within
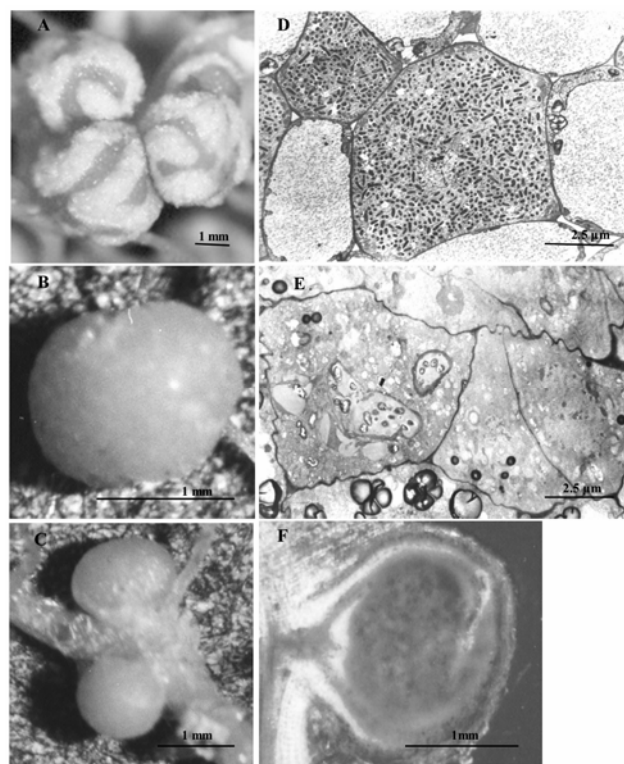

Fig. 3. Macroscopic aspect of nodules induced by strains **A,** CIAT899, **B,** mutant 899-*sycA*Δ1, and **C,** mutant 899-*olsC*Δ1 on *Phaseolus vulgaris* plants (21 days postinoculation). Electron micrographs reveal drastic differences in the levels of invasion presented by **D,** parent strain and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

bacteroids, and presence of amyloplasts in the host cells, whereas the parent strain was able to fully invade plant cells (Fig. 3D and E). The symbiotic proficiency of mutant 899-*sycA*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 *sycA*. 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 (Ndv⁻) and lacked lenticels (Fig. 3C). These nodules also showed reduced levels of nitrogen fixation (about 50%), when compared with the wild-type strain CIAT899, as determined by acetylene reduction assays (Fig. 2B). When 899-*olsC*Δ1 was coinocu-

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[a]

Lipid	Composition (%total ^{14}C)			
	CIAT899	899-*olsC*Δ1	899-*olsC*Δ1/pBBR-1,6BE	899-*olsC*Δ1/899-*olsC*Δ1/pBBR-MCS5
PC	40.3 ± 4.7	47.8 ± 6.0	32.5 ± 1.5	37.4 ± 5.5
Phosphatidylglycerol (PG)	11.9 ± 2.1	10.7 ± 1.9	12.7 ± 1.3	11.2 ± 0.2
Cardiolipin (CL)	2.9 ± 2.0	2.6 ± 0.8	2.9 ± 0.3	3.2 ± 1.4
Phosphatidylethanolamine (PE)	23.4 ± 3.8	16.3 ± 4.9	33.4 ± 0.4	26.2 ± 1.7
S1[b]	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.

[a] Values are the mean ± standard deviations of three independent experiments. n.d. = not detected.
[b] S1, S2, P1, and P2 denote four additional components detected in the chromatogram of *R. tropici* CIAT899.
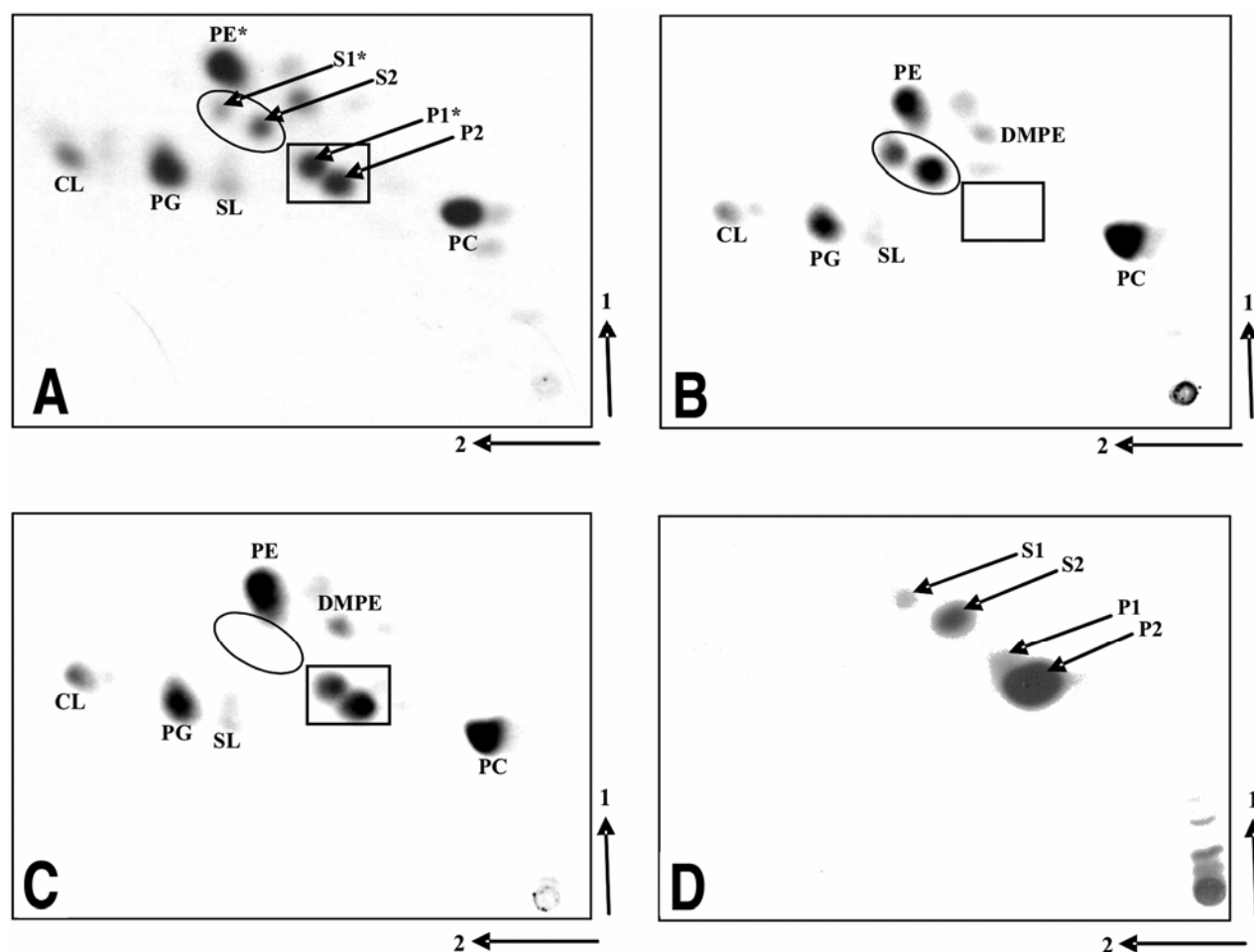

Fig. 4. Membrane lipid analysis of *Rhizobium tropici* strains. Separation of [14C]acetate-labeled lipids from **A,** *R. tropici* CIAT899, **B,** mutant 899-*olsC*Δ1, and **C,** complemented mutant 899-*olsC*Δ1/pBBR-1,6BE as well as of **D,** [14C]ornithine-labeled lipids from *R. tropici* CIAT899, using two-dimensional thin-layer chromatography. The lipids cardiolipin (CL), phosphatidylglycerol (PG), sulphoquinovosyl diacylglycerol (SL), phosphatidylethanolamine (PE), dimethylphosphatidylethanolamine (DMPE), and phosphatidylcholine (PC) are indicated. In A, B, and C, 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) that are enclosed in rectangular boxes. Asterisks indicate ninhydrin-positive lipids.

lated 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 those achieved by the parent strain CIAT899, although not as drastic as observed for mutant 899-*sycA*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 1,660-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 to 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 overexpression (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 *R. 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 2D-TLC, and individual lipids were quantified (Table 1). Rhizobial membrane phospholipids like phosphatidylethanolamine (PE), dimethylphosphatidylethanolamine, cardiolipin, phosphatidylglycerol, sulphoquinovosyl diacylglycerol, and phosphatidylcholine (PC) were identified based on their relative mobilities and in comparison to the well-characterized lipid profile of *S. meliloti* 1021 (Gao et al. 2004; Weissenmayer et al. 2002). As found for *S. meliloti* 1021, PC constitutes 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*

Table 2. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Source or reference
Rhizobium tropici strains		
CIAT899	Acid tolerant, Ap^r, Nal^r	Martínez-Romero et al. 1991
899-PV9	CIAT899 derivative (*sycA::*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-*sycA*Δ1	CIAT899 carrying a 991 bp non-polar deletion in *sycA*	This study
899-*sycA*Δ1/pPV9cos2	899-*sycA*Δ1 carrying the complementing cosmid of 899-PV9, Tc^r	This study
899-*olsC*Δ1	CIAT899 carrying a 211-bp nonpolar 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
Escherichia coli strains		
DH5α	*recA*1, Δ*lacU*169, Φ80dlacZΔM1	Stratagene, La Jolla, CA, U.S.A.
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, Carlsbad, CA, U.S.A.
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	12-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
P859PV9E6-SK	pSK construct containing the 6.2-kb *Eco*RV fragment hit by the Tn*5* insertion in 899-PV9	This study
pPV9cos2	Cosmid complementing 899-PV9, Tc^r	This study
pKRΔ02	Integrative mutagenizing plasmid based on pK18*mobsacB* used to construct strain 899-*sycA*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

[a] Ap, Nal, Sm, Km. Sp, Tc, and Tp = ampicillin, nalidixic acid, streptomycin, kanamycin, spectinomycin, tetracycline, and trimethoprim.

(Gao et al. 2004; Weissenmayer et al. 2002). 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-*sycA*Δ1, both defective in the predicted chloride channel protein, were indistinguishable from that of the wild type (data not shown). 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 suggest that OlsC is required for the formation of lipids P1 and P2.

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 distinct classes of OL of *R. tropici* CIAT899.

DISCUSSION

In this work, we report two novel genes of *Rhizobium tropici* CIAT899 that 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-*sycA*Δ1, which carries a deletion in the putative chloride channel encoded by *sycA*, 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 homolog 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 *E. coli*, the molecular and physiological functions of dozens of prokaryotic ClC homologs 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 as 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 double 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, *Brucella melitensis* 16M, *Bradyrhizobium japonicum* USDA110, and *Mesorhizobium loti* MAFF303099 but not *S. 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 (*sycA*)

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 *sycA* have a direct or indirect effect on the symbiotic phenotype observed in strains 899-PV9 and 899-*sycA*Δ1. At this point and based on the evidence gained from TEM data, we cannot state whether 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 *R. leguminosarum* mutants blocked in amino acid transporters, present poorly developed nodules and 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-*sycA*Δ1 displayed a similar phenotype to that observed for the *R. leguminosarum* mutant, it is tempting to speculate that SycA 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 bacteroids 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.

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 *S. 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 to 60% of the lipids in membranes of *S. meliloti* or *Bradyrhizobium japonicum* and OL 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.

R. tropici CIAT899 produces four different classes of OL (S1, S2, P1, and P2). 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-TLC, we expect that S1 presents a similar or identical structure as sinorhizobial OL. In contrast, the S2, P1, and P2 classes of OL, 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.

Mutant 899-*olsC*Δ1 forms more S1 and S2 than wild type and lacks P1 and P2, whereas the complemented mutant 899-*olsC*Δ1/pBBR-1,6BE presented mainly P1 and P2 and a near-complete lack of S1 and S2. These data show that OlsC is required for the formation of P1 and P2 and suggest that OlsC might consume S1 and S2. This is 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 seem to function as in vivo substrates for the OlsC reaction, one might expect that S1 and S2 would have similar chemical structures and that, therefore, S2 might be a modified version of the OL known to date. The nonreactivity 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 might be both products of an 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 near-complete lack of S1 and S2 in the case of 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.

Mutant 899-*olsC*Δ1 is acid-tolerant but symbiotically defective. In contrast, the complemented mutant 899-*olsC*Δ1/pBBR-1,6BE was able to restore the symbiotic proficiency but was acid-sensitive. The latter suggests 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 OL 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 *R. 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 OL and that the product encoded by *olsC* is necessary to convert lipids S1 and S2 to P1 and P2, presumably by hydroxylation at a still-unknown position. Lipids P1 and P2, which presumably carry a hydroxyl group at a still-unkown position, are required for an effective symbiotic interaction with bean plants, while a lack of lipids S1 and S2 was correlated with acid sensitivity. 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). Although the molecular functions of OL modifications in *R. tropici* are not understood at this point, 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 (MM) (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, Cleveland, OH, U.S.A.). *E. 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, 100 μg/ml; streptomycin, 150 μg/ml; ampicillin (Ap), 50 μg/ml; tetracycline, 7.5 μg/ml; gentamicin (Gm), 10 μg/ml; nalidixic acid (Nal), 20 μg/ml.

Random transposon mutagenesis of *R. 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 and tissues DNA isolation kit (Amersham Biosciences, Buckinghamshire, U.K.), following the manufacturer's instructions. Plasmid DNA from *E. coli* cultures was isolated with the High pure plasmid isolation kit (Roche, Mannheim, Germany). Restriction endonucleases were purchased from New England Biolabs (Beverly, MN, U.S.A.) and were used according to standard procedures (Sambrook et al. 1989). PCR amplifications were carried out in a Gene Amp PCR system 2700 (Applied Biosystems, Foster City, CA, U.S.A.), 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, was transferred to a nylon membrane, and was hybridized with a DIG-labeled probe generated by the incorporation of DIG-UTP (Roche) into the *nptII* marker of Tn*5* via PCR, as previously described (Vinuesa et al. 2003). The single hybridizing fragment (approximately 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* insertion elements. The amplification product was cloned into pSK, yielding p899PV9E-PCR, and was 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 Prism 3700 automated sequencer, using the universal M13f and M13r primers (Applied Biosystems). PCR primers were subsequently designed to obtain an approximately 8× coverage of overlapping plasmid subclones and PCR products from which a contig was assembled using SeqManII from the DNASTAR package (Lasergene, Madison, WI, U.S.A.).

DNA sequence analyses.

ORF with high coding probability were identified on the contig sequence using FrameD, with the *S. meliloti* codon usage table and the pentanucleotide aagga as ribosomal binding site. The BLASTX and BLASTP programs from the nonredundant sequence databases at the National Center for Biotechnology Information were used to search for homology. 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.

Determination of operon structure by RT-PCR.

RNA from *R. 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, Hilden, Germany). This cDNA was used as template for PCR amplification of the IGS between *sycA* and *olsC*, using primers PV9-2000f (5′gcagcggc cataccagcatc) 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 nonpolar mutants 899-sycAΔ1 and 899-*olsC*Δ1.

To construct a nonpolar mutant in *sycA*, PCR amplification of two nonoverlapping approximately 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) and PV9-971r-B (5′cggatccggcaacgggcataagaaag) and PV9-1962f-B (5′ccggatcctcgctgtcgcgtgctt) and PV9-2272r-E (5'ccgaattcct gccatcggagcgtc) 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′ccaagcttcctcccg gaccgcac) and PV9-2960r-B (5′ccggatccagcgggtgtcggtgg), and PV9-3171f-B (5′cggatccgcgtcgacaatcacg) and PV9-3622r-E (5′cgcgaattcggtggcggcatgacg). The PCR products were digested with *Hin*dIII plus *Bam*HI and *Bam*HI plus *Eco*RI, respectively, and were ligated to *Hin*dIII plus *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 nonpolar mutants, hereafter named 899-sycAΔ1 and 899-*olsC*Δ1, were obtained.

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

A 1,660-bp *Eco*RI-*Bam*HI fragment was amplified with primers PV9-1962f-B and PV9-3622r-E and was cloned into pBBR-MCS5 (Gmʳ), 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 an additional 534 bp upstream of the predicted start codon with the region carrying the putative promoter sequence predicted by the NNPP server (Fig. 1). This fragment was cloned in the 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, Ap, and Nal. 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.

P. vulgaris seeds were surface-sterilized with 1.2% sodium hypochlorite and were 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 were inoculated with about 10^5 CFU per plant. Plants were grown in a controlled growth chamber and harvested 21 dpi. Nitrogenase activity of nodulated roots was determined by acetylene reduction assay. Competition experiments were performed by coinoculating the mutant strain together with a *gusA*-tagged reported strain, CIAT899-G1, at a low-inoculum titer (approximately 500 cells per plantlet) to minimize nodule coinfections, as previously described (Vinuesa et al. 2003). Plants were harvested 21 dpi, and blue nodules were counted after β-glucuronidase 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 MM for 48 h. Cultures (2 ml) were inoculated from precultures to an initial optical density at 600 nm 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°C with appropriate shaking. At the end of the growth period, cells were harvested by centrifugation and resuspended in 100 μl of water, and lipid extracts were obtained according to Bligh and Dyer (1959). Aliquots of the lipid extracts were spotted on high-performance TLC silica gel 60 (Merck, Poole, U.K.) plates and were separated in two dimensions using chloroform/methanol/water (140:60:10, vol/vol/vol) as a mobile phase for the first dimension and chloroform/methanol/acetic acid (130:50:20, vol/vol/vol) 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°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).

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AUTHOR-RECOMMENDED INTERNET RESOURCES

Genopole's FrameD gene finder program:
genopole.toulouse.inra.fr/bioinfo/FrameD/FD

The Berkeley Drosophila Genome Project NNPP server:
www.fruitfly.org/seq_tools/promoter.html