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Mutations in lipopolysaccharide biosynthetic genes impair maize rhizosphere and root colonization of *Rhizobium tropici* CIAT899

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Summary

Three transposon mutants of *Rhizobium tropici* CIAT899 affected in lipopolysaccharide (LPS) biosynthesis were characterized and their maize rhizosphere and endophytic root colonization abilities were evaluated. The disrupted genes coded for the following putative products: the ATPase component of an O antigen ABC-2 type transporter (*wzt*), a nucleotide-sugar dehydratase (*lpsβ2*) and a bifunctional enzyme producing GDP-mannose (*noeJ*). Electrophoretic analysis of affinity purified LPS showed that all mutants lacked the smooth LPS bands indicating an O antigen minus phenotype. In the *noeJ* mutant, the rough LPS band migrated faster than the parental band, suggesting a truncated LPS core. When inoculated individually, the *wzt* and *noeJ* mutants colonize the rhizosphere and root to a lower extent than the parental strain while no differences were observed between the *lpsβ2* mutant and the parental strain. All mutants were impaired in competitive rhizosphere and root colonization. Pleiotropic effects of the mutations on known colonization traits such as motility and growth rate were observed, but they were not sufficient to explain the colonization behaviours. It was found that the LPS mutants were sensitive to the maize antimicrobial 6-methoxy-2-benzoxazolinone (MBOA). Only the combined effects of altered growth rate and susceptibility to maize antimicrobials could account for all the observed colonization phenotypes. The results suggest an involvement of the LPS in protecting *R. tropici* against maize defence response during rhizosphere and root colonization.

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Introduction

Rhizobia are best known as the nodule-inducing bacterial symbionts of legume plants. However, an increasing number of studies are uncovering their lifestyles as rhizosphere inhabitants and as endophytes of several plant families. Various rhizobial species and strains are able to colonize the rhizosphere or the interior of wheat (Sabry *et al*., 1997), cultivated and wild rice (Yanni *et al*., 1997; Chaintreuil *et al*., 2000), barley and canola (Lupwayi *et al*., 2004), maize (Chabot *et al*., 1996; Gutierrez-Zamora and Martínez-Romero, 2001), *Arabidopsis* (Stone *et al*., 2001) and poplar (Doty *et al*., 2005). Plant growth promotion has been reported in several cases opening the possibility to use rhizobia as inoculants for non-legume plants. Rhizobia have the advantage in that they have been safely used as plant inoculants in agriculture over a period of more than 100 years (Gutierrez-Zamora and Martínez-Romero, 2001).

Rhizobium tropici CIAT899 was isolated from a common bean nodule but has a broad legume host range (Martínez-Romero *et al*., 1991; Hernandez-Lucas *et al*., 1995; Acosta-Duran and Martínez-Romero, 2002). Recently, we have found that CIAT899 is a good maize rhizosphere and root endophytic colonizer, promoting growth and being as competitive as *Rhizobium etli* Ch24–10, one of the best maize endophytes (Rosenblueth and Martínez-Romero, 2004). It appears that *R. tropici* strains are well adapted to endophytic colonization as they have been isolated inside poplar trees as well (Doty *et al*., 2005). CIAT899 is highly resistant to acid soil conditions and to aluminum (Graham *et al*., 1994; Vinuesa *et al*., 2003), and it is tolerant to salinity, high temperatures and heavy metals (Martínez-Romero *et al*., 1991; Riccillo *et al*., 2000). These characteristics make it a useful strain to be used as inoculant under environmental stress conditions (Bernal *et al*., 2004). *Rhizobium tropici* strains are successfully used as bean inoculants in Brazil (Hungria *et al*., 2000). As bean and maize are intercropped

by small farmers in Brazil (M. Hungria, pers. comm.) *R. tropici* could be used as inoculant for both crops.

The potential environmental benefits of using plant growth promoting (PGP) bacteria to reduce the use of agrochemicals have encouraged research directed to identify traits required for a successful rhizosphere or endophytic root colonization (Lugtenberg *et al*., 2001). Several molecular determinants involved in this process have been identified by the analysis of colonization impaired mutants. Most studies on this topic have focused on fluorescent pseudomonads, with work reported also on *Enterobacter cloacae* (Lohrke *et al*. 2002; Roberts *et al*., 2006), *Azospirillum brasilense* (Jofre *et al*., 2004), *Azoarcus* (Dorr *et al*., 1998; Reinhold-Hurek *et al*., 2006), and recently on *Burkholderia vietnamensis* (O'Sullivan *et al*. 2007). On the contrary, nothing is known about rhizobial factors required for rhizosphere or root colonization of non-legume plants.

The lipopolysaccharides (LPS) are complex glycolipids, which are the major components of the external leaflet of the outer membrane (OM) of Gram-negative bacteria. They can be divided in three structural regions: the lipid A that anchors the LPS to the OM, the core which is a short oligosaccharide attached to the lipid A, and a distal polysaccharide known as the O antigen (Raetz and Whitfield, 2002). Given its location, the LPS are important for the interaction with the environment and eukaryotic hosts. In microbe–plant interactions, several roles have been proposed for the LPS. In the rhizobia–legume association, they may act as communication signals, they can mask bacterial cell surface components that can be detrimental to the symbiosis, contribute to cell surface adaptation for optimal cell–cell contact within the symbiosome, or be a protective barrier against plant antimicrobials (Lerouge and Vanderleyden, 2002; Becker *et al*., 2005). The latter has been suggested as well as a function of LPS during plant–pathogen interactions (Newman *et al*., 2001). In the highly specialized symbiosis between *Sinorhizobium meliloti* and *Medicago*, the LPS are involved in the suppression of plant defence responses (Scheidle *et al*., 2005; Tellstroem *et al*., 2007).

A role for the LPS during rhizosphere and endophytic root colonization of different crops by PGP bacteria has been suggested by studies performed on *Pseudomonas fluorescens* and *A. brasilense*. O antigen minus mutants of several biocontrol strains of *P. fluorescens* showed reduced colonization and the same was reported for an *A. brasilense* mutant with an altered LPS core (Simons *et al*., 1996; Duijff *et al*., 1997; Dekkers *et al*., 1998; Jofre *et al*., 2004). However, these mutants showed pleiotropic effects that could account for their impaired colonization. While the *Pseudomonas* mutants had reduced growth rates, the *A. brasilense* mutant was affected in exopolysaccharide (EPS) production. Nevertheless, a *Pseudomonas* mutant with a shortened O antigen did not have pleiotropic effects but still showed reduced competitiveness, pointing to a direct role of LPS in colonization (Dekkers *et al*., 1998). The defective phenotype was observed on tomato, radish and wheat suggesting a non-host-specific function of the LPS.

We identified three *R. tropici* mutants with LPS defects and found that they were affected in maize rhizosphere and root endophytic colonization. By analysing the genetic nature of the mutations and considering pleiotropic effects but also possible direct roles of the LPS we aimed at understanding the involvement of LPS in maize colonization by *R. tropici* CIAT899.

Results

Identification of LPS defective mutants

Auto-agglutination in unshaken liquid medium is a common phenotype displayed by rhizobia with LPS defects (de Maagd *et al*., 1989; Priefer, 1989; Garcia-de los Santos and Brom, 1997). Three *R. tropici* mutants, designated CIAT899-E1, CIAT899-E2 and CIAT899-E3, showing this phenotype, were identified by screening a transposon mutant library (Fig. S1).

The LPS from the parental and mutant strains were extracted with the proteinase K and hot phenol-water methods, resolved by polyacrylamide gel electrophoresis (PAGE) in the presence of SDS, and silver stained. The extract obtained from the parental strain by the proteinase K method produced a LPS profile showing a low molecular weight (LMW) band and a series of high molecular weight (HMW) bands in a ladder-like pattern (Fig. 1A). Comparisons with known LPS profiles from rhizobia and other bacteria suggested that the LMW band corresponded to the rough LPS (lipid A plus core) and the HMW bands to the smooth LPS (complete LPS molecules with different number of attached O antigen units) (Carlson, 1984; Vinuesa *et al*., 1999). The mutants did not produce the smooth LPS bands and showed an accumulation of a rough LPS band with the same (CIAT899-E1 and CIAT899-E2) or higher (CIAT899-E3) electrophoretic mobility than the parental band (Fig. 1A).

When the LPS were extracted with the hot phenol-water procedure, the parental rough LPS band was recovered in the water phase while the smooth LPS bands appeared mainly in the phenol phase (Fig. 1B), consistent with the use of this latter phase as the starting material for the isolation and structural characterization of the *R. tropici* CIAT899 O antigen by Gil-Serrano and colleagues (1995). The phenol phase of all mutants lacked any stainable material in concordance with an O antigen minus phenotype (Fig. 1B). The rough LPS bands from mutants CIAT899-E1 and CIAT899-E2 showed the same partition-

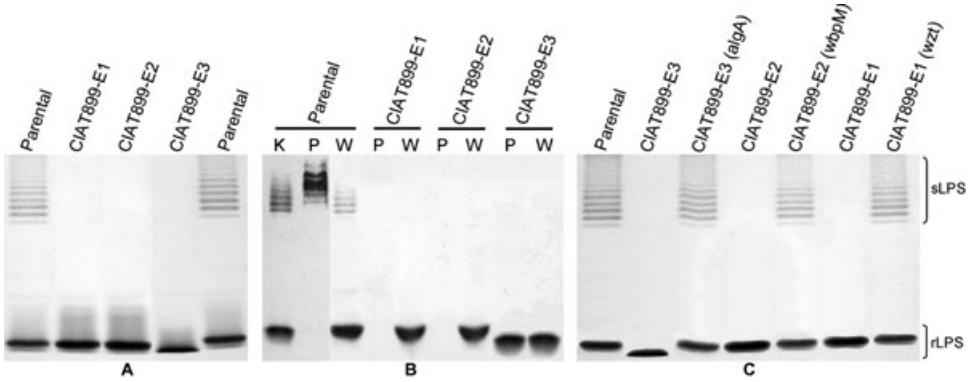

Fig. 1. Silver-stained PAGE profiles of the LPS present in the proteinase K (A), hot phenol-water extracts (B) or affinity purified material (C) of the *R. tropici* parental strain, its LPS mutants or complemented mutants. In B, extracts from the phenol (P) or water (W) phase are indicated above lanes together with a proteinase K extract (K) for comparison. sLPS, smooth LPS; rLPS, rough LPS.

ing behaviour as the parental band (Fig. 1B). In contrast, the corresponding band from mutant CIAT899-E3 partitioned in both phases (Fig. 1B). The higher electrophoretic mobility and different partitioning behaviour of the CIAT899-E3 rough band, together with the genetic evidence (see below), suggest that it represents a rough LPS with a truncated core.

To confirm that the bands detected by silver staining correspond to LPS molecules, affinity purification was performed as described by Valverde and colleagues (1997). This method uses the specific interaction between polymyxin B and the lipid A portion of the LPS. When the affinity-purified material was visualized by silver staining, the same bands previously observed were detected for the parental and mutant strains, confirming that they represent LPS molecules (Fig. 1C).

R. tropici CIAT899 EPS mutants are known to produce non-gummy colonies (Milner *et al*., 1992). In contrast, the mutants identified here were as gummy as those produced by the parental strain in PY, YEM and minimal media (MM) (Fig. S2A). Additionally, the mutants and parental strain showed similar fluorescent intensities under UV when grown on PY containing calcofluor (Fig. S2B) indicating normal production of succinoglycan-like EPS (Gil-Serrano *et al*., 1990; Andrade *et al*., 2002). Gel fixation with Alcian Blue prior to silver staining, a method used to visualize acidic capsular polysaccharides (Corzo *et al*., 1991), did not reveal any difference between the mutants and parental strain (data not shown).

The *R. tropici* derivatives were symbiotically defective when inoculated on common bean plants. CIAT899-E3 did not induce nodulation, while CIAT899-E1 and CIAT899-E2 induced the formation of scattered, small, Fix$^-$ pseudonodules. These phenotypes are similar to those reported for *R. etli* LPS mutants in common bean (Noel *et al*., 1984; Garcia-de los Santos and Brom, 1997; Lerouge *et al*., 2001). In contrast, EPS mutants of CIAT899 are known to induce the formation of normal and Fix$^+$ nodules (Milner *et al*., 1992).

Genetic characterization of the LPS mutants

Blots of restricted total DNA hybridized with a labelled *gusA* probe showed single transposon insertions in all mutants (data not shown). A fragment of the transposon along with *R. tropici* DNA flanking the *gusA* border was cloned from each mutant and partially sequenced.

In CIAT899-E1, the transposon disrupted a gene similar to *wzt* from several bacteria (Table 1). These genes code for the ATPase component of ABC-2 type transporters which translocate the O antigen from the cytoplasmic face of the inner membrane to the periplasmic face where it is ligated to the lipid A-core (Raetz and Whitfield, 2002). A cosmid restoring normal nodulation of common bean was isolated and shown by polymerase chain reaction (PCR) to contain the same genetic region cloned from mutant CIAT899-E1 (data not shown). Partial sequencing of this cosmid revealed the complete *wzt* gene (1359 nt) and an 831 nt-long ORF upstream of *wzt* with an 11 nt overlap (Fig. 2). The *in silico* translated product of the second ORF was similar to proteins known as Wzm which correspond to the transmembrane components of the O antigen ABC transporters (data not shown). A partially sequenced ORF (402 nt) was identified downstream of *wzt* (Fig. 2). The putative product was similar to β-glycosyltransferases present in O antigen gene clusters of other bacteria (data not shown).

Sequencing of the region flanking the transposon in CIAT899-E2 revealed that the transposon disrupted an ORF whose *in silico* translated product was highly similar (> 84%) to LpsB from *Rhizobium leguminosarum* 3841 and to Lpsβ2 from *R. etli* CFN42, both implicated in O antigen biosynthesis (Table 1, Fig. 2) (Garcia-de los Santos and Brom, 1997; Young *et al*., 2006). The ORF

Table 1. Similarity of the products of the genes disrupted in the *R. tropici* mutants with homologous proteins as determined with the BLASTX program.

Mutant (length of sequence compared)	Similar protein/organism	Identity/ similarity (%)	E[a]	Product	Accesion number
CIAT899-E1 (1359 nt)	Wzt/*Raoultella terrigena* ATCC 33257	37/53	3e-65	Putative ATP-binding protein of ABC transport system[b]	AAQ82930
	Wzt/*Klebsiella pneumoniae*	38/54	8e-64	ATP-binding protein of ABC-2 type transport system[b]	AAN06493
	Wzt/*Serratia marcescens* N28b	36/54	9e-63	putative ATP-binding protein of ABC transport system[b]	AAC00182
	Wzt/*Escherichia coli* F492	35/53	4e-56	ATP-binding component of ABC transporter[b]	BAA28325
	Wzt/*Rhizobium etli* CE3	38/53	7e-52	ATP-binding component of ABC transporter[c]	AAK51165
CIAT899-E2 (1913 nt)	LpsB/*Rhizobium leguminosarum* 3841	72/85	0	Putative dTDP-glucose 4,6-dehydratase	CAE00204
	Lpsβ2/*Rhizobium etli* CFN42	72/84	0	Putative dTDP-glucose 4,6-dehydratase	AAB66669
	WbpM/*Pseudomonas aeruginosa* PA01	51/67	1e-158	UDP-GlcNAc C_6 dehydratase/ C4 reductase	AAC45867
CIAT899-E3 (624 nt)	NoeJ/*Rhizobium tropici* CIAT899	100/100	3e-92	Putative mannose-1-phosphate guanylyltransferase	CAC38770
	NoeJ/*Rhizobium etli* CFN42	82/93	2e-99	Putative mannose-1-phosphate guanylyltransferase	ABC92008
	NoeJ/*Sinorhizobium* sp. NGR234	67/82	6e-78	Putative mannose-1-phosphate guanylyltransferase	AAB91607
	ManC/*Bradyrhizobium japonicum* USDA110	56/73	5e-61	Putative GDP-mannose-1-phosphate guanylyltransferase	BAC51184
	AceF/*Gluconacetobacter xylinus* C1	52/67	5e-56	Phosphomannose isomerase/ mannose-1-phosphate guanylyltransferase	CAA72316
	AlgA/*Pseudomonas aeruginosa*	51/70	2e-54	Phosphomannose isomerase/ mannose-1-phosphate guanylyltransferase	AAA25972

a. Expect value reported by the BLASTX program.
b. Located inside an O antigen biosynthetic gene cluster.
c. Located inside the LPS α region.

disrupted in CIAT899-E2 was named *lpsβ2* because of its similarity to and functional complementation by the CFN42 gene (see below). The most similar protein with known function was WbpM from *Pseudomonas aeruginosa* an enzyme-producing UDP-D-acetylquinovosamine (UDP-D-QuiNAc) from UDP-*N*-acetylglucosamine (UDP-GlcNAc), and essential for the biosynthesis of the B-band O antigen in some *P. aeruginosa* serotypes (Creuzenet and Lam, 2001). No other ORF was detected in the sequenced 100 nt upstream region of the CIAT899 *lpsβ2* gene.

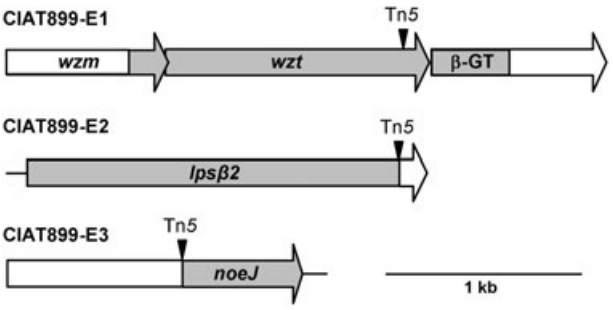

Fig. 2. Genetic organization of the regions disrupted in the *R. tropici* LPS mutants. The site of transposon (Tn*5*) insertion is indicated. The shaded areas represent the sequenced regions submitted to GenBank. The lengths of the partially sequenced ORFs were assumed to be equal to the most similar genes in the GenBank database according to BLAST searches. β-GT, β-glycosyltransferase.

Mutant CIAT899-E3 had an insertion in a gene coding for a protein similar to phosphomannose isomerases (PMI) known as NoeJ, ManC, AceF or AlgA in different Gram-negative bacteria (Table 1). These enzymes belong to the type II PMIs which, in addition to the PMI activity, have guanosine diphospho-D-mannose pyrophosphorylase (GMP) activity. Different domains in the protein code for these functions which are required in non-consecutive steps in the biosynthesis of the GDP-D-mannose used for mannosylation of surface polysaccharides (Jensen and Reeves, 1998). The position of the transposon insertion (Fig. 2) suggests that this mutant produces an incomplete protein with a partial GMP and no PMI domain. The two most similar proteins with experimentally tested function were AceF from *Gluconacetobacter xylinus* and AlgA from *P. aeruginosa* (Table 1). Because of the Nod⁻ phenotype of CIAT899-E3 the interrupted gene was named *noeJ*. Nogales and colleagues (2002) reported a Nod⁻ CIAT899

Table 2. Maize rhizosphere and endophytic root colonization by the *R. tropici* parental strain, its LPS mutants and complemented mutants.

Strain	Log_{10} (cfu g^{-1} fresh weight)	
	Rhizosphere	Root
CIAT899	9.7 ± 0.4 a	5.5 ± 0.3 a
CIAT899-E1	8.8 ± 0.2 c	4.8 ± 0.2 c
CIAT899-E2	9.6 ± 0.3 a	5.4 ± 0.2 a
CIAT899-E3	8.6 ± 0.2 c	4.9 ± 0.1 c
CIAT899-E1 (*wzt*)	9.4 ± 0.2 b	5.4 ± 0.3 a
CIAT899-E2 (*wbpM*)	9.2 ± 0.4 b	5.2 ± 0.1 b
CIAT899-E3 (*algA*)	9.6 ± 0.3 a	5.3 ± 0.1 a

Data are presented as means ± SD. Numbers in the same column followed by different letters are significantly different at $P = 0.05$ according to Duncan's multiple-range test. Similar results were obtained in two independent experiments performed with five replicates per treatment. Data from one experiment are shown.

transposon mutant with an insertion in a partially sequenced gene with 100% identity to the sequence obtained from CIAT899-E3 but its LPS was not analysed. The ATG start codon of a partially sequenced ORF was found 183 nt downstream of the *noeJ* gene. The *in silico* translated product showed significant similarities with putative transglutaminase-like proteins of undefined function (Makarova *et al.*, 1999).

Effects of LPS defects on maize rhizosphere and root colonization

To evaluate the influence of the LPS defects on colonization, the bacterial numbers recovered from the rhizosphere (including the rhizoplane) and the root interior of maize plants 2 weeks after inoculation were determined. To test whether the sonication treatment used to liberate bacteria from the root has any effect on viability, mid log cultures were suspended in sterile water to 10^5 cfu ml^{-1} and sonicated for 15 min (three times longer than the treatment used in the colonization experiments). No effects on viability were observed for the parental strain or mutants (as shown in Table S1).

Higher bacterial numbers were recovered from the rhizosphere than from the root interior in agreement with previous reports for *R. tropici* and other endophytic bacteria (Rosenblueth and Martínez-Romero, 2004; 2006). In experiments where each strain was inoculated individually, CIAT899-E1 and CIAT899-E3 were recovered from both compartments at significantly lower numbers when compared with the parental strain (Table 2). In contrast, no significant difference was observed between the colonization of CIAT899-E2 and the parental strain (Table 2).

All mutants were significantly impaired in competitive root colonization with the parental strain when coinoculated in a 1:1 ratio (Fig. 3). CIAT899-E1 and CIAT899-E3 constituted only 4.9% or 4.3% and 9.7% or 7.1% of the bacteria recovered from the rhizosphere or root interior, respectively. Mutant CIAT899-E2 also showed a diminished competitiveness, albeit not as severe as the other mutants, representing 25.8% and 27.1% of the total bacteria in the rhizosphere and root, respectively (Fig. 3).

The lower recovery of the LPS mutants could not be attributed to their auto-agglutination as phase-contrast microscopic observations showed that our routinary homogenization of the bacterial suspensions by vortexing (four 15 s intervals at maximum speed) was sufficient to disaggregated the clumps (data not shown). The same approach was used by others that reported reduced root colonization by clumping *Rhizobium* mutants (Araujo *et al.*, 1994). Additionally, sonication performed during the

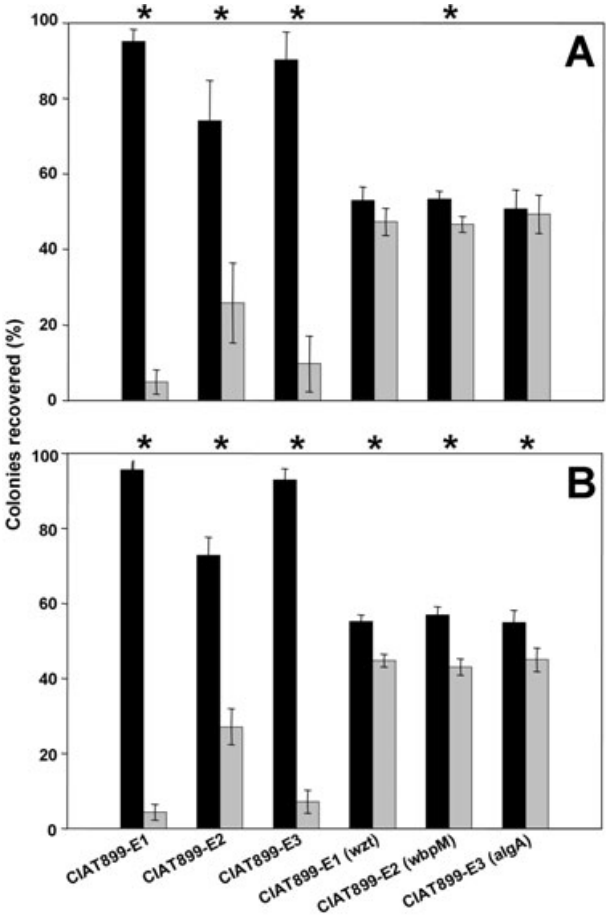

Fig. 3. Competitive maize rhizosphere (A) and root endophytic (B) colonization by the *R. tropici* LPS mutants and complemented strains in 1:1 coinoculation experiments with the parental strain. Results are shown as means ± SD of the percentage of recovered colonies. Grey bars represent values from the mutants or complemented mutants, while black bars represent the corresponding values for the parental strain. Similar results were obtained in two independent experiments performed with five replicates per treatment. Data from one experiment are shown. Asterisks indicate significant differences at $P = 0.05$ between the competing strains based on Log cfu g^{-1} of fresh weight.

Table 3. Susceptibility to antimicrobials expressed as the minimal inhibitory concentration (MIC), and swimming motility of the *R. tropici* parental strain, its LPS mutants and complemented mutants.

	MIC					
Strain	Novobiocin (μg ml^{-1})	Captan (μg ml^{-1})	SDS (%)	MBOA (mg ml^{-1})	Polymyxin B (μg ml^{-1})	Motility[a] (mm)
CIAT899	8	75	> 0.125	> 0.25	> 105	17.2 ± 0.2 a
CIAT899-E1	7.5	50	0.075	0.25	35	15 ± 0.3 c
CIAT899-E2	7.5	50	0.075	0.25	35	15.2 ± 0.3 c
CIAT899-E3	6	25	0.0125	0.18	20	15.3 ± 0.4 c
CIAT899-E1 (*wzt*)	8	75	> 0.125	> 0.25	> 105	16.1 ± 0.2 b
CIAT899-E2 (*wbpM*)	8	75	> 0.125	> 0.25	> 105	16.4 ± 0.4 b
CIAT899-E2 (*lpsβ2*)	8	75	> 0.125	> 0.25	> 105	16.3 ± 0.4 b
CIAT899-E3 (*algA*)	8	75	> 0.125	> 0.25	> 105	16.5 ± 0.5 b

a. Data are presented as means ± SD. Numbers in the same column followed with different letters are significantly different at $P = 0.05$ according to Duncan's multiple-range test.
The same MIC values were obtained in at least two independent experiments. Similar motilities were observed in three independent experiments. Data from one experiment are presented.

extraction of bacteria could contribute to clump dispersion (Falcioni *et al*., 2006).

The mutants show increased sensitivity to some antibiotics including a maize antimicrobial compound

The LPS are important as a protective barrier against hydrophobic and lipophilic antimicrobial compounds, and to detergents (Nikaido, 2003). Thus, the minimal inhibitory concentration (MIC) of the hydrophobic antibiotic novobiocin, the lipophilic captan fungicide [*N*-(trichloromethylthio)-4-cyclohexene-1,2-dicarboximide], and the SDS detergent was determined. Captan was included because it is commonly used as a maize seed dressing to protect against fungal pathogens but is often detrimental to bacteria as well (Bernal *et al*., 2004). Additionally, the susceptibility to the cationic antimicrobial peptide polymyxin B was evaluated. The mutants showed higher sensitivities to all the compounds in comparison to the parental strain (Table 3). CIAT899-E1 and CIAT899-E2, affected only in O antigen biosynthesis, had the same levels of sensitivity while CIAT899-E3, which appears to be affected also in core integrity, showed the highest level of susceptibility. These results suggest an increased accessibility of all the compounds to the cell as the LPS structure becomes shorter. No differences were observed between the mutants and parental strain in their sensitivities to ampicillin and streptomycin, antibiotics which do not interact with LPS and traverse the OM by porin channels (data not shown).

Besides captan, it is unlikely that the above mentioned compounds occur in the rhizosphere or roots but plants do produce antimicrobials in response to microbial invaders. Maize synthesizes heterocyclic benzoxazinones as secondary metabolites having antifungal and antibacterial activities (Niemeyer, 1988). 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA), the primary benzoxazinone produced by maize, is unstable and quickly decomposes to the more stable 6-methoxy-2-benzoxazolinone (MBOA) which also has antimicrobial activity (Corcuera *et al*., 1978). To explore if the inability to cope with maize antimicrobials was contributing to the colonization impaired phenotypes of the LPS mutants, we evaluated their sensitivity to MBOA. At 0.1 mg ml^{-1}, MBOA reduced the growth of the parental strain (not shown) and LPS mutants, but had a greater deleterious effect on the latter (Fig. 4). As observed for the previously tested antimicrobials, CIAT899-E1 and CIAT899-E2 behaved similarly, while mutant CIAT899-E3 was more sensitive. The growth of the mutants was completely inhibited by 0.25 mg ml^{-1} of MBOA while the parental strain was still able to growth (Table 3). The effect of hydrogen peroxide, a general plant antimicrobial and signaling molecule was also evaluated. The mutants and the parental strain were equally sensi-

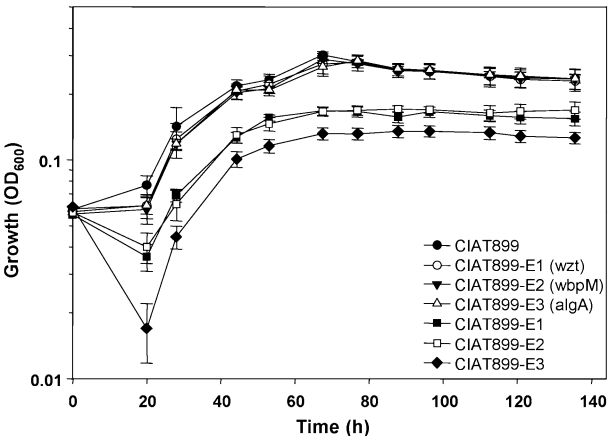

Fig. 4. Effect of the maize antimicrobial MBOA at 0.1 mg ml^{-1} on the growth of the *R. tropici* parental strain, its LPS mutants and complemented strains. Data are presented as OD_{600} means ± SD. The experiments were performed two times giving similar results. The data presented correspond to one experiment.

Table 4. Generation times (g) of the *R. tropici* parental strain and its LPS mutants in PY medium.

	g (h^{-1}) determined by	
Strain	OD_{600}	cfu ml^{-1}
CIAT899	2.81 ± 0.02 a	5.34 ± 0.03 a
CIAT899-E1	2.97 ± 0.06 b	5.86 ± 0.04 b
CIAT899-E2	2.41 ± 0.03 c	4.86 ± 0.06 c
CIAT899-E3	2.74 ± 0.07 a	5.29 ± 0.02 a

Data are presented as means ± SD. Numbers in the same column followed with different letters are significantly different at $P = 0.05$ according to Duncan's multiple-range test. Experiments were performed three times giving similar results. Data from one experiment are shown.

tive according to the disc diffusion method used, suggesting that the LPS are not involved in protection against this compound (data not shown). A recently published work on the *Azorhizobium caulinodans–Sesbania rostrata* interaction suggests that EPS are more likely involved in protection against hydrogen peroxide (D'Haeze *et al*., 2004).

The mutants show pleiotropic effects in some traits related to root colonization

The LPS defects are often correlated with reductions in motility (Priefer, 1989; Garcia-de los Santos and Brom, 1997) and sometimes in growth rate (de Maagd *et al*., 1989; Dekkers *et al*., 1998), traits known to play roles in rhizosphere and root colonization (Lugtenberg *et al*., 2001). The *R. tropici* LPS mutants showed slight but significant reduction in swimming motilities in comparison to the parental strain (Table 3). Despite having a different LPS defect, no significant differences were observed in the diameter of the swimming haloes between CIAT899-E3 and the other mutants. Swarming motility could not be observed in the parental strain or mutants under the conditions tested (data not shown). No difference was observed between the growth rate of CIAT899-E3 and the parental strain in PY (Table 4), YEM and MM (data not shown). In contrast, CIAT899-E1 and CIAT899-E2 grew slower and faster, respectively, than the parental strain in all media tested (Table 4 and data not shown).

The behaviour of the mutants in other traits related to root colonization was also evaluated. Besides the differences in growth rate, all mutants could grow in MM with ammonium nitrate as sole nitrogen source, indicating that they were not auxotrophs. Qualitative assays did not reveal differences between the parental and mutants in vitamin requirements, in the utilization of the 49 carbon sources present in the API 50 CH gallery, or in the catabolism of glutamine, asparagine, alanine, glycine, serine, arginine and proline, which are the prevalent amino acids detected in maize root exudates (Kraffczyk *et al*., 1984).

Genetic complementation of the LPS mutants

The *R. tropici* CIAT899 wild-type *wzt* gene complemented CIAT899-E1 for the production of smooth LPS (Fig. 1C). *R. tropici* CIAT899 DNA cosmids complementing the nodulation phenotype of mutants CIAT899-E2 and CIAT899-E3 were isolated. These cosmids had distinct restriction patterns, contained the regions disrupted in the corresponding mutants as judged by PCR amplifications, and complemented the mutants for their LPS defective phenotypes (data not shown). Given that the genes disrupted in mutants CIAT899-E2 and CIAT899-E3 coded for putative enzymes we chose to perform heterologous complementation experiments with genes having experimentally confirmed function. The *P. aeruginosa* PAO1 *wbpM* gene complemented CIAT899-E2 for the production of smooth LPS bands having wild-type electrophoretic mobilities (Fig. 1C). As expected owing to its high similarity, the *R. etli* CFN42 *lpsβ2* gene also complemented the LPS defects in this mutant (not shown). CIAT899-E3 was complemented for the production of a parental LPS profile by the *P. aeruginosa* PAO1 *algA* gene (Fig. 1C). The complemented mutants also recovered parental phenotypes for auto-agglutination and susceptibility to antimicrobials (Table 3, Fig. 4 and data not shown). The swimming motility was not fully restored to parental level but a significant increase was observed in comparison to the mutants (Table 3).

All the complemented mutants recovered the ability to induce the formation of well developed, nitrogen fixing nodules on common bean (not shown). When inoculated individually on maize, they showed the same or only slightly reduced abilities to colonize the rhizosphere and root in comparison to the parental strain (Table 2). In competition, the complemented CIAT899-E1 and CIAT899-E3 mutants showed the same competitiveness as the parental strain to colonize the rhizosphere (Fig. 3A). The complemented CIAT899-E2 mutant showed reduced competitiveness relative to the parental but it was higher than the mutant without *wbpM* (Fig. 3A). None of the complemented mutants reached the same numbers as the parental strain in the root interior but the introduction of the complementing genes resulted in an increase in their recovery in comparison to the mutants (Fig. 3B).

Discussion

The three *R. tropici* mutants with LPS defects were found to be negatively affected in maize rhizosphere and root endophytic colonization. Nevertheless, a direct correlation between the LPS defects and the colonization phenotypes could not be established. As a first step to untangle the involvement of LPS in maize colonization, we began by analysing the nature of the genetic lesions. The puta-

tive product of the mutated gene in CIAT899-E1, an O antigen transporter, was clearly related to its LPS phenotype (Lerouge *et al*., 2001; Raetz and Whitfield, 2002). In CIAT899-E2, the functional complementation by the *P. aeruginosa wbpM* gene indicated that the *R. tropici lpsβ2* gene is involved in UDP-D-QuiNAc biosynthesis (Creuzenet and Lam, 2001). QuiNAc is the first O antigen sugar residue in *R. etli* CFN42 and *Burkholderia caryophylli* NCPP 2151 (Lerouge *et al*., 2001; De Castro *et al*., 2005). It is conceivable that a deficiency of this primer sugar would prevent O antigen biosynthesis. Supporting this notion, a *lpsβ2* mutant of *R. etli* CFN42 lacked the O antigen (Garcia-de los Santos and Brom, 1997). Given the LPS phenotype of CIAT899-E2, it may be hypothesized that the CIAT899 LPS have QuiNAc in an equivalent position as the above-mentioned strains. The LPS of mutant CIAT899-E3 not only lacked the O antigen but also seems to have a truncated core. The putative function of the disrupted gene in this mutant and the functional complementation by the *algA* gene suggest that mannose, or a sugar derived from it, is required for the synthesis of the LPS core in *R. tropici* CIAT899. Actually, mannose is a sugar residue commonly present in the LPS core of rhizobia (Kannenberg *et al*., 1998). The enzymatic function affected in CIAT899-E3 would also block O antigen biosynthesis given that GDP-D-mannose is a precursor for the biosynthesis of the GDP-D-talose and GDP-L-fucose, required as donors for two of the three sugar residues present in the O antigen repeating unit of CIAT899 (Gil-Serrano *et al*., 1995; Maki and Renkonen, 2004).

We searched for pleiotropic effects of the mutations on several traits playing a role in plant colonization and found differences with the parental strain in motility and growth rate. In PGP *P. fluorescens*, non-motile as well as mutants with reduced motility are impaired in competitive colonization of the rhizosphere of several plants (Lugtenberg *et al*., 2001). Motility is important for migration towards and along the root to niches where nutrients are exuded and thus contributes to an effective colonization of the root (de Weert *et al*., 2002; Capdevila *et al*., 2004). The three *R. tropici* LPS mutants showed equal levels of motility reduction relative to the parental strain, a factor likely contributing to their impaired colonization abilities but not explaining their distinct behaviours. Lipopolysaccharide defects are thought to influence swimming motility indirectly owing to alterations in the OM that interfere with flagella biogenesis or function (Abeyrathne *et al*., 2005; Canals *et al*., 2006). It is worth mentioning that the effects on motility were very mild in *R. tropici* when compared with the complete abolition of motility in LPS mutants of *R. etli* and *R. leguminosarum* bv. viciae (de Maagd *et al*., 1989; Priefer, 1989; Garcia-de los Santos and Brom, 1997).

Dekkers and colleagues (1998) found a correlation between O antigen deficiency and reduced growth rate but this relationship was not discussed further. We found that the two *R. tropici* mutants with normal rough LPS but lacking O antigen showed altered growth rate but, unexpectedly, only CIAT899-E1 was a slow grower while CIAT899-E2 grew faster than the parental strain. These phenotypes were observed in all media tested, suggesting that they reflect a general metabolic effect similar to the one described in an adenylate cyclase mutant of *E. cloacae* that showed reduced growth rates in several media and was impaired in cucumber root colonization (Roberts *et al*., 2006). In CIAT899-E1, O antigen units are likely being accumulated in the cytoplasmic face of the inner membrane owing to a non-functional *wzm-wzt* ABC transporter. Growth inhibitions have been observed in *wzm* and *wzt* mutants of *Escherichia coli* O8 and O9a (Cuthbertson *et al*., 2005) probably caused by the sequestration of the undecaprenyl carriers or perturbation of membrane function as a result of the accumulation of O antigen units. It is possible that a similar effect is occurring in CIAT899-E1 resulting in a reduced growth rate. In CIAT899-E2, complementation by the *wbpM* gene suggests that the CIAT899 Lpsβ2 protein uses UDP-GlcNAc as substrate (Creuzenet and Lam, 2001). This nucleotide sugar is also used as an essential precursor for the biosynthesis of peptidoglycan and lipid A (Ramos-Aires *et al*., 2004). It can be speculated that the reduced consumption of this key nucleotide sugar in CIAT899-E2 is causing a favourable carbon and energy balance that can boost growth rate. A fast growth could give a selective advantage for nutrient competition in the rhizosphere and root apoplast where the availability of photosynthate is a main limiting factor for growth (Simons *et al*., 1996; Rosenblueth and Martínez-Romero, 2006). Interestingly, CIAT899-E2 was recovered in higher numbers than the other mutants, supporting the notion that a higher growth rate can be beneficial for root colonization.

Even when interacting with non-pathogenic bacteria, some plant defence mechanisms are activated (Azpilicueta *et al*., 2004; Compant *et al*., 2005), and it has been suggested that defence responses could regulate colonization by these microorganisms (Iniguez *et al*., 2005; Miche *et al*., 2006). One major component of plant defence is the production of antimicrobial compounds (Castro and Fontes, 2005). Symbionts have to withstand potentially toxic concentrations of these molecules in order to successfully colonize plants (Eisenschenk *et al*., 1994; Gonzalez-Pasayo and Martínez-Romero, 2000; D′Haeze and Holsters, 2004). In maize, antimicrobial benzoxaninones are accumulated in plant tissues as low toxicity glycosides and are released by glucosidases in reaction to pathogens or tissue damage (Niemeyer, 1988). Nevertheless, active root exudation has been

reported in non-injured plants (Zhang *et al*., 2000), and it is probable that field-grown maize plants liberate considerable amounts of benzoxazinones owing to stress factors that induce defence reactions, making it likely that these compounds affect *R. tropici* during its interaction with maize. Surface polysaccharides can provide protection against antimicrobials during microbial–plant interactions (D'Haeze and Holsters, 2004), and in particular, the LPS are well known as an effective barrier against hydrophobic and lipophilic molecules (Nikaido, 2003). Interestingly, maize benzoxazinones have a lipohilic character (Chiapusio *et al*., 2004). Because the *R. tropici* LPS mutants were more sensitive to MBOA than the parental strain, it is possible that their impaired colonization was partly due to the inability of their altered LPS to properly exclude the maize benzoxazinones. Although we did not test for susceptibility towards maize antimicrobial peptides (Duvick *et al*., 1992), the mutants were more sensitive to polymyxin B. Given that LPS are involved in resistance to these compounds, the *R. tropici* mutants may be also more sensitive to the antimicrobial peptides present in maize.

In some rhizobial-legume symbioses, cytological evidence for activation of plant defences have been described after inoculation with LPS mutants (Stacey *et al*., 1991; Perotto *et al*., 1994; Niehaus *et al*., 1998). These observations suggest an alternative scenario for the involvement of maize defence mechanisms in which the *R. tropici* mutants may be eliciting an active plant defence response that affects colonization. In addition, their higher susceptibilities to maize antimicrobials may further limit their spread within maize tissues. We considered that this situation is unlikely because, when inoculated individually, all mutants reached high numbers in the rhizosphere (> log 8.6) and roots (> log 4.8). Lower numbers, especially within the root, may be expected if they were being recognized as pathogens thereby triggering complete plant defence responses. As a comparison, LPS mutants of bacterial pathogens not only show reduced virulence but their numbers in plant tissues rapidly decline (Newman *et al*., 2001). The observations made for *R. tropici* are similar to those reported for an O antigen mutant of the biocontrol *P. fluorescens* WSC147r strain that colonized tomato roots to a lower extent than the wild type but did not induce a different defence response (Duijff *et al*., 1997).

We conclude that the diminished colonization phenotypes of the *R. tropici* LPS mutants in comparison to the parental strain were caused by the effects of the mutations on susceptibility to maize antimicrobials, growth rate, and, to a less extent, on motility. The latter two represent pleiotropic effects while the first, reflecting the LPS function as a protective barrier, would indicate a direct role of the LPS in colonization. The combined effects of altered growth rate and MBOA sensitivity explained the observed differences between the colonization behaviours of the mutants. It was interesting to find that the analysis of a single molecular trait supports the conclusion of Latour and colleagues (2004) that colonization has a multifactorial determinism.

Experimental procedures

Bacterial strains and culture conditions

R. tropici CIAT899cp is a spontaneous mutant of the wild-type CIAT899 strain (Martínez-Romero *et al*., 1991) selected for higher resistance to fungicide Captan to be used as a common bean inoculant on Captan-dressed seeds. This strain was referred as the parental strain in this study. *R. tropici* were routinely grown in the complex PY medium at 30°C (Noel *et al*., 1984). YEM (Vincent, 1970) and the MM described by Kingsley and Bohlool (1992) using 10 mM ammonium nitrate as nitrogen source were used for some tests. *Escherichia coli* strains were cultivated in Luria–Bertani medium at 37°C. The following antibiotics were added to the media when required (μg ml^{-1}): nalidixic acid (30), kanamycin (45), neomycin (60), gentamycin (10), carbenicillin (100) and tetracycline (7.5 for rhizobia, 10 for *E. coli*).

Molecular techniques

Transposon mutagenesis was performed with an mTn*5gusA-oriV-pgfp* (Rosconi *et al*., 2006) using the procedures described by Xi and colleagues (1999). Lipopolysaccharide defective derivatives were identified by screening for mutants showing auto-agglutination in PY liquid medium. To clone the insertions, genomic DNA from each mutant was digested with XhoI, ligated and used to transform *E. coli* DH5α. Plasmid DNA was isolated from the kanamycin-resistant colonies. The *R. tropici* DNA fragments adjacent to the transposon were sequenced using primer RHI128 (Xi *et al*., 1999) directed outwards from the 5′ end of *gusA*. A primer walking strategy was used to extend the sequences further. ORF prediction was performed with GeneMark (Besemer and Borodovsky, 1999). Putative functions were assigned based on homology to proteins retrieved from the GenBank database with the BLASTX program, and on the presence of conserved domains detected with InterProScan (http://www.ebi.ac.uk/InterProScan).

For complementation *in trans*, fragments containing complete ORFs were PCR amplified from genomic DNA using the PfuUltra DNA polymerase. The following primer pairs were used (introduced restriction sites underlined): H91wzt (5′-ttttaagcttttcgacgttcttggcatctat-3′) – wzt189B (5′-ttttggatccggatggcgtcacgaggga-3′) for the *R. tropici* CIAT899 wild-type *wzt* gene, H240_lpsBeta2 (5′- ttttaagcttaaggtcgttcgccgtaag-3′) – lpsBeta2_54B (5′-ttttggatcctccgatattcgcctatatggt-3′) for the *R. etli* CFN42 *lpsβ2* gene using pAGS10 plasmid DNA as template (Garcia-de los Santos and Brom, 1997), and H67wbpM (5′-tttaagcttgttgccatggttatctgt-3′) – wbpM51B (5′-tttggatccccggaaacatcgta-3′) and H28algA (5′-ttttaagcttcagacgcatcgcttcaa-3′) – algA83B (5′-ttttggatccggccagggaaagtcaatc-3′) for the *P. aeruginosa* PAO1 *wbpM* and

algA genes, respectively. The PCR products were TA cloned into the pCR4-TOPO vector (Invitrogen) after addition of 3′ A-overhangs by incubating the PCR reaction with Taq polymerase (Invitrogen) and 200 μM dATP for 60 min at 72°C. The amplified genes were excised from the plasmids as HindIII-BamHI fragments, gel purified, and cloned into the broad host range vector pBBR1MCS-5 (Kovach *et al*., 1995). The resulting plasmids were transferred into the mutants by triparental matings using a helper strain carrying the pRK2013 plasmid.

Nucleotide sequence accession numbers

The nucleotide sequences determined in this study have been deposited in the GenBank database under accession numbers DQ311659–DQ311661.

LPS isolation and analysis

Three different methods were used to isolate LPS: (i) the proteinase K method as described by Campbell and colleagues (2003) (ii) the hot phenol-water extraction procedure as modified for rhizobia by Carlson and colleagues (1978), and (iii) the affinity purification method developed by Valverde and colleagues (1997). Lipopolysaccharide extracts were subjected to 18% PAGE and visualized by silver staining (Hitchcock and Brown, 1983). To reveal acidic polysaccharides, gels were stained with Alcian Blue prior to silver staining (Corzo *et al*., 1991).

Phenotypic characterization

Production of EPS was visually evaluated by observing gum production on solid media. Calcofluor-binding EPS were revealed by fluorescence under UV on media supplemented with 0.02% calcofluor. To evaluate motility, late log cultures ($OD_{600} = 0.8$) were inoculated at the centre of PY plates containing 0.3% or 0.6% agar for swimming or swarming motility, respectively. The plates were incubated for 3 days and the diameter of the growth zone was determined. Utilization of amino acids as sole nitrogen sources at 10 mM was evaluated in MM. Carbon source utilization was determined using the API 50 CH gallery as instructed by the manufacturer (Biomerieux). Sensitivity to hydrogen peroxide was tested by a disc diffusion assay spreading 10^8 cfu of mid log phase cultures ($OD_{600} = 0.4$) onto PY plates and placing filter paper discs saturated with increasing concentrations of hydrogen peroxide in the surface of the plate. After 3 days of incubation, the diameter of the inhibition zone was recorded. To test for sensitivity to other antimicrobial compounds, mid log phase cultures ($OD_{600} = 0.4$) were diluted in fresh PY and 20 μl (10^4 cfu) was spotted onto PY plates containing different concentrations of polymyxin B, novobiocin, SDS, ampicillin, streptomycin or captan. The lowest concentration that completely inhibited growth was recorded as the MIC. To determine the effect of MBOA on growth, cultures were diluted to an $OD_{600} = 0.05$ in PY containing 0.1 or 0.25 mg ml^{-1} MBOA, and the growth was spectrophotometrically recorded for 140 h. Additionally, strains were grown for 72 h at 0.1, 0.18 or 0.25 mg ml^{-1} to determine MICs of MBOA, the MIC was recorded as the concentration where the OD_{600} did not increase more than 10% over the 0.05 initial value. At least two independent experiments with five replicates were performed for each variable evaluated. Clump disaggregation of auto-agglutinated cells was determined by microscopic examinations using a Nikon Diaphot phase contrast inverted microscope.

Plant experiments

Nodulation and nitrogen fixation were evaluated with common bean plants (*P. vulgaris* cv. Negro Xamapa) 4 weeks after inoculation as previously described (Martínez-Romero *et al*., 1991). Experiments with maize were performed with the cultivar Criollo de Amatlán. The sterilization and germination of maize seeds were performed as previously described (Rosenblueth and Martínez-Romero, 2004). After germination, two seedlings were transferred to 1 L of pots filled with sterile vermiculite and watered with 150 ml of Fahraeus solution (Fahraeus, 1957), and incubated overnight in the dark at 30°C. Bacteria were grown to late exponential phase ($OD_{600} = 0.8$), harvested by centrifugation and re-suspended in 10 mM $MgSO_4.7H_2O$. Appropriate volumes of these suspensions were mixed with 150 ml of Fahraeus solution and this volume was homogeneously distributed in each pot. A total of 10^8 or 0.5×10^8 cfu were used for single or double strain inoculations, respectively. Plants were grown in a greenhouse under natural illumination. Two weeks after inoculation, rhizospheric and endophytic bacteria were obtained as previously described (Rosenblueth and Martínez-Romero, 2004). Briefly, roots were manually cleaned of gross vermiculite particles and sonicated in an ice bath for 5 min using a Bransonic 1510 (Branson) ultrasonic cleaner to liberate bacteria from the vermiculite and root surface, and the resulting suspension was considered to contain bacteria from the rhizosphere (but also contained bacteria from the rhizoplane). Sonicated roots were washed with water, sterilized by immersion in 1.2% sodium hypochlorite for 3 min with agitation, rinsed in 2% sodium thiosulfate to eliminate chlorine, and thoroughly rinsed with water. Surface sterility was checked by rolling the roots over PY agar plates and incubating the plates for 5 days. The roots were mixed with nine times their weight of water, cut into small pieces and ground with a mortar and pestle for 3 min to release endophytic bacteria. The rhizosphere and endosphere suspensions were serially diluted with 10 mM $MgSO_4.7H_2O$ and plated on PY, and PY with the proper antibiotics. Colonies growing in PY containing kanamycin were checked for GFP fluorescence under UV to confirm that were the inoculated mutants. Colonization was expressed as cfu g^{-1} of fresh weight. The experimental unit consisted of one pot with two plants. The experiments were independently performed two times with five replicate pots per treatment. For statistical analyses, data were logarithmically transformed and subjected to an analysis of variance. Means were compared by the Duncan's multiple-range test.

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Supplementary material

The following supplementary material is available for this article online:

Fig. S1. Auto-agglutination phenotypes of the *R. tropici* LPS mutants.

Fig. S2. Gum production (A) and Calcofluor fluorescence (B) shown by the *R. tropici* parental strain and LPS mutants in PY, YEM and MM media.

Table S1. Effect of the sonication treatment on viability.

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