Molecular Microbiology (2011) 79(6), 1496–1514 ■
doi:10.1111/j.1365-2958.2011.07535.x
First published online 23 January 2011

Hydroxylated ornithine lipids increase stress tolerance in *Rhizobium tropici* CIAT899

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Summary

Ornithine lipids (OLs) are widespread among Gram-negative bacteria. Their basic structure consists of a 3-hydroxy fatty acyl group attached in amide linkage to the α-amino group of ornithine and a second fatty acyl group ester-linked to the 3-hydroxy position of the first fatty acid. OLs can be hydroxylated within the secondary fatty acyl moiety and this modification has been related to increased stress tolerance. *Rhizobium tropici*, a nodule-forming α-proteobacterium known for its stress tolerance, forms four different OLs. Studies of the function of these OLs have been hampered due to lack of knowledge about their biosynthesis. Here we describe that OL biosynthesis increases under acid stress and that OLs are enriched in the outer membrane. Using a functional expression screen, the OL hydroxylase OlsE was identified, which in combination with the OL hydroxylase OlsC is responsible for the synthesis of modified OLs in *R. tropici*. Unlike described OL hydroxylations, the OlsE-catalysed hydroxylation occurs within the ornithine moiety. Mutants deficient in OlsE or OlsC and double mutants deficient in OlsC/OlsE were characterized. *R. tropici* mutants deficient in OlsC-mediated OL hydroxylation are more susceptible to acid and temperature stress. All three mutants lacking OL hydroxylases are affected during symbiosis.

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Introduction

Membranes of the Gram-negative model organism *Escherichia coli* only contain three major phospholipids, that is phosphatidylethanolamine, phosphatidylglycerol and cardiolipin (Heath *et al*., 2002). Some other bacteria also form the membrane lipids phosphatidylinositol or phosphatidylcholine (Jackson *et al*., 2000; Sohlenkamp *et al*., 2003). In addition to phospholipids, many bacteria also present phosphorus-free membrane lipids such as ornithine lipids (OLs), diacylglyceryl-*N*, *N*, *N*-trimethylhomoserine (DGTS) or sulpholipids (SLs) in their membranes (López-Lara *et al*., 2003; Geiger *et al*., 2010). In some cases, like for example *Rhodobacter sphaeroides* or *Sinorhizobium meliloti*, the formation of these phosphorus-free membrane lipids is induced by phosphate-limiting growth conditions (Benning *et al*., 1995; Geiger *et al*., 1999). Some bacteria such as *Brucella abortus* (Comerci *et al*., 2006; Bukata *et al*., 2008) or *Rhizobium tropici* (Rojas-Jiménez *et al*., 2005; Sohlenkamp *et al*., 2007) also form significant amounts of OLs during growth in standard laboratory media such as LB which contain phosphate in concentrations that are not growth-limiting.

Ornithine lipids are widespread among Gram-negative bacteria and have also been reported in some Gram-positive bacteria, like *Mycobacterium* and *Streptomyces* species, but seem to be absent from Archaea and Eukarya (López-Lara *et al*., 2003; Geiger *et al*., 2010). OLs contain a 3-hydroxy fatty acyl group that is attached in amide linkage to the α-amino group of ornithine. A second fatty acyl group is ester-linked to the 3-hydroxy position of the first fatty acid. It has been reported that in some bacteria the ester-linked fatty acid is hydroxylated at the 2 or 3 position (Asselineau, 1991). The genes *olsB* and *olsA* encoding the two enzymes essential for OL biosynthesis from ornithine and acyl-ACPs have been first described in *S. meliloti* (Weissenmayer *et al*., 2002; Gao *et al*., 2004). Although OLs are probably found in both membranes of Gram-negative bacteria, they seem to be enriched in the outer membrane (OM) as was shown in the acid-resistant species *Thiobacillus thiooxidans* (Dees and Shively, 1982). Therefore, Dees and Shively speculated about a role of OLs in acid resistance (Dees and Shively, 1982).

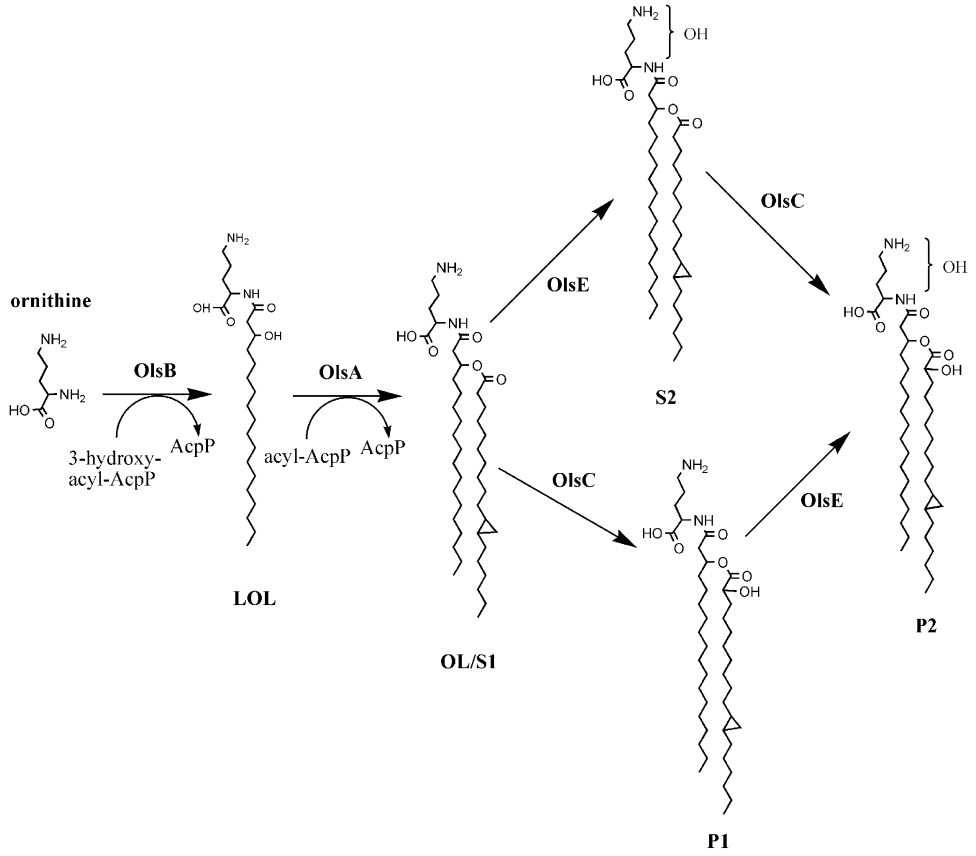

Fig. 1. Biosynthesis of ornithine lipids in *Rhizobium tropici* CIAT899. The genes coding for OlsB and OlsA have been first identified in *Sinorhizobium meliloti*, whereas the gene encoding the OL hydroxylase OlsC has been described first in *R. tropici*. Here we describe that the hydroxylation introduced by OlsC is in the 2 position of the secondary fatty acid. We also describe the identification of the gene encoding the OL hydroxylase OlsE introducing a hydroxyl group in the ornithine moiety of OL. Lyso-ornithine lipid (LOL), ornithine lipid (OL).

Rhizobium tropici CIAT899 is highly tolerant to many environmental stresses such as acidity or high temperatures. It can grow on acidified media down to pH 4.0, and it is a good competitor for nodule occupancy in *Phaseolus vulgaris* (common bean) and other hosts under acidic conditions (Martínez-Romero *et al*., 1991). A gene responsible for the hydroxylation of OL has been isolated in *R. tropici* using a transposon mutagenesis approach looking for mutants affected in their capacity to grow at pH 4.5 (Vinuesa *et al*., 2003; Rojas-Jiménez *et al*., 2005). Rojas-Jiménez *et al*. described the presence of four different species of OL in *R. tropici* membranes which were called S1, S2, P1 and P2. They showed that the putative hydroxylase OlsC is responsible for the formation of P1 and P2, presumably from OLs S1 and S2 functioning as substrates (Fig. 1), but they did not investigate on the position of the OlsC-dependent hydroxylation. No acid growth phenotype was observed for the *olsC*-deficient mutant, but constitutive expression of *olsC* was associated with the inability of the strain to grow at pH 4.5. Upon inoculation of the *olsC* mutant onto bean plants only poorly developed nodules were observed (Ndv^-) 21 days after inoculation of the plants (Rojas-Jiménez *et al*., 2005). In an earlier study, Taylor *et al*. (1998) had observed an increased formation of hydroxylated OLs at an elevated temperature in *Burkholderia cepacia*. These two previous results indicated a role of modified OLs in stress tolerance, and prompted us to investigate the synthesis of modified OLs and their role in stress tolerance in *R. tropici* in more detail. In this study we describe the isolation of the OL hydroxylase OlsE and the construction of *R. tropici* mutants deficient in the hydroxylation of OLs. We show that OlsC is introducing a hydroxyl group in the 2 position of the secondary fatty acid of OLs and that OlsE introduces a hydroxylation in the ornithine moiety of OLs. The characterization of these mutants shows that hydroxylated OLs are important for adaptation to stress conditions in *R. tropici*.

Results

Stress conditions alter the amount of modified OLs in R. tropici *indicating a role of OLs in stress adaptation*

Rhizobium tropici CIAT899 is a nodule-forming rhizobium well known for its ability to resist stress conditions such as acidic pH or high temperatures (Martínez-Romero *et al*., 1991). In an earlier study Rojas-Jiménez *et al*. (2005) had observed that *R. tropici* forms four different OLs. In addition to the unmodified OL which was named S1 (for substrate 1) three additional modified OLs probably derived from S1 are present. Taylor *et al*. (1998) had observed an increase in the relative amounts of hydroxylated OL when *B. cepacia* was grown at increased temperatures. To find out if the modification of OL also occurs as a stress response in *R. tropici* and if these modifications might have a role in stress adaptation, *R. tropici* CIAT899 was grown at 30°C, 37°C and 42°C and its lipid composition was analysed (Fig. 2A–C, Table 1). At the standard growth temperature of 30°C, all four OLs can be detected, with P1 being the most abundant OL. An increase in growth temperature to 37°C causes a decrease in the OLs S2 and P2 and a simultaneous increase in S1. When grown at 42°C the amounts of S1 and P1 decrease slightly. The OLs S2 and P2 cannot be detected in cells grown at 42°C. An unknown lipid which migrates similarly as the sulpholipid sulphoquinovosyl diacylglycerol is apparently formed at 42°C but not at lower growth temperatures. The decrease in OLs is accompanied by changes in the phospholipid composition: phosphatidylethanolamine (PE) decreases whereas phosphatidylcholine (PC), phosphatidylglycerol (PG) and cardiolipin (CL) increase. *R. tropici* CIAT899 was also grown in complex TY medium adjusted to different pH values (compare Fig. 2A, D and E, Table 2). In *R. tropici* cells grown at pH 4.5 the OLs S1 and S2 are not detectable, whereas P2 is

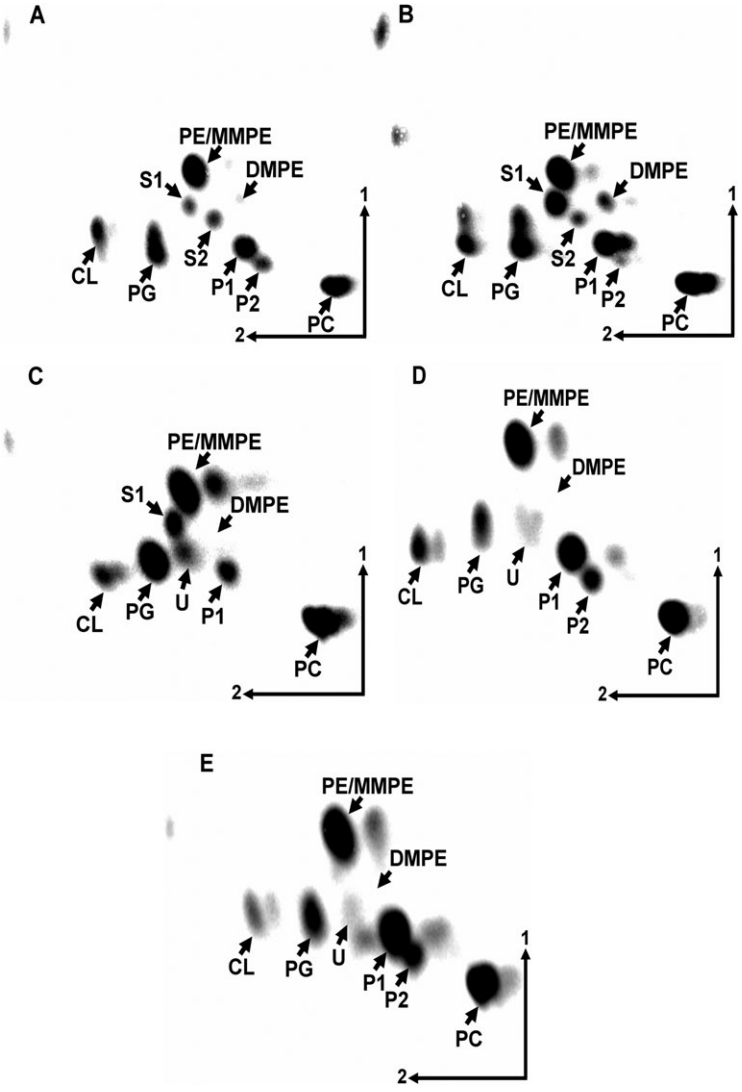

Fig. 2. Separation of [^{14}C]acetate-labelled lipids from *Rhizobium tropici* CIAT899 grown in complex TY medium at 30°C (A), at 37°C (B), at 42°C (C), at 30°C at pH 4.5 (D) or at 30°C at pH 4.0 (E) by two-dimensional thin-layer chromatography. The phospholipids phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylglycerol (PG), cardiolipin (CL), monomethyl PE (MMPE), dimethyl PE (DMPE) and the ornithine lipids (OLs) S1, S2, P1 and P2 are indicated. U, unknown lipid.

Table 1. Membrane lipid composition of *Rhizobium tropici* wild-type CIAT899, *olsE*-deficient mutant MAV04, *olsC*-deficient mutant 899-*olsCΔ1* and *olsC/olsE*-deficient double mutant MAV05 after growth on complex TY medium at 30°C, 37°C or 42°C.

	Composition (% of total ^{14}C)											
	30°C				37°C				42°C			
Lipid	CIAT899	MAV04	899-*olsCΔ1*	MAV05	CIAT899	MAV04	899-*olsCΔ1*	MAV05	CIAT899	MAV04	899-*olsCΔ1*	MAV05
PC	22.6 ± 0.6	24.8 ± 2.9	20.7 ± 0.7	24.8 ± 0.1	28.9 ± 4.0	27.2 ± 4.8	23.5 ± 4.3	24.6 ± 1.2	26.8 ± 1.2	28.5 ± 1.6	27.0 ± 2.0	27.5 ± 2.1
PE	25.0 ± 0.1	24.6 ± 1.4	24.9 ± 2.1	25.6 ± 0.6	23.5 ± 5.0	23.2 ± 3.7	19.1 ± 1.0	20.4 ± 4.8	16.2 ± 1.3	17.9 ± 1.9	11.9 ± 1.3	11.8 ± 1.1
DMPE	1.1 ± 0.1	1.7 ± 0.2	1.6 ± 0.6	1.6 ± 0.4	1.4 ± 0.6	1.1 ± 0.2	1.4 ± 0.4	1.3 ± 0.2	0.4 ± 0.0	0.4 ± 0.1	0.6 ± 0.2	0.7 ± 0.1
PG	15.8 ± 0.8	13.9 ± 2.8	17.4 ± 1.5	16.0 ± 0.6	12.5 ± 0.9	11.8 ± 1.2	18.8 ± 1.7	16.9 ± 1.6	23.8 ± 0.3	22.1 ± 0.8	33.9 ± 1.0	29.2 ± 1.1
CL	4.0 ± 0.1	4.8 ± 0.1	3.0 ± 0.1	5.6 ± 0.3	5.3 ± 1.3	4.8 ± 1.0	8.0 ± 2.0	6.7 ± 2.4	7.5 ± 0.5	8.2 ± 0.3	11.1 ± 2.5	10.7 ± 1.3
S1	2.9 ± 0.2	6.8 ± 1.0	26.5 ± 1.7	26.4 ± 1.8	7.3 ± 1.0	9.0 ± 1.4	27.7 ± 0.9	30.1 ± 5.6	5.6 ± 0.2	4.7 ± 0.9	10.8 ± 0.8	16.0 ± 1.5
S2	3.5 ± 0.5	n.d.	5.9 ± 0.5	n.d.	1.6 ± 0.3	n.d.	1.5 ± 0.2	n.d.	n.d.	n.d.	n.d.	n.d.
P1	20.6 ± 0.3	23.4 ± 1.8	n.d.	n.d.	18.2 ± 2.8	22.9 ± 0.1	n.d.	n.d.	15.2 ± 0.5	15.2 ± 0.7	n.d.	n.d.
P2	4.5 ± 0.3	n.d.	n.d.	n.d.	1.3 ± 0.4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
U	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	4.5 ± 0.5	3.0 ± 0.7	4.7 ± 0.7	4.1 ± 1.0

The values shown are mean values ± standard deviation derived from at least three independent experiments.
PC: phosphatidylcholine; PE: phosphatidylethanolamine; MMPE: monomethyl phosphatidylethanolamine; DMPE: dimethyl phosphatidylethanolamine; PG: phosphatidylglycerol; CL: cardiolipin; S1: substrate 1, unmodified ornithine lipid; S2, P1, P2: hydroxylated ornithine lipids; U: unidentified lipid; n.d.: not detected.

Table 2. Membrane lipid composition of *Rhizobium tropici* wild-type CIAT899, *olsE*-deficient mutant MAV04, *olsC*-deficient mutant 899-*olsCΔ1* and *olsC/olsE*-deficient double mutant MAV05 after growth on complex TY medium adjusted to pH 7.0, pH 4.5 or pH 4.0.

	Composition (% of total ^{14}C)											
	pH 7.0				pH 4.5				pH 4.0			
Lipid	CIAT899	MAV04	899-*olsCΔ1*	MAV05	CIAT899	MAV04	899-*olsCΔ1*	MAV05	CIAT899	MAV04	899-*olsCΔ1*	MAV05
PC	27.6 ± 0.7	25.0 ± 0.2	21.3 ± 0.3	24.6 ± 0.4	27.7 ± 0.2	28.1 ± 1.7	23.3 ± 2.2	23.8 ± 3.8	26.2 ± 0.3	26.6 ± 0.4	19.5 ± 0.5	18.1 ± 2.0
PE	26.7 ± 0.3	26.5 ± 0.1	21.7 ± 0.1	24.7 ± 0.5	27.4 ± 0.3	27.3 ± 2.1	12.8 ± 1.5	10.9 ± 0.4	22.3 ± 0.4	25.6 ± 0.3	14.9 ± 0.4	12.3 ± 2.0
DMPE	0.4 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.9 ± 0.0	0.8 ± 0.1	0.5 ± 0.1	1.0 ± 0.2	1.0 ± 0.1	0.8 ± 0.2	0.2 ± 0.0	0.2 ± 0.1	0.6 ± 0.2
PG	12.2 ± 0.4	10.0 ± 0.1	13.1 ± 0.2	11.5 ± 0.1	8.2 ± 0.2	4.7 ± 0.4	10.1 ± 0.9	9.2 ± 0.9	6.8 ± 0.1	7.4 ± 1.3	12.4 ± 1.2	12.9 ± 1.1
CL	4.4 ± 0.2	5.7 ± 0.4	5.5 ± 0.3	3.7 ± 0.3	6.9 ± 0.3	2.4 ± 0.2	4.2 ± 0.7	4.3 ± 0.1	3.7 ± 0.5	4.2 ± 1.0	5.7 ± 0.4	4.7 ± 0.7
S1	2.9 ± 1.7	8.9 ± 0.2	32.5 ± 0.2	34.6 ± 0.2	n.d.	2.0 ± 0.5	40.6 ± 2.4	46.0 ± 2.9	n.d.	1.6 ± 0.3	40.7 ± 1.1	44.6 ± 3.6
S2	3.3 ± 0.3	n.d.	5.3 ± 0.5	n.d.	n.d.	n.d.	2.5 ± 0.1	n.d.	n.d.	n.d.	n.d.	n.d.
P1	18.0 ± 0.4	23.3 ± 0.1	n.d.	n.d.	19.4 ± 0.3	32.0 ± 1.0	n.d.	n.d.	30.2 ± 0.1	31.9 ± 2.5	n.d.	n.d.
P2	4.5 ± 0.4	n.d.	n.d.	n.d.	8.3 ± 0.2	n.d.	n.d.	n.d.	8.1 ± 0.0	n.d.	n.d.	n.d.
U	n.d.	n.d.	n.d.	n.d.	1.3 ± 0.1	3.0 ± 0.3	5.5 ± 1.0	4.8 ± 0.5	1.9 ± 0.1	2.5 ± 0.4	6.6 ± 0.6	6.8 ± 0.5

The values shown are mean values ± standard deviation derived from at least three independent experiments. For abbreviations see Table 1.

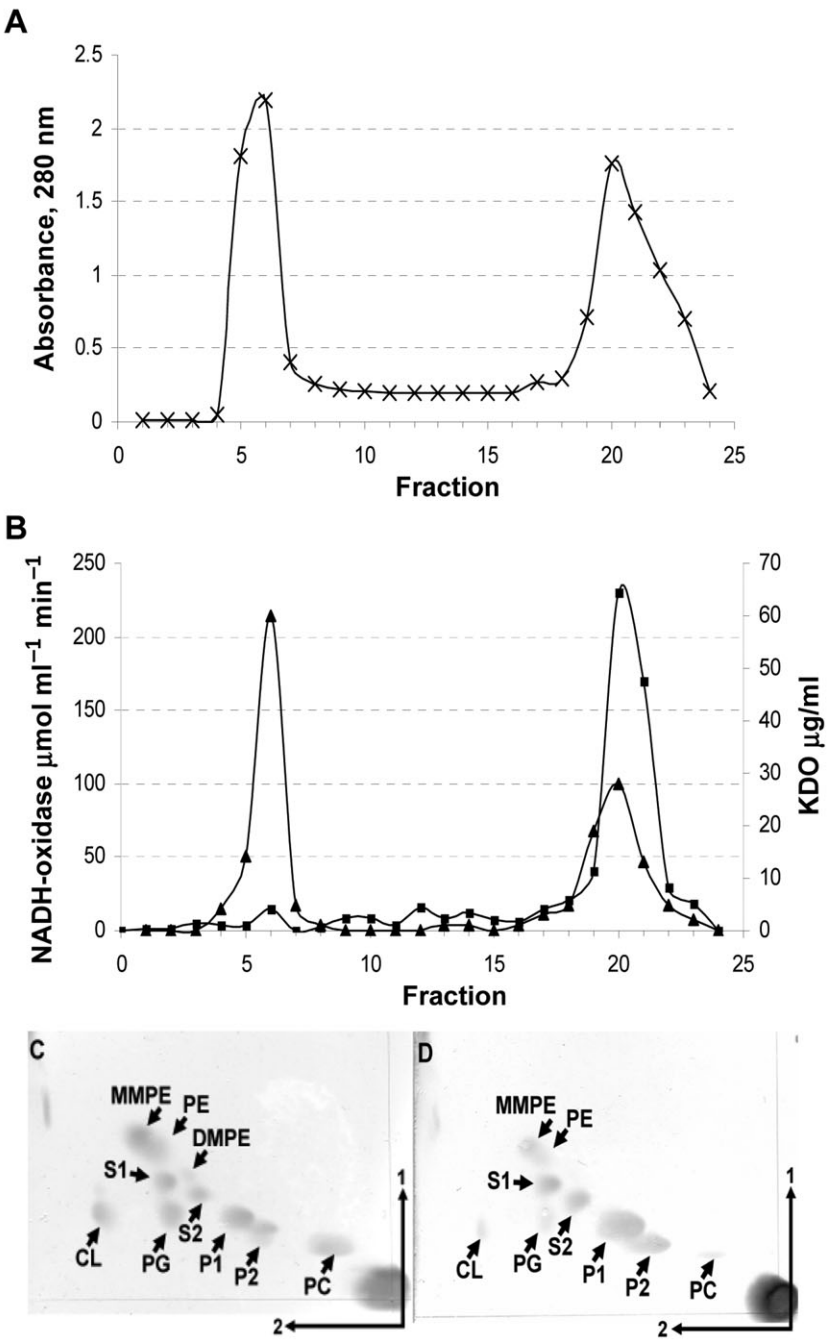

Fig. 3. Localization of OLs in membranes of wild-type *Rhizobium tropici* CIAT899.
A and B. Results of a sucrose density gradient centrifugation of cell membranes of *R. tropici* CIAT899. (A) A280 readings of the gradient fractions. (B) 2-Keto-3-oxyoctonate content (closed triangles) and NADH oxidase (closed squares) activity of the fractions.
C and D. Separation of membrane lipids extracted from the inner (C) and outer membrane (D). Fractions corresponding to the inner and outer membranes were pooled, lipids were extracted with 1-butanol and subsequently analysed using two-dimensional TLC. Lipids were visualized by spraying with ceric sulphate in sulphuric acid. The phospholipids phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylglycerol (PG), cardiolipin (CL), monomethyl PE (MMPE), dimethyl PE (DMPE) and the ornithine lipids (OLs) S1, S2, P1 and P2 are indicated. A quantification of the lipids is shown in Table 3.

increased and no changes are detected for P1. When grown at pH 4.0 again OLs S1 and S2 cannot be detected, but P1 increases drastically and becomes the major membrane lipid (Fig. 2E).

OLs are enriched in the OM of R. tropici *CIAT899*

Dees and Shively (1982) had shown that in the acid-resistant species *T. thiooxidans* OL is present mainly in the OM and they had therefore speculated that it might play a role in conferring acid resistance to these bacteria. If such a hypothesis were true one would expect an accumulation of OLs also in the OM of the acid-tolerant bacterium *R. tropici*. Inner membrane (IM) and OM from *R. tropici* were separated and the lipids of both membranes were extracted and separated using two two-dimensional TLC (Fig. 3). The protein content of the fractions was estimated using absorption measurements at 280 nm. The protein-enriched fractions formed two peaks corresponding to the IM and OM (Fig. 3A). KDO (2-keto-3-deoxyoctanoate)

Table 3. Membrane lipid composition of the inner and outer membrane of *R. tropici* CIAT899.

Lipid	Inner membrane	Outer membrane
PC	23.9	9.1
PE	8.2	6.6
MMPE	5.4	5.9
DMPE	4.8	5.0
PG	17.2	7.7
CL	12.2	6.7
S1	6.2	11.4
S2	5.0	11.0
P1	11.1	25.3
P2	6.0	11.3

The data were obtained from the TLC plates shown in Fig. 3C and D using the program ImageQuant. Numbers present per cent of total lipids present in the TLC. For abbreviations see Table 1.

content and NADH oxidase activity that were used as markers for the OM and IM, respectively, indicated that the IM was contaminated to some extent by the OM, but that the OM was almost free of contamination by the IM (Fig. 3B). The TLC analysis of the Bligh-Dyer extracts showed that phospholipids are the major membrane lipids of the IM but are present in much smaller relative amounts in the OM (Fig. 3C and D, Table 3). A quantification of the lipids showed that phospholipids form more than 70% of the membrane lipids of the IM but only about 40% of the membrane lipids of the OM, excluding lipopolysaccharide (LPS). OLs form less than 30% of the membrane lipids of the IM but about 60% of the membrane lipids of the OM (again excluding LPS). Taking the contamination of the IM fractions with OM material into account the result overestimates the real concentration of OLs in the IM. Assuming that the outer leaflet of the OM is composed mainly of the lipid A moiety of LPS, this result indicates that the major proportion of the inner leaflet of the OM is composed of OLs.

Expression cloning of the OL-modifying enzyme OlsE from R. tropici

The experiments described earlier indicated a possible role for the different OLs in the *R. tropici* stress response. In *S. meliloti* only one type of OL is present. In contrast, four different types of OLs called S1, S2, P1 and P2 are present in *R. tropici* CIAT899 (Fig. 1). The gene *olsC* encoding the enzyme OlsC responsible for the synthesis of OLs P1 and P2 from the substrates S1 and S2 has been described earlier (Rojas-Jiménez *et al.*, 2005). It was not known, however, which gene encodes the hypothetical enzyme OlsE responsible for the synthesis of S2 and possibly also for the synthesis of P2 (Fig. 1). We suspected that S1, corresponding to the OL present in *S. meliloti*, was a substrate for the OlsE-catalysed reaction. The *S. meliloti* strain CS111.pNG25 lacking the ninhydrin-positive lipid PE and producing increased amounts of the OL S1 was constructed and transconjugants of CS111.pNG25 harbouring cosmids containing *R. tropici* CIAT899 genomic DNA were assayed for the presence of a second ninhydrin-positive lipid in addition to S1. In the transconjugant referred to as CS111.pNG25.pCos94, two ninhydrin-positive lipids with the expected R_f values for S1 and S2 were detected. A restriction analysis of pCos94 showed that it contains about 18–20 kb of inserted DNA. Restriction fragments of the pCos94 insert were subcloned into a broad-host-range vector and again conjugated into CS111.pNG25. The resulting transconjugants were analysed as described above for the cosmid bank (data not shown). A plasmid conferring the formation of the OL S2 was identified and its insert was sequenced. In addition to three predicted complete ORFs it contained two incomplete ORFs (GenBank Accession No. HM010770). BLAST searches using the NCBI database with the amino acid sequences of the three complete ORFs as query were made (Altschul *et al.*, 1997). The first ORF was annotated as a putative acetyltransferase, the second ORF as a putative aminoglycoside N(6′) acetyltransferase and the third ORF as a putative hydroxylase. The three candidate ORFs were cloned into a broad-host-range plasmid and the resulting plasmids were conjugated into CS111.pNG25. Labelling of the lipids of the three transconjugants with [^{14}C]acetate showed that ORF3 codes for the putative hydroxylase OlsE which is responsible for the formation of S2 (Fig. 4).

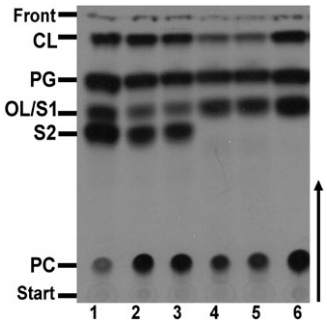

Fig. 4. Expression cloning of *olsE* from *R. tropici*. Lipids of *Sinorhizobium meliloti* CS111.pNG25 containing different plasmids or cosmids were radiolabelled with [^{14}C]acetate and separated by one-dimensional TLC. The following strains were analysed: CS111.pNG25.pCos94 (cosmid, lane 1), CS111.pNG25.pERMAV04 (ORF1 to 3/3.5 kb insert, lane 2), CS111.pNG25.pERMAV13 (ORF3, lane 3), CS111.pNG25.pERMAV12 (ORF2, lane 4), CS111.pNG25.pERMAV11 (ORF1, lane 5) and CS111.pNG25.pERMAV06 (negative control, lane 6). The phospholipids phosphatidylcholine (PC), phosphatidylglycerol (PG), cardiolipin (CL) and the ornithine lipids (OLs) S1 and S2 are indicated.

OlsE belongs to the fatty acyl hydroxylase superfamily and is responsible for the hydroxylation of OL within the ornithine moiety

The gene *olsE* encodes a very hydrophobic protein of 330 amino acids predicted to form between four and six transmembrane helices. An analysis of the amino acid sequence shows that OlsE belongs to the fatty acyl hydroxylase superfamily (cl01132) which is characterized by the presence of two copies of the HXHH motif. This superfamily includes fatty acid and carotene hydroxylases, sterol desaturases (Mitchell and Martin, 1997), C-5 sterol desaturase (Arthington *et al*., 1991) and C-4 sterol methyl oxidase (Bard *et al*., 1996; Kennedy *et al*., 2000). A similar motif ($HX_{3-4}H$, $HX_{2-3}HH$, $HX_{2-3}H$) can be found in membrane-bound fatty acid desaturases such as OLE1 from *Saccharomyces cerevisiae* and is also present in bacterial alkane hydroxylase (Kok *et al*., 1989) and xylene monooxygenase (Suzuki *et al*., 1991). In these proteins the conserved histidine residues act to co-ordinate an oxo-bridged diiron cluster (Fe–O–Fe) that functions as part of the reaction centre (Fox *et al*., 1993; Shanklin *et al*., 1994).

The annotation of OlsE as a fatty acyl hydroxylase indicates that OlsE introduces a hydroxyl group into OL at an unknown position. To localize the hydroxyl group on the OL S2, lipids were extracted according to Bligh and Dyer (1959) from a 1 l culture of the *olsC*-deficient *R. tropici* mutant *899-olsCΔ1*. OLs S1 and S2 were purified from the total lipid extract and analysed by normal-phase LC-coupled electrospray ionization (ESI) mass spectrometry (MS) in the negative ion mode. Prior to fragmentation ions with *m/z* 691 and 707 corresponding to OLs S1 and S2 were detected. The molecular ion was shifted in case of S2 to an *m/z* 16 amu higher in comparison with S1 indicating the presence of an additional oxygen suggesting the presence of an additional hydroxyl group. Comparing the fragmentation patterns of S1 and S2 it was observed that the modification present in S2 is located within the ornithine moiety and not in the fatty acyl chains (Fig. 5). When assaying the two-dimensional TLC plates with *R. tropici* lipids with ninhydrin it was noticed that S2 and P2 react with delay in comparison with S1 and P1, and that the developed colour is different. While S1 and P1 upon reaction with ninhydrin develop a red to purple colour, the reaction of S2 and P2 causes the formation of an orange colour.

OlsC introduces a hydroxyl group at the 2 position of the secondary fatty acid of OL

OlsC is a homologue of the hydroxylase LpxO from *Salmonella typhimurium* that is responsible for the addition of a 2-hydroxy group to the myristate residue present at the 3′ position of lipid A. Rojas-Jiménez *et al*. (2005) had discovered the gene *olsC* and had shown that OlsC is a putative hydroxylase responsible for the formation of the OLs P1 and P2 from the OLs S1 and S2 in *R. tropici* (Fig. 1). However, it was not known in what part of the OL structure the OlsC-dependent hydroxylation occurs. To localize the hydroxyl group on the OL P1, OLs S1 and P1 were purified from the total lipid extracts and analysed by normal-phase LC-coupled ESI-MS in the negative ion mode. Prior to fragmentation ions with *m/z* 691 and 707 corresponding to OLs S1 and P1 were detected. The molecular ion of P1 was shifted to an *m/z* 16 amu higher in comparison with S1 indicating the presence of an additional oxygen suggesting the presence of an additional hydroxyl group. Comparing the fragmentation patterns of S1 and P1 it was observed that the modification present in P1 is located within the secondary fatty acyl chain (data not shown) which in case of S1 is mainly lactobacillic acid and in case of P1 hydroxy lactobacillic acid. In order to determine the position of the OlsC-dependent hydroxylation in P1 its fatty acids were transmethylated before the hydroxyl groups were derivatized to trimethylsilyl (TMS) ethers similar to the procedures described by Gibbons *et al*. (2008). Alpha- and beta-hydroxy fatty acid standards of 16 and 18 carbons were processed in parallel with the samples (Fig. S1A–D). GC/MS analysis of the derivatized fatty acids shows the presence of three peaks present in the samples derived from P1 that are not present in the samples derived from S1 (Fig. S1E and F). Their fragmentation pattern indicates that the OlsC-dependent hydroxylation occurs in the 2 position (Fig. S1G).

Lipid composition analysis of olsE *and* olsE/olsC *mutants*

To study the role of OLs in *R. tropici* in more detail, mutants deficient in *olsE* and double mutants deficient in *olsC* and *olsE* were constructed. Their lipid compositions were compared with the wild-type strain CIAT899 and the OlsC-deficient mutant 899-*olsCΔ1* (Fig. 6, Table 1). As expected the *olsE*-deficient mutant MAV04 lacked the OLs S2 and P2, the *olsC*-deficient mutant 899-*olsCΔ1* lacked P1 and P2 and in the double mutant MAV05 (Δ*olsC*Δ*olsE*) no S2, P1 or P2 were detectable. Apparently, the amount of OLs, being the sum of S1, S2, P1 and P2, is more or less stable between 20% and 35% when *R. tropici* is grown in complex TY medium at 30°C. No significant differences in the relative amounts of the phospholipids PE, PC, PG and CL were observed between the different strains. To show that the observed phenotypes were caused by the absence of the deleted genes, mutants MAV04 (Δ*olsE*) and MAV05 (Δ*olsC*Δ*olsE*) were also complemented. When *olsE* was present *in trans* in MAV04 again formation of S2 and P2 was detected and

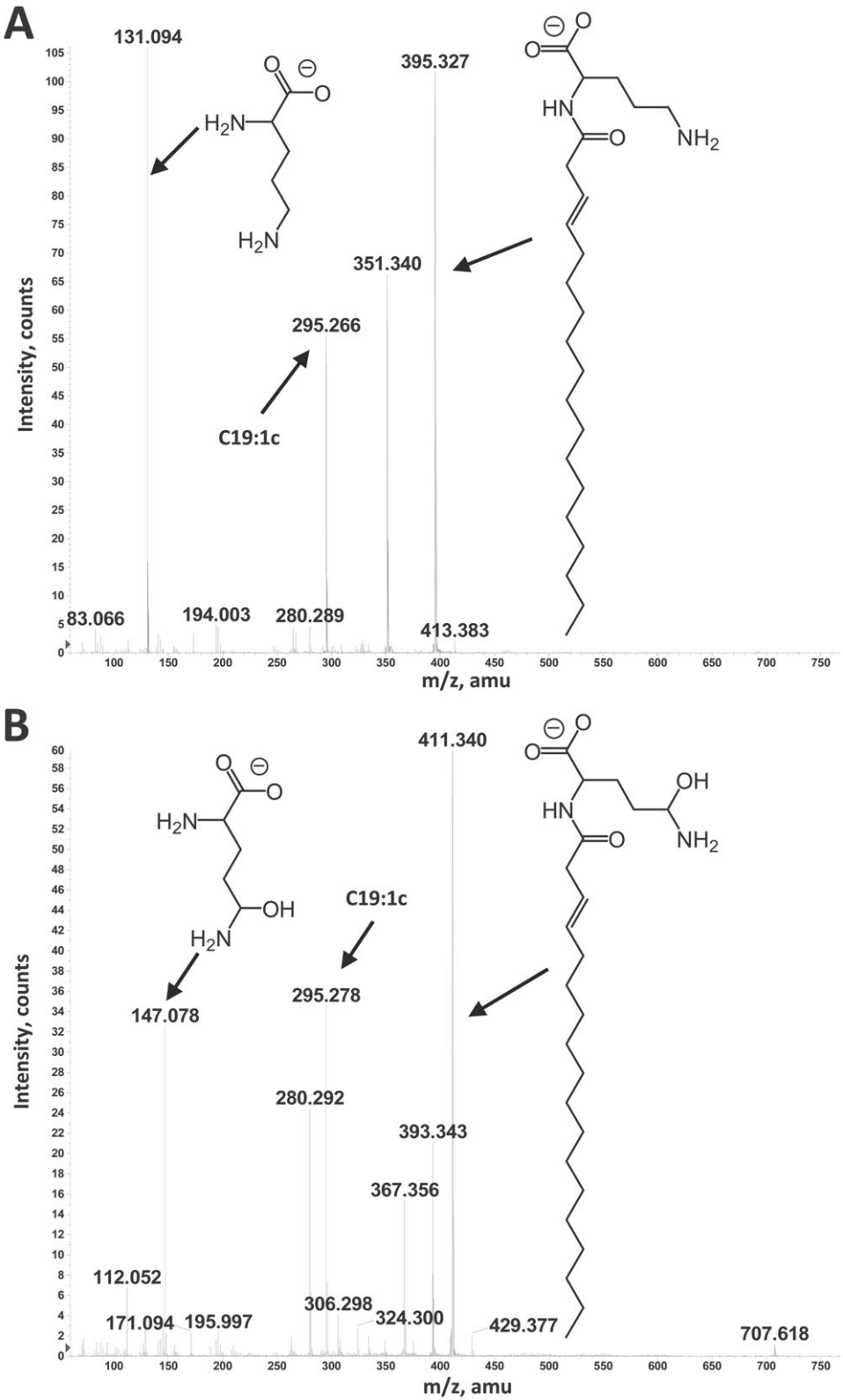

Fig. 5. Collision-induced dissociation mass spectra of ornithine lipids S1 and S2 detected in lipid extract of *R. tropici* mutant 899-*olsC*Δ*1*. Negative ion collision induced dissociation mass spectra of $[M\text{-}H]^-$ ions at *m/z* 671 (A) obtained from OL S1 and *m/z* 707 (B) obtained from OL S2. The structures of major fragment ions are indicated. The position of the hydroxyl group introduced in the ornithine moiety is assigned tentatively. Complete structures of the OLs are shown in Fig. 1.

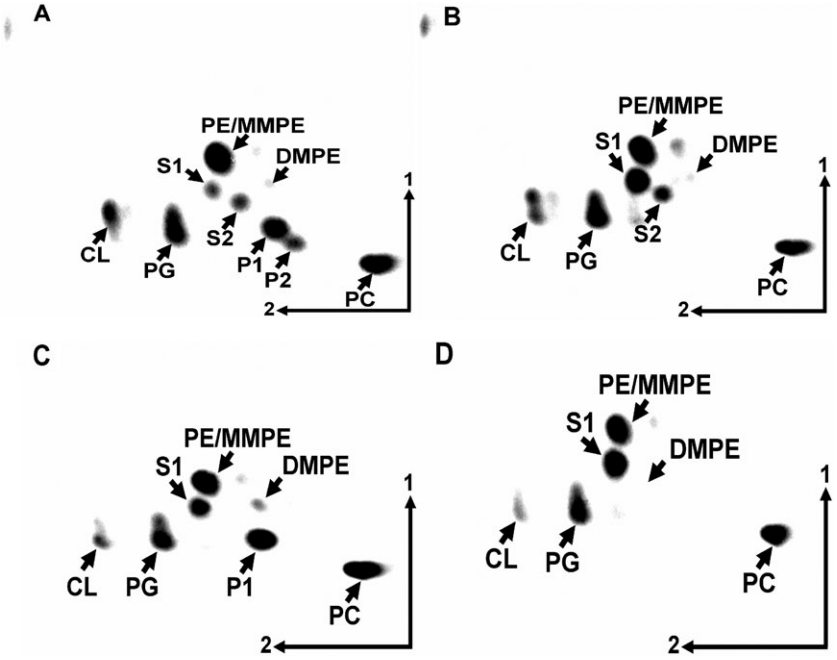

Fig. 6. Analysis of membrane lipid composition of *R. tropici* wild-type CIAT899 (A), *olsC*-deficient mutant 899-*olsC*Δ*1* (B), *olsE*-deficient mutant MAV04 (C) and *olsC/olsE*-deficient double mutant MAV05 (D). Lipids were labelled with [^{14}C]acetate during growth in complex TY medium at 30°C and separated using two-dimensional TLC. The phospholipids phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylglycerol (PG), cardiolipin (CL), monomethyl PE (MMPE), dimethyl PE (DMPE) and the ornithine lipids (OLs) S1, S2, P1 and P2 are indicated.

when mutant MAV05 was complemented with *olsE* the OLs S2 and P2 could be detected, whereas S1 and P1 did not accumulate (data not shown). Constitutive expression of *olsC* and *olsE* together in MAV05 caused the accumulation of P2 while only trace amounts of the other OLs were observed (data not shown). Such an over-complementation leading to the accumulation of the reaction product(s) while almost completely consuming the substrate(s) had also been observed earlier for the complementation of the *olsC*-deficient mutant 899-*olsC*Δ*1* (Rojas-Jiménez *et al*., 2005).

*Growth and characterization of the lipid composition of mutants 899-*olsCΔ1*, MAV04 and MAV05: OlsC is important in conferring stress resistance*

An earlier study in *B. cepacia* (Taylor *et al*., 1998) had shown an increase in formation of hydroxylated OL when the bacteria were grown at higher temperature. In this study we have shown that the relative amounts of the different OLs shift in response to a change in growth temperature or pH of the medium. Rojas-Jiménez *et al*. (2005) had observed that a *R. tropici* strain constitutively expressing *olsC* was not able to grow at pH 4.5 any more. Therefore, it was expected that the *R. tropici* mutants deficient in OL hydroxylation would show a phenotype under conditions of acid or temperature stress. The wild-type *R. tropici* CIAT899 and the three mutants 899-*olsC*Δ*1*, MAV04 and MAV05 were cultivated in complex TY medium at pH 4.0, 4.5 and 7.0. At pH 7.0 all four strains divide at a similar rate (Fig. 7A). At pH 4.5 the wild-type CIAT899 and the mutant MAV04 (Δ*olsE*) grow at a similar rate compared with pH 7.0 whereas the other two mutants seem to present a longer generation time (Fig. 7B). At pH 4.0 the wild-type CIAT899 and the mutant MAV04 (Δ*olsE*) grow significantly slower than at pH 4.5 but still both cultures reach a final optical density larger than 1.0, whereas the mutants 899-*olsC*Δ*1* and MAV05 (Δ*olsC*Δ*olsE*) at most undergo one single division (Fig. 7C). To determine if the observed differences are related to changes in lipid composition, wild-type and mutant cells were grown and labelled in the corresponding media and analysed by TLC in two dimensions (Table 2). At pH 7.0 all four strains show similar concentrations of phospholipids and the distinct patterns of the different OLs typical for each mutant described above. At pH 4.5 both OlsC-deficient mutants (899-*olsC*Δ*1* and MAV05) show a drastic reduction in PE content and a strong increase in S1 to up to more than 40%. At pH 4.0 again, both OlsC-deficient mutants show a very similar lipid composition with S1 being the major membrane lipid and PE being drastically reduced. The wild-type CIAT899 apparently forms more P1 under these conditions. It seems that low-pH conditions cause the accumulation of OLs in all strains: in the wild-type and the mutant MAV04 (Δ*olsE*) the major lipid accumulated is P1, whereas in the mutants 899-*olsC*Δ*1* and MAV05 (Δ*olsC*Δ*olsE*) the major lipid is S1.

When the wild-type CIAT899 and the three mutants deficient in OL hydroxylation were cultivated in TY medium at 30°C, no differences in generation time can be observed between them (Fig. 7D). At 37°C, both strains lacking *olsC* (899-*olsC*Δ*1* and MAV05) seem to grow slightly slower than the other two strains (Fig. 7E). At 42°C

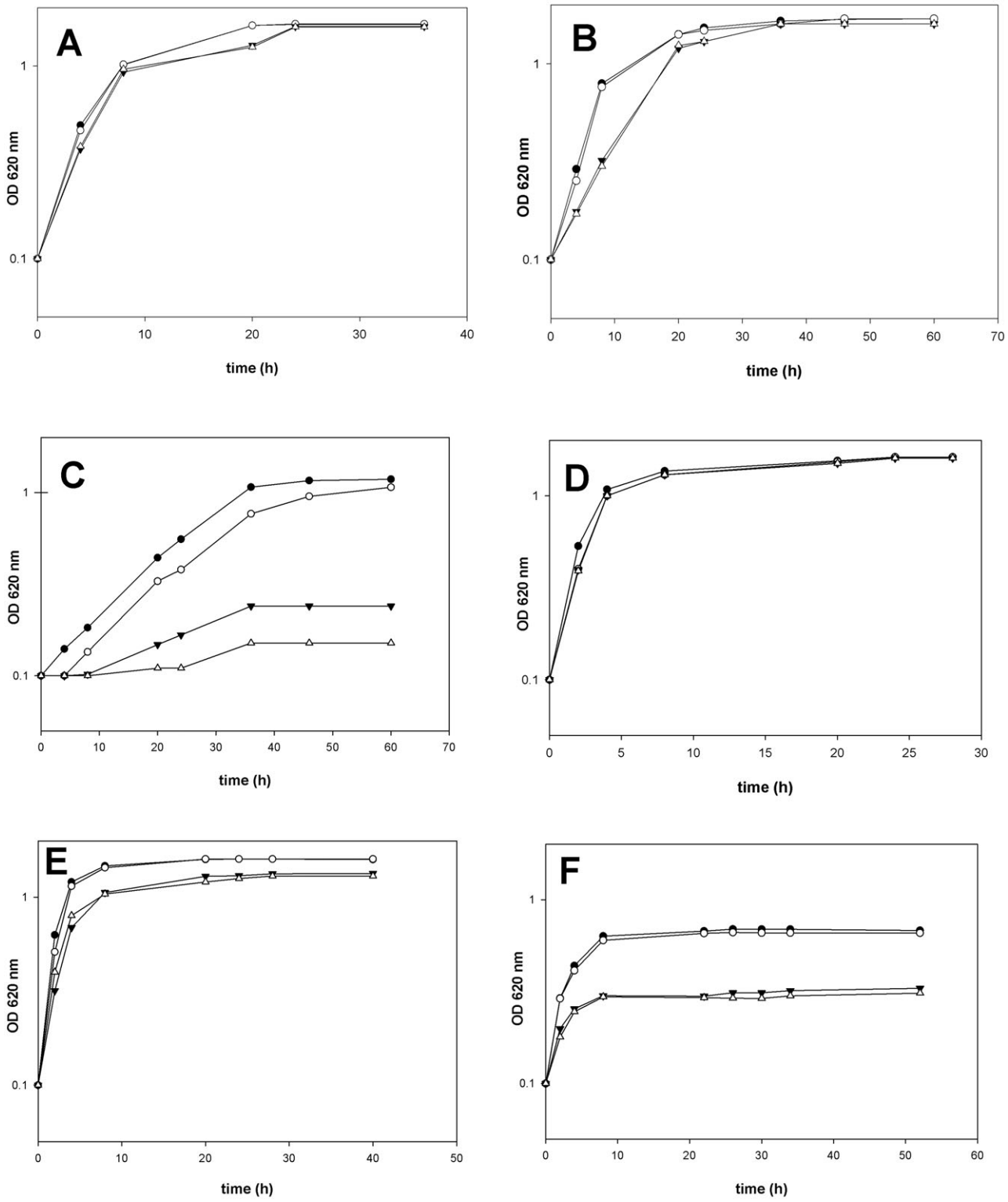

Fig. 7. Growth of *R. tropici* mutants lacking *olsC* is affected under stress conditions. *R. tropici* wild-type CIAT899 and mutants were grown in complex TY medium adjusted to pH 7.0 (A), pH 4.5 (B) or pH 4.0 (C) at 30°C or in complex TY medium at 30°C (D), 37°C (E) or 42°C (F). The result of a typical experiment is shown. CIAT899 – closed circles, MAV04 – open circles, MAV05 – open triangles, 899-*olsCΔ1* – closed triangles.

wild-type CIAT899 and mutant MAV04 (Δ*olsE*) grow slower than at the lower temperature and reach a final OD_{620} of only 0.65–0.68 (Fig. 7F). The mutants 899-*olsCΔ1* and MAV05 (Δ*olsC*Δ*olsE*) divide distinctly slower at 42°C than the two former strains and reach a final OD_{620} of only 0.3.

The lipid composition of the three mutants deficient in OL hydroxylation and the wild-type CIAT899 was also analysed at the different temperatures (Table 1). For each of the four strains the lipid compositions are very similar at 30°C and 37°C. At 42°C the amount of PG is increased by about 10–15% and also CL seems to be a bit more abundant at the higher temperature. The total of the four OLs is decreasing in all four strains. Whereas at 30°C and 37°C the sum of S1, S2, P1 and P2 is about 30%, at 42°C the strains contain only between 10% and 20% OLs.

R. tropici *mutants deficient in OlsC cause an increase in nodule number that is reverted by the deletion of* olsE

The *R. tropici* mutant deficient in OlsC (899-*olsCΔ1*) formed nodules on bean plants that were poorly developed 21 days after inoculation with the bacteria, lacked lenticels and presented a twofold reduction in nitrogen fixation (Rojas-Jiménez *et al.*, 2005). These results suggested that the *R. tropici* mutants MAV04 deficient in *olsE* and double mutants MAV05 deficient in *olsC* and *olsE* might also show nodulation phenotypes. Nodulation assays were performed in an agar-based medium in order to be able to observe the kinetics of nodule formation over time. While wild-type *R. tropici* CIAT899 and MAV04 (Δ*olsE*) produced reproducibly between 80 and 100 nodules per plant, the mutant 899-*olsCΔ1* caused the formation of more than 160 nodules per plant. When the *olsC*-deficient mutant was complemented with the *olsC* gene, it again formed nodules in numbers similar to the wild-type (Fig. 8A). Surprisingly, the double mutant MAV05 (Δ*olsC*Δ*olsE*) formed a similar number of nodules as the wild-type. The nodules were sectioned and while the wild-type caused almost exclusively the formation of nodules that were red inside indicating the formation of leghaemoglobin, while the mutants caused formation of many small nodules that were whitish on the inside indicating the absence of leghaemoglobin (Fig. S2). Roots from plants infected with the *olsC*-deficient mutant 899-*olsCΔ1* presented few red nodules and many whitish nodules and roots from plants infected with the *olsE*-deficient mutant MAV04 presented even less red and more white nodules. On roots infected with the *olsC/olsE*-deficient double mutant MAV05 almost no red nodules were formed (Fig. S2). Nitrogen fixation per hour and nodule fresh weight was affected in all three mutants in comparison with the wild-type (Fig. 8B). These results indicate that the absence of hydroxylated OLs strongly interferes with the development of functional nodules during *R. tropici*–bean symbiosis.

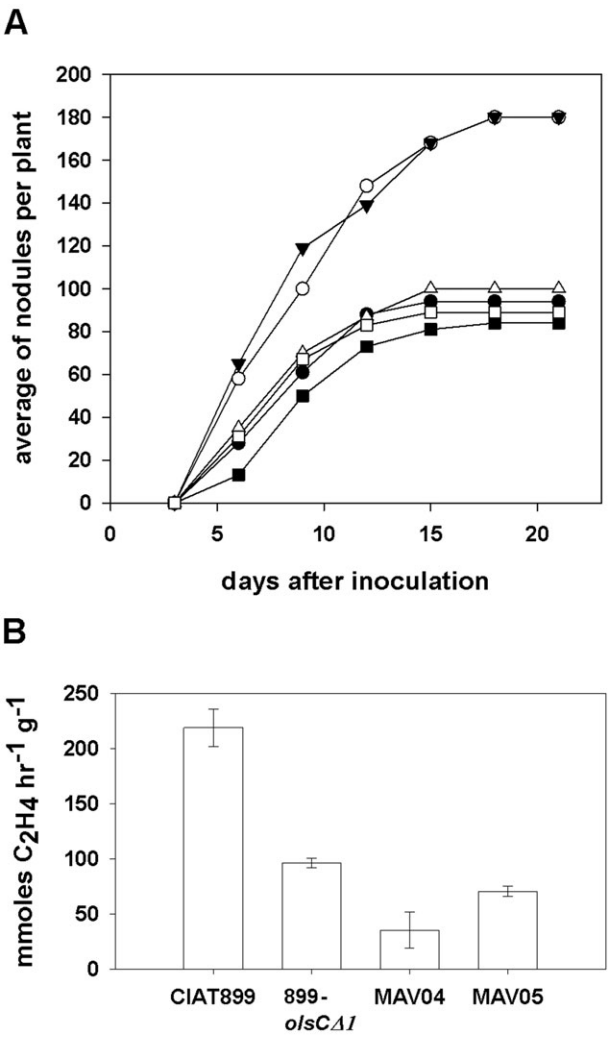

Fig. 8. Symbiotic phenotypes of *R. tropici* wild-type CIAT899 and strains deficient in OL modification on bean plants.
A. Nodulation assay. Nodules were counted every second or third day. Plants were harvested 21 days post inoculation; nodules were assayed for nitrogen fixation activity. The experiment was repeated three times with five plants for each strain. The result of a typical experiment is shown. CIAT899 (closed circles), 899-*olsCΔ1* (open circles), MAV04 (closed squares), MAV05 (open squares), 899-*olsCΔ1*.pERMAV05 (closed triangles), 899-*olsCΔ1*.pERMAV15 (open triangles). Uninoculated plants did not develop nodules.
B. Mean acetylene reduction of nodulated bean roots inoculated with wild-type *R. tropici* CIAT899 and mutants 899-*olsCΔ1*, MAV04 and MAV05. Values are the mean ± SD of three repetitions.

Discussion

Although OLs are widespread in eubacteria (López-Lara *et al.*, 2003; Geiger *et al.*, 2010) the genes *olsB* and *olsA* responsible for OL biosynthesis were only recently described in *S. meliloti* (Weissenmayer *et al.*, 2002; Gao

et al., 2004). In addition to the unmodified OL consisting of a 3-hydroxy fatty acid linked in an amide bond to the α-amino group of ornithine and a second fatty acid bound in an ester linkage to the first, several hydroxylated forms of OL have been described in organisms diverse as *B. cepacia* (Taylor *et al*., 1998), *R. tropici* (Rojas-Jiménez *et al*., 2005), *Flavobacterium* (Kawai *et al*., 1988), *Thiobacillus* (Knoche and Shively, 1972), *Streptomyces* (Asselineau, 1991) and some *Ralstonia* (Galbraith *et al*., 1999) species. The fact that the genes coding for the enzymes responsible for the hydroxylation of OLs have not been identified except for the case of *R. tropici* where the gene *olsC* was described (Rojas-Jiménez *et al*., 2005) has made it difficult to study the function of these hydroxylated forms of OL.

Apparently OL and especially their hydroxylated forms play a role in stress response as has been observed by Rojas-Jiménez *et al*. (2005) and Taylor *et al*. (1998). *R. tropici* mutants deficient in the formation of the hydroxylated OL P1 (899-*olsCΔ1* and MAV05) are affected in growth at low pH and at high temperature in comparison with the wild-type. It has to be mentioned that in an earlier study the mutant 899-*olsCΔ1* grew as well as the wild-type (Rojas-Jiménez *et al*., 2005). The explanation for this difference is unknown, but possibly slight differences in the pH of the medium cause drastic differences in the growth behaviour of the mutant. At pH 4.0 and 4.5 a drastic increase in the formation of OLs was observed when compared with growth at neutral pH. In the wild-type CIAT899 and in the mutant MAV04 (Δ*olsE*) especially P1 is increased, whereas in the *olsC*-deficient mutants unable to form P1 the substrate S1 is accumulating. This probably means that under acid growth conditions OL biosynthesis via OlsB and OlsA is induced. It is less clear what happens at the elevated growth temperature. Although the concentration of OLs is decreased during growth at 42°C in comparison with 30°C, again the presence of P1 seems to be of importance as *olsC*-deficient mutants show a growth phenotype under this condition. The elevated temperature also seems to interfere with OlsE activity as S2 and P2 cannot be detected.

Dees and Shively (1982) made the observation that in the extreme acid-tolerant bacterium *Thiobacillus oxidans* OLs are accumulated in the OM and therefore speculated about a role for OL in acid resistance in this organism (Dees and Shively, 1982). From the growth phenotype of the mutants unable to form P1 it is apparent that the hydroxylation at the 2 position of the secondary fatty acid is of importance under acid growth conditions. Our localization study confirms that although OLs seem to be present in both membranes, they show a higher relative abundance in the OM. Both studies therefore agree that OLs play a role in acid resistance, but it is not clear by which mechanism this effect of OLs is exerted. The hydroxyl group introduced by OlsC in the 2 position of the secondary fatty acid may increase hydrogen bonding between neighbouring OL molecules similarly as has been suggested for LpxO-hydroxylated lipid A in *Salmonella* and hydroxylated sphingolipids (Nikaido, 2003; Murata *et al*., 2007). These additional hydrogen bonds should result in bilayer stabilization and a decrease in membrane permeability which could explain the decrease in acid and temperature resistance of OlsC-deficient mutants.

In this study we identified the OL hydroxylase OlsE using a functional expression screening. OlsE belongs to the fatty acyl hydroxylase superfamily, unlike the other OL hydroxylase OlsC from *R. tropici* which belongs to the aspartyl-/asparaginyl β-hydroxylase protein family to which also the lipid A-myristate β-hydroxylase LpxO from *S. typhimurium* belongs (Gibbons *et al*., 2000; 2008). The closest homologues to OlsC from *R. tropici* are present in the α-proteobacteria *Agrobacterium radiobacter*, *Agrobacterium vitis*, *Ochrobactrum anthropi*, *Brucella* species, and in several cyanobacteria. Unlike other hydroxylations described in OL, the hydroxylation introduced by OlsE seems to be unique because it occurs in the ornithine moiety, but not in the fatty acid moieties as has been described for example in *T. thiooxidans, B. cepacia* or *R. tropici* (this study). Unrelated ornithine hydroxylases like for example PvdA from *Pseudomonas aeruginosa* have been described and studied in some detail (Visca *et al*., 1994; Meneely *et al*., 2009). PvdA is involved in pyoverdin biosynthesis and introduces a hydroxyl group in the δ-amino group of ornithine but is unrelated on sequence level to OlsE. It is not clear yet in which position the OlsE-catalysed hydroxylation occurs, but apparently the newly introduced hydroxyl group is close enough to the δ-amino group to change its reactivity with ninhydrin. As other members of the fatty acyl hydroxylase superfamily introduce hydroxyl groups at carbon atoms but not at nitrogen atoms OlsE possibly introduces a hydroxyl group at the δ-carbon. It is not clear how the OlsE-dependent hydroxylation might affect membrane characteristics. Possibly the OlsE-dependent hydroxylation enables the OLs S2 and P2 to form a lactone ring within the ornithine headgroup, the presence of which should change its biophysical properties drastically.

The closest OlsE homologues are present in some α-proteobacteria and more distant homologues are present in several actinobacteria, a few γ-proteobacteria and a few other α-proteobacteria. Possibly several of the closer homologues also function as OL hydroxylases. For the OlsE homologue Atu0318 from *Agrobacterium tumefaciens* we could show that is responsible for the formation of the OL S2 (data not shown). Distant OlsE homologues such as the one in *Bradyrhizobium japonicum* may use distinct substrates. One example for

bacterial lipids that are frequently hydroxylated is the hopanoids. In *B. japonicum*, an α-proteobacteria that forms hopanoids but no OL (Perzl *et al*., 1998; López-Lara *et al*., 2003) the OlsE homologue might be responsible for the hydroxylation of hopanoids.

The *R. tropici* mutants deficient in OL hydroxylation showed nodulation phenotypes, indicating that an adequate concentration of the correct OLs is required for the establishment of a successful symbiosis. It is possible that the nodulation phenotype is partly a consequence of the acid sensitivity phenotype, as during establishment of the symbiosis between rhizobia and legumes the bacteria are exposed to low-pH conditions in the rhizosphere and later again inside symbiosomes (Udvardi and Day, 1997). Other aspects, however, seem to be important as well as the OlsE-deficient mutant grows like the wild-type in media at pH 4.0, but still presents a severe nodulation phenotype. Modification of OL might be also of importance for the animal pathogen *Brucella* that has to survive acid pH conditions in the range of 4.0–4.5 inside phagosomes (Kohler *et al*., 2002). *Brucella* species form OL in a constitutive manner (Comerci *et al*., 2006; Bukata *et al*., 2008) and additionally have a close homologue to OlsC from *R. tropici* which makes it probable that they can form the hydroxylated OL P1. If hydroxylated OLs really play a role in conferring acid resistance then *Brucella* mutants deficient in their OlsC homologue might be affected in their survival inside phagosomes.

The exact function of OL S1 and its hydroxylated forms is still not known, although our data argue for an important role in stress resistance. The knowledge of the complete scheme of OL biosynthesis in *R. tropici* should facilitate future functional studies on the role of OLs. In addition, the phenomenon of over-complementation described above allows the construction of *R. tropici* strains principally accumulating one specific class of OL. Characterization of these strains should make it possible to assign roles to the different forms of OL.

Experimental procedures

Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in the present work and their relevant characteristics are shown in Table 4. *R. tropici* strains were grown in complex TY medium that contained 10 mM $CaCl_2$ (Beringer, 1974) at 30°C, 37°C or 42°C. Acidic media at pH 4.0 and 4.5 were buffered with 25 mM Homopipes (Research Organics, Cleveland, OH, USA) adjusted to the respective pH with NaOH, and media at pH 7.0 were buffered with 25 mM HEPES (Sigma). *E. coli* strains were grown in Luria–Bertani (LB) medium at 37°C (Sambrook and Russell, 2001). When needed, antibiotics were added at the following final concentrations (μg ml^{-1}): kanamycin (Km) 50; carbenicillin (Cb) 100; tetracycline (Tc) 10; nalidixic acid (Nal) 20; and chloramphenicol (Cm) 60.

DNA manipulations

Recombinant DNA techniques were performed according to standard protocols (Sambrook and Russell, 2001). The cosmid subclone containing *olsE* and PCR products were sequenced at Eurofins Medigenomix by the chain termination method. The DNA region containing *olsE* was analysed using the NCBI (National Center for Biotechnology Information) BLAST network server (Altschul *et al*., 1997). Oligonucleotide sequences are listed in Table S1.

Expression cloning of the R. tropici *OL hydroxylase gene* olsE

A cosmid library of *R. tropici* CIAT899 made in pVK102 using partially digested HindIII genomic DNA fragments (Vargas *et al*., 1990) was mobilized into *S. meliloti* CS111.pNG25 by triparental mating using pRK2013 as the helper plasmid (Figurski and Helinski, 1979). CS111.pNG25 was used to facilitate the screening: CS111 is a phosphatidylserine synthase-deficient mutant (Sohlenkamp *et al*., 2004) derived from the wild-type 1021 which is constitutively expressing the gene *olsB* from *Burkholderia cenocepacia*. CS111.pNG25 will form increased amounts of S1 which is one of the suspected substrates of OlsE while lacking the ninhydrin-positive membrane lipid phosphatidylethanolamine. Plasmid pNG25 was constructed as follows: the oligonucleotide primers oLOP111 and oLOP112, introducing NdeI and HindIII sites, respectively, were used in the PCR to amplify the gene *olsB* from *B. cenocepacia* J2315 using genomic DNA as template. After digestion of the PCR product the obtained fragment was cloned into the plasmid pET17b previously digested with the same enzymes to yield the plasmid pNG23. To obtain plasmid pNG25, the BglII/HindIII fragment containing *olsB* of *B. cenocepacia* together with the T7 promoter of pET17b was subcloned from pNG23 and cloned into BamHI/HindIII-digested pBBR1-MCS. Via diparental mating using *E. coli* S17-1 as a donor strain, pNG25 was introduced into *S. meliloti* CS111 to obtain CS111.pNG25 which was used as a receptor strain for the cosmid bank. Cosmid transconjugants were selected on TY containing the following antibiotics: tetracycline 10 μg ml^{-1}; nalidixic acid 20 μg ml^{-1}; chloramphenicol 60 μg ml^{-1}. Four hundred individual *S. meliloti* transconjugants harbouring random fragments of the library were picked and streaked for subsequent lipid analysis in small patches (1 cm by 1 cm) on fresh plates. After growth for 3 days, cells from each patch were collected with a toothpick and swirled in 60 μl of chloroform–methanol (1:1, v/v) as described previously (Benning and Somerville, 1992). After the addition of 20 μl of 1 M KCl-0.2 N H_3PO_4, the tubes were vortexed and centrifuged to separate the organic and aqueous phases. A 10 μl aliquot from the lipid-containing lower phase was spotted on a HPTLC silica gel 60 plate (Merck). The TLC was developed in one dimension using the solvent system chloroform–methanol–glacial acetic acid (130:50:20, v/v). Under these conditions unmodified OL was readily separated from the modified OL we were looking for and from other polar lipids such as PC, PG and CL. Lipids were detected first with iodine and subsequently primary amine containing lipids were visualized by spraying the plates with a solution of 0.2% ninhydrin in acetone and heating the

Table 4. Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant characteristics	Reference
Rhizobium tropici strains		
CIAT899	Wild-type; acid-tolerant, Nal^r	Martínez-Romero *et al.* (1991)
899-*olsC*Δ*1*	CIAT899 carrying a 211 bp non-polar deletion in *olsC*	Rojas-Jiménez *et al.* (2005)
MAV04	CIAT899 carrying a deletion in *olsE*	This work
MAV05	CIAT899 carrying deletions in *olsC* and *olsE*	This work
Sinorhizobium meliloti strains		
CS111	*pssA*-deficient mutant of wild-type 1021	Sohlenkamp *et al.* (2004)
Burkholderia cenocepacia strains		
J2315	Wild-type	Holden *et al.* (2009)
Escherichia coli strains		
DH5α	*recA*1, Φ80 *lacZ*Δ*M15*; cloning strain	Hanahan (1983)
S17-1	*thi pro recA hsdR*$^-$ *hsdM*$^+$ RP4 integrated in the chromosome, 2-Tc::Mu, Km::Tn*7*(Tp^r/Sm^r)	Simon *et al.* (1983)
Plasmids		
pET17b	Expression vector, Cb^r	Studier (1991)
pET9a	Expression vector, Kan^r	Studier (1991)
pRK404	Broad-host-range vector, tetracycline-resistant	Ditta *et al.* (1985)
pBBR1MCS	Broad-host-range plasmid, chloramphenicol-resistant	Kovach *et al.* (1994)
pUC18	Cloning vector, ampicillin-resistant	Yanisch-Perron *et al.* (1985)
pRK2013	Helper plasmid; Km^r	Ditta *et al.* (1985)
pVK102	Cosmid vector	Vargas *et al.* (1990)
pK18mob*sacB*	Conjugative suicide vector, kanamycin-resistant	Schäfer *et al.* (1994)
pNG23	*olsB* of *B. cenocepacia* cloned in pET17b	This work
pNG25	*olsB* of *B. cenocepacia* subcloned as a BglII/HindIII fragment from pNG23 into BamHI/HindIII-digested pBBR1MCS	This work
pCCS98	*olsC* of *R. tropici* in pET9a	This work
pCos94	pVK102 derivative containing the *olsE* gene	This work
pEMAV01	1 kb fragment upstream of *olsE*, cloned as SmaI/BamHI fragment in pUC18	This work
pEMAV02	1 kb fragment downstream of *olsE*, cloned as BamHI/HindIII fragment in pUC18	This work
pEMAV03	1 kb upstream and 1 kb downstream sequences flanking *olsE*, cloned into pUC18	This work
pPMAV04	Suicide vector for construction of mutant MAV04 and MAV05	This work
pURMAV03	*olsE*-containing 3.5 kb fragment of pCos94 cloned as PstI/PstI fragment in pUC18	This work
pERMAV04	*olsE*-containing 3.5 kb fragment of pCos94 cloned as PstI/PstI fragment in pRK404	This work
pERMAV06	pET9a cloned as a BamHI fragment into pRK404	This work
pEMAV07	ORF1 in pET9a	This work
pEMAV08	ORF2 in pET9a	This work
pEMAV09	*olsE* in pET9a	This work
pERMAV11	pEMAV07 cloned as a BamHI fragment into pRK404	This work
pERMAV12	pEMAV08 cloned as a BamHI fragment into pRK404	This work
pERMAV13	pEMAV09 cloned as a BamHI fragment into pRK404	This work
pERMAV15	pCCS98 cloned as a BamHI fragment into pRK404	This work
pEMAV16	*olsC* cloned as a BamHI/BglII fragment into BamHI-digested pEMAV09	This work
pERMAV17	pEMAV16 cloned as a BamHI fragment into pRK404	This work

plates at 120°C. A transconjugant containing a gene modifying S1 should have two ninhydrin-positive lipids being either S2 and S1 similar to the lipid profile of the *R. tropici* mutant 899-*olsC*Δ*1* or S1 and P1. Once *S. meliloti* CS111.pNG25. pCos94 had been identified, cosmid pCos94 was isolated and re-introduced by conjugation into CS111.pNG25 to confirm that the lipid phenotype was caused by the presence of the cosmid and not by an independent mutation leading to the activation of an endogenous *S. meliloti* gene. In this independent transconjugant again the presence of S2 was observed. Next, the insert of pCos94 was digested with PstI. The resulting PstI/PstI fragments were subcloned into the broad host vector pRK404 and again mobilized into CS111. pNG25 repeating the lipid analysis described above. A pRK404-derived plasmid containing an approximately 3.5 kb insert was identified (pERMAV04) and its insert sequenced after subcloning into pUC18.

Expression of the three candidate ORFs from R. tropici *CIAT899*

The three candidate ORFs from plasmid pERMAV04 were separately amplified using genomic DNA from *R. tropici* CIAT899 as a template and XL-PCR polymerase (Applied Biosystems). Specific oligonucleotide primers incorporating

NdeI and BamHI sites into the final PCR products were used (oORF1-01 and oORF1-02 for ORF1; oORF2-01 and oORF2-02 for ORF2; oORF3-01 and oORF3-02 for ORF3). After digestion with the respective enzymes, the PCR products were cloned as NdeI/BamHI fragments into pET9a to yield the plasmids pEMAV07, pEMAV08 and pEMAV09 respectively. These three plasmids and pET9a were linearized with BamHI and were cloned into the BamHI site of pRK404, similarly to an earlier description (Gao *et al*., 2004) yielding the plasmids pERMAV11, pERMAV12, pERMAV13 and pERMAV06 respectively. Plasmids were mobilized into *S. meliloti* CS111.pNG25 and the lipids of the transconjugants were assayed as described above.

Deletion of the olsE *gene from* R. tropici *CIAT899*

Oligonucleotide primers oOlsX899ar1 and oOlsX899ar2 were used in a PCR (XL-PCR kit; Applied Biosystems) to amplify about 1.0 kb of genomic DNA upstream of the putative *olsE* gene from *R. tropici* CIAT899, introducing SmaI and BamHI sites into the PCR product. Similarly, primers oOlsX899ab1 and oOlsX899ab2 were used to amplify about 1.0 kb of genomic DNA downstream of the putative *olsE* gene from *R. tropici* CIAT899, introducing BamHI and HindIII sites into the PCR product. After digestion with the respective enzymes, PCR products were cloned as SmaI/BamHI or BamHI/HindIII fragments into pUC18 to yield the plasmids pUMAV01 and pUMAV02 respectively. Then, the BamHI/HindIII fragment from pUMAV02 was subcloned into pUMAV01 to yield pUMAV03. Plasmid pUMAV03 was digested with SmaI and HindIII to subclone the regions usually flanking the rhizobial *olsE* gene into the suicide vector pK18*mobsacB* (Schäfer *et al*., 1994) to yield pPMAV04. Via diparental mating using *E. coli* S17-1 (Simon *et al*., 1983) as a mobilizing strain, pPMAV04 was introduced into the wild-type strain *R. tropici* CIAT899. Transconjugants were selected on TY medium containing neomycin to select for single recombinants in a first step. The plasmid pK18*mobsacB* contains the *sacB* gene (Selbitschka *et al*., 1993), which confers sucrose sensitivity to many bacteria. Growth of the single recombinants on high sucrose will therefore select for double recombinants and the loss of the vector backbone of pK18*mobsacB* from the bacterial genome. Single recombinants were grown under non-selective conditions in complex medium for 1 day before being plated on TY medium containing 12% (w/v) sucrose. Several large and small colonies grew after 5 days, and the membrane lipids of eight candidates were analysed by *in vivo* labelling during growth on complex medium with [^{14}C]acetate and subsequent TLC (data not shown). Four clones lacking S2 and P2 were identified. Southern blot analysis confirmed that the S2- and P2-deficient strains were indeed double recombinants in which the gene *olsE* was deleted (data not shown).

Construction of a double mutant deficient in olsE *and* olsC

To construct a *R. tropici* double mutant deficient in *olsE* and *olsC*, the suicide plasmid pPMAV04 was conjugated into the *olsC*-deficient mutant 899-*olsC*Δ*1* (Rojas-Jiménez *et al*., 2005). The selection for double recombinants was performed in two steps as described above. Ten isolated colonies were chosen and their lipids were labelled with [^{14}C]acetate (see below). We used *R. tropici* CIAT899 and the mutant *899-olsC*Δ*1* as control strains. The lipids were analysed by TLC. One strain presented the expected phenotype which is the absence of the OLs S2, P1 and P2. Therefore this colony was called MAV05. Southern blot analysis confirmed that MAV05 was indeed a double recombinant in which the genes *olsC* and *olsE* were deleted (data not shown).

Complementation of the R. tropici *mutants MAV04, MAV05 and 899-*olsCΔ1

To show that the observed mutant phenotypes were caused by the introduced deletion and not by a secondary independent mutation, the mutants were complemented. The *olsE*-deficient mutant MAV04 was complemented with the plasmid pERMAV13. In this construct *olsE* is expressed under control of the T7 promoter. In earlier work we had observed constitutive expression from this promoter in different *Rhizobiaceae*. In the study published by Rojas-Jiménez *et al*. (2005) the mutant 899-*olsC*Δ*1* was complemented by *olsC* under its endogenous promoter, but in order to be able to compare the results from the complementation of the *olsC*-deficient mutant with the complementations of the mutants MAV04 and MAV05 a new plasmid was constructed.

The gene *olsC* was amplified using genomic DNA from *R. tropici* CIAT899 as a template and XL-PCR polymerase (Applied Biosystems). Specific oligonucleotide primers incorporating NdeI and BamHI sites into the final PCR product were used (o5B_olsC and o3_olsC). The digested PCR product was cloned into pET9a to yield the plasmid pCCS98. Plasmid pCCS98 was linearized with BamHI and cloned into BamHI-digested pRK404 to yield pERMAV15. To complement the double mutant MAV05 a plasmid containing both *olsC* and *olsE* under the control of the T7 promoter was constructed. A DNA fragment containing *olsC* under the control of the T7 promoter was subcloned from pCCS98 as BamHI/BglII fragment into the BamHI-digested pEMAV09 yielding plasmid pEMAV16. Plasmid pEMAV16 therefore contains the genes *olsC* and *olsE*, both under the control of separate T7 promoters. Subsequently, pEMAV16 was linearized with BamHI and cloned into BamHI-linearized pRK404 to yield pERMAV17.

In vivo *labelling of* S. meliloti *and* R. tropici *with [^{14}C]acetate and quantitative analysis of lipid extracts*

The lipid compositions of bacterial strains were determined following labelling with [1-^{14}C]acetate (Amersham Biosciences). Cultures (1 ml) of wild-type and mutant strains were inoculated from pre-cultures grown in the same medium. After addition of 0.5 μCi of [^{14}C]acetate (60 mCi mmol^{-1}) to each culture, the cultures were incubated for 4 h. The cells were harvested by centrifugation, washed with 500 μl of water 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, UK) plates and

were separated in two dimensions using chloroform/methanol/water (140:60:10, v/v) as a mobile phase for the first dimension and chloroform/methanol/glacial acetic acid (130:50:20, v/v) for the second dimension. Primary amine-containing lipids were visualized by spraying the plates with a solution of 0.2% ninhydrin in acetone and subsequent treatment at 120°C for 10 min. To visualize the membrane lipids, developed two-dimensional TLC plates were exposed to autoradiography film (Kodak) or to a PhosphorImager screen (Amersham Biosciences). The individual lipids were quantified using ImageQuant software (Amersham Biosciences).

Separation of IM and OM and determination of their respective lipid compositions

Membrane separation was performed as described previously (de Maagd and Lugtenberg, 1986; Klüsener *et al*., 2009), with minor modifications. A 400 ml culture *R. tropici* CIAT899 was grown in TY medium at 30°C overnight to an OD_{600} of 0.5–0.6. Cells were harvested by centrifugation at 10 000 *g*, 4°C, for 10 min. The cells were resuspended in 24 ml of lysis buffer [50 mM Tris-HCl, pH 7.5, 20% (w/v) sucrose, 0.2 M KCl, 0.2 mM dithiothreitol (DTT), 0.2 mg ml^{-1} DNase I, 0.2 mg ml^{-1} RNase A] and disrupted by two passages through a pre-chilled French pressure cell at 16 000 lb in^{-2}. The lysate was treated with 0.5 mg ml^{-1} lysozyme for 1 h on ice and centrifuged at 10 000 *g* for 20 min, 4°C, to remove the unbroken cells. The supernatant was centrifuged at 150 000 *g* (SW40Ti), 4°C, for 1 h to collect the membranes. The resulting membrane pellet was carefully resuspended in 2 ml of 20% (w/v) sucrose containing 5 mM EDTA, pH 7.5, and 0.2 mM DTT. Material that was not completely suspended was removed by centrifugation for 5 min at 16 000 *g*. The gradient was prepared by layering 7.5 ml of 53% (w/v) sucrose over a cushion of 2.5 ml of 70% (w/v) sucrose. Both sucrose solutions contained 5 mM EDTA, pH 7.5. The membrane suspension was layered on the top of the gradient, and sucrose density gradient ultracentrifugation was carried out at 100 000 *g* (SW40Ti), 4°C, for 16 h. After ultracentrifugation, the separated membranes were fractionated in 500 µl aliquots. For each fraction the protein concentration was estimated, and the density, the NADH activity and the 2-keto-3-deoxyoctonate (KDO) content were determined. The protein distribution was estimated using absorption measurements at 280 nm (Scopes, 1987). The NADH oxidase activity was determined by the method of Osborn *et al*. (1972) and the KDO content was determined as described earlier after the fractions had been precipitated twice with 10% (w/v) TCA (Karkhanis *et al*., 1978). NADH oxidase activity and KDO content were used as marker for the IM and OM respectively. Fractions corresponding to the IM and the OM were pooled and the lipids were extracted with 1-butanol (Bremer, 1963). Lipids were analysed using two-dimensional TLC as described above and the lipids were detected by oxidative charring using ceric sulphate in sulphuric acid (Villaescusa and Pettit, 1972). The lipid spots were quantified using the program ImageQuant (Applied Biosystems).

ESI-MS/MS analysis of lipids S1 and S2

In order to identify in which part of the OL S2 the modification is encountered, a 1 l culture of the mutant 899-*olsCΔ1* (Rojas-Jiménez *et al*., 2005) was grown to an optical density at 620 nm of 1.0 in TY medium, and lipids were extracted according to a modified Bligh-and-Dyer procedure (Bligh and Dyer, 1959). Lipids were fractionated using a silica column and chloroform/methanol/water (140:60:8, v/v) as a mobile phase. Fractions were analysed by one-dimensional TLC using chloroform/methanol/water (140:60:8, v/v) as a mobile phase. Fractions containing OLs were identified by iodine and ninhydrin staining as described above. OL-containing fractions were dried under N_2 stream and re-dissolved in methanol/chloroform (1:1, v/v). LC-ESI/MS of lipids was performed using an Agilent 1200 Quaternary LC system coupled to a QSTAR XL quadrupole time-of-flight tandem mass spectrometer (Applied Biosystems, Foster City, CA). An Ascentis® Si HPLC column (5 µm, 25 cm × 2.1 mm) was used. Mobile phase A consisted of chloroform/methanol/aqueous ammonium hydroxide (800:195:5, v/v). Mobile phase B consisted of chloroform/methanol/water/aqueous ammonium hydroxide (600:340:50:5, v/v). Mobile phase C consisted of chloroform/methanol/water/aqueous ammonium hydroxide (450:450:95:5, v/v). The elution programme consisted of the following: 100% mobile phase A was held isocratically for 2 min and then linearly increased to 100% mobile phase B over 14 min and held at 100% B for 11 min. The LC gradient was then changed to 100% mobile phase C over 3 min and held at 100% C for 3 min, and finally returned to 100% A over 0.5 min and held at 100% A for 5 min. The total LC flow rate was 300 µl min^{-1}. The post-column splitter diverted ~10% of the LC flow to the ESI source of the Q-Star XL mass spectrometer, with MS settings as follows: IS = –4500 V, CUR = 20 psi, GS1 = 20 psi, DP = –55 V and FP = –150 V. Nitrogen was used as the collision gas. Data acquisition and analysis were performed using Analyst QS software version 1.1.

Determination of the position of the hydroxyl group introduced by OlsC into OLs

Large cultures (4 l) of the *R. tropici* mutant MAV05 and the strain MAV05.pERMAV15 were grown to an OD_{620} of 0.9 in TY medium. MAV05 only forms S1 and MAV05.pERMAV15 forms preferentially P1. Cells were harvested and lipids were extracted from the cell pellets according to a modified Bligh-and-Dyer method. OLs S1 and P1 were purified using preparative TLC using Si500F plates (Baker) in two steps. First chloroform/methanol/water (140:60:10, v/v) was used as a mobile phase and the OLs were purified from the silica. Enriched OLs were further purified by a second preparative TLC using chloroform/methanol/glacial acetic acid (130:50:20, v/v) as mobile phase. ESI-MS/MS analysis of S1 and P1 was performed as described above.

The derivatization of the lipids was performed essentially as described by Gibbons *et al*. (2008). Purified OLs S1 and P1 were hydrolysed in acidic methanol, and then converted to TMS ethers. Hydroxy fatty acid standards (α- and β-hydroxy palmitic acid, α- and β-hydroxy stearic acid) were processed and analysed in parallel with the samples. Typically, about 1 mg of sample was dried in a Reacti-vial and samples were hydrolysed by adding 300 µl of 1 M HCl in methanol and heated at 80°C for 16 h. The reactions were cooled and solvents were evaporated under a stream of nitrogen. Next, 200 µl Tri-Sil HTP reagent (Thermo) was added to the dried

samples. After incubation for 1 h at 25°C a 20 µl aliquot was diluted 1:6 in hexane and transferred to a new vial for GC/MS analysis.

GC/MS was performed using a Clarus 600T MS instrument coupled to a Clarus 600 gas chromatography system (Perkin Elmer). The column was a Elite-5 MS (0.32 mm internal diameter and 0.25 µm phase thickness) from Perkin Elmer. The temperature programme of the GC was as follows: the column oven temperature was initially held at 140°C for 6 min, increased to 250°C at a rate of 4°C min^{-1} and finally held at 250°C for 5 min. The total run time was 38.5 min. The injector was operated in the split mode, and the temperature of the injector was kept at 250°C. Helium was the carrier gas at a constant pressure of 7 psi. The instrument was operated in the electron impact (EI) mode with the electron energy set at 70 eV.

Plant tests

Phaseolus 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 220 ml of nitrogen-free nutrient solution (Fahraeus, 1957) containing agar at 0.7% and were inoculated with about 50 000 cfu ml^{-1} per plant. Plants were grown in a controlled growth chamber at 28°C with a 15 h day/9 h night cycle and harvested 21 days after inoculation. Nitrogenase activity of nodulated roots was determined by the acetylene reduction assay as described previously (Martínez *et al*., 1985). Nitrogen fixation activity per plant was normalized with respect to the nodule fresh weight per plant.

Acknowledgements

This research was supported by grants from CONACyT-Mexico (46020-N) and DGAPA/UNAM (IN217907 and IN201310). Z.G. and the mass spectrometry facility in the Department of Biochemistry, Duke University Medical Center, were supported by a LIPID MAPS glue grant (GM-069338) from the National Institutes of Health. M.Á.V.-G. is a PhD student from the Programa de Doctorado en Ciencias Biomédicas, Universidad Nacional Autónoma de México and is a recipient of a scholarship from the Consejo Nacional de Ciencia y Tecnología, Mexico. We are grateful to Alfonso Leija Salas for his support in nodule analysis.

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