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FEMS Microbiology Letters 231 (2004) 267–275

FEMS MICROBIOLOGY Letters

www.fems-microbiology.org

Disruption of dTDP-rhamnose biosynthesis modifies lipopolysaccharide core, exopolysaccharide production, and root colonization in *Azospirillum brasilense*

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Received 15 October 2003; received in revised form 23 December 2003; accepted 24 December 2003

First published online 26 January 2004

Abstract

The interaction between *Azospirillum brasilense* and plants is not fully understood, although several bacterial surface components like exopolysaccharides (EPS), flagella, and capsular polysaccharides are required for attachment and colonization. While in other plant–bacteria associations (*Rhizobium*–legume, *Pseudomonas*–potato), lipopolysaccharides (LPS) play a key role in the establishment of an effective association, their role in the root colonization by *Azospirillum* had not been determined. In this study, we isolated a Tn*5* mutant of *A. brasilense* Cd (EJ1) with an apparently modified LPS core structure, non-mucoid colony morphology, increased EPS production, and affected in maize root colonization. A 3790-bp region revealed the presence of three complete open reading frames designated *rmlC*, *rmlB* and *rmlD*. The beginning of a fourth open reading frame was found and designated *rmlA*. These genes are organized in a cluster which shows homology to the cluster involved in the synthesis of dTDP-rhamnose in other bacteria. Additionally, the analysis of the monosaccharide composition of LPSs showed a diminution of rhamnose compared to the wild-type strain.
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Keywords: Lipopolysaccharide; Exopolysaccharide; Gas-liquid chromatography-mass spectrometry; dTDP-rhamnose; *Azospirillum brasilense*

1. Introduction

Azospirillum species are known to associate with many agriculturally important crops (for a review see [1–3]). Upon inoculation, bacteria attach to plant roots, proliferate, and in some cases subsequently invade and colonize the internal tissues of the roots. The association results in a positive effect of the bacteria on plant growth [2]. *Azospirillum* spp. is currently one of the best-studied plant growth-promoting rhizobacteria. The plant growth-promoting effects have been attributed to mechanisms such as nitrogen fixation, phytohormone production, and nitrate reduction among others [4,5].

The *Azospirillum*–plant interaction is not yet fully understood, nevertheless some bacterial surface components have been demonstrated to be involved in the early stages of the colonization process [6–10]. Colonization of plant roots is preceded by a two-step process, starting with a rapid and reversible bacterial adsorption onto the root system, followed by an irreversible anchoring of the bacteria to the root surface [11]. The adsorption of *Azospirillum brasilense* to wheat roots was shown to be highly dependent on the presence of the polar flagellum, whereas the anchoring (second step) was associated with the biosynthesis of a polysaccharidic fibrillar material. Two different loci have been shown to be associated with polysaccharide biosynthesis in *A. brasilense*. One of them was identified by complementation of *Sinorhizobium meliloti exoB* and *exoC* mutants with *A. brasilense* DNA. Tn*5* insertions in the *A. brasilense exoB* and *exoC* loci showed that these mutants failed to produce the wild-type high-molecular-mass exopolysaccharides (EPS), producing only EPSs of lower molecular mass with a fluorescent calcofluor phenotype similar to the wild-type strain [12]. The second locus (Cal) known to be associated with polysaccharide production was shown to affect calcofluor staining

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doi:10.1016/S0378-1097(04)00003-5

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of the cells but not EPS production. It was thus suggested that fluorescence of *A. brasilense* with calcofluor is caused by a polysaccharide different from EPS. *A. brasilense* Cal^- mutants were unable to flocculate and were deficient in the production of calcofluor-binding extracellular fibrils [13]. Several authors have demonstrated that fibrillar material is involved in the physical interaction of *A. brasilense* with plant roots (for a review see [14]). Michiels et al. [11] showed that Cal^- mutants were indeed affected in their anchoring to wheat roots.

As occurs in different plant–bacteria associations, other surface polysaccharides such as lipopolysaccharide (LPS) could also be involved in the colonization of roots by *A. brasilense*. No well-characterized LPS mutants have been available to compare their colonization phenotype with the wild-type strains. The O-specific polysaccharide structure has been studied in only one strain of *A. lipoferum* and recently in *A. brasilense* Sp245 [15]. In several associative and symbiotic plant–bacteria interactions, LPS molecules were shown to play key roles during the establishment of an effective association [16–18]. It has been demonstrated that the O-antigen from LPS is necessary for an efficient colonization of potato roots by *Pseudomonas putida* [19]. LPS mutants of several rhizobia were also shown to be modified in their symbiotic associative behavior with their host legumes, as it was reported for LPS rough mutants of *Rhizobium leguminosarum* bv. *phaseoli* which remained in the infection threads without developing into nitrogen-fixing bacteroids [20].

In this work we report on the isolation and characterization of an *A. brasilense* LPS mutant generated by Tn*5* insertion, and its effect on maize root colonization.

2. Materials and methods

2.1. Bacterial strains, plasmids and media

The genotypes of the bacterial strains and plasmids used in this study are listed in Table 1. *A. brasilense* was grown at 33°C in Luria–Bertani (LB) medium supplemented with 2.5 mM $CaCl_2$ and 2.5 mM $MgSO_4$ [21] or minimal medium lactate (MML [22]). *Escherichia coli* strains were grown at 37°C in LB medium. Antibiotics were added at the following concentrations when required: ampicillin (Ap), 25 μg ml^{-1}; chloramphenicol (Cm), 25 μg ml^{-1}; kanamycin (Km), 25 μg ml^{-1}; and tetracycline (Tc), 5 μg ml^{-1}.

2.2. DNA manipulations

Plasmid and total DNA preparations, agarose gel electrophoresis, restriction endonuclease digestion and cloning were performed according to standard protocols [23]. *E. coli* transformation was carried out by electroporation with a Gene Pulser apparatus (Bio-Rad Laboratories). Southern hybridizations were performed using a non-radioactive probe labeled with 14-dCTP-biotin, and a chemiluminescence method was used to detect hybridization bands according to the instructions of the manufacturer (Gibco BRL, Life Technologies). The probe was obtained from *Eco*RI digestion of pEJ1. Thus, an *Eco*RI fragment containing Tn*5* and flanking DNA from *A. brasilense* EJ1 was obtained from agarose gel, and purified using the Wizard® DNA Clean-Up System kit (Promega).

2.3. Mutagenesis

Random transposon Tn*5* mutagenesis of *A. brasilense* was carried out using the mobilizable pGS9 suicide plasmid [24]. The kanamycin-resistant (Km^r) transconjugants were selected on MML agar medium and screened for increased sensitivity to sodium dodecyl sulfate (SDS), a hydrophobic compound. 0.01% SDS was the highest concentration that did not affect the growth of *A. brasilense*. Colonies showing impairment of grow on 0.01% SDS were selected for LPS analysis. A growth curve of the selected Tn*5* mutant was followed in MML medium supplemented with Km. Cultures were grown at 33°C on a rotary shaker at 100 rpm. Bacterial growth was monitored spectrophotometrically at 600 nm.

2.4. Cloning of the Tn*5*-interrupted region

Total DNA from *A. brasilense* EJ1 was digested with *Eco*RI and ligated to pSUP102. The ligation mixture was used to transform electrocompetent *E. coli* S17-1 cells. Transformant clones were selected on LB medium containing Km and Tc and screened for the presence of IS50 sequences by a polymerase chain reaction (PCR) assay using the primers IS1 and IS2 that led to a PCR product of 1.2 kb. One of the plasmids (Tc^r, Km^r) was designated pEJ1.

2.5. Subcloning, and sequencing outward the transposon in both directions

Plasmid pEJ1 was digested with *Bam*HI-*Eco*RI and *Bam*HI, the products were ligated to pBluescript SK. The recombinant plasmids were used to transform electrocompetent *E. coli* JM109 cells. Clones were selected on LB medium containing Ap and Km and LB medium containing Ap and X-gal. Plasmids were isolated and purified using Wizard DNA Clean-Up System kit (Promega). DNA was sequenced using Taq FS DNA polymerase and fluorescent dideoxy terminators in a cycle sequencing method and the resultant DNA fragments were electrophoresed and analyzed using an automated Applied Biosystems 377 DNA sequencer. Sequencing service was done by HHMI/Keck DNA Sequencing Facility, USA. The amino acid sequences deduced from the nucleotide sequence were compared to the GenBank database, using

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Table 1
Strains and plasmids used

	Relevant characteristics	Reference or source
Strains		
A. brasilense Cd	ATCC 29710 wild-type	[45]
A. brasilense EJ1	Tn*5* mutant of *A. brasilense* Cd, with modified LPS	This work
E. coli JM109	*recA endA1 gyrA96 thi hsdR17 supE44* Δ(*lac-proAB*) *relA1*	[23]
E. coli S17-1	*pro recA hsdR*, *thi* RP4-2-Tc::Mu-Km::Tn*7* integrated into the chromosome	[46]
Plasmids		
pGS9	Cm^r, Km^r, (Tn*5*) p15A replicon N-*tra* Tn*5* donor	[47]
pSUP102	Tc^r, Cm^r, vector-mobilizable pACYC184 derivative	[48]
pBluescript SK	Cloning and sequencing vector, Ap^r, phagemid, M13 derivative, f1 origin of replication	Stratagene, La Jolla, CA, USA
pEJ1	15-kb *Eco*RI fragment containing Tn*5* insertion and flanking DNA from *A. brasilense* EJ1 cloned into pSUP102; Km^r Tc^r	This work
pEJ2	3.7-kb *Eco*RI-*Bam*HI fragment, containing flanking DNA, IS*50*L and *nptII*, from pEJ1 cloned into pBluescript SK; Km^r Ap^r	This work
pEJ3	9.0-kb *Bam*HI fragment containing IS*50*R and flanking DNA, from pEJ1, cloned into pBluescript SK; Ap^r *lac*$^-$	This work

Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Km^r, kanamycin resistance; Tc^r, tetracycline resistance.

the PHI- and PSI-BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST) [32].

2.6. Nucleotide sequence accession number

The nucleotide sequence has been submitted to the GenBank database under the accession number AF349575.

2.7. PCR

PCR was carried out using primers IS1 (5′-GCTCGATCTAGAACGTTCATGATAACTTCTGC-3′) and IS2 (5′-TCTGCGGACTGGCTTTCTAC-3′) in an Idaho 1605 Air Thermo Cycler for capillary tubes (Idaho Technology) using reaction mixtures as recommended by the manufacturer. Cycling conditions were as follows: 94°C for 2 min, 35 cycles of 94°C for 30 s, 52°C for 20 s, and 72°C for 45 s. A final step at 72°C for 3 min was performed.

2.8. LPS isolation and analysis

Cultures were grown in LB broth at 33°C. LPS were isolated using polymyxin B agarose immobilized (Affiprep polymyxin support, Bio-Rad Laboratories) according to Valverde et al. [25]. LPSs were separated by 20% polyacrylamide gel electrophoresis (PAGE) using an SDS-Tricine buffer system and visualized by silver staining [26].

2.9. Analysis of LPSs by gas-liquid chromatography-mass spectrometry (GLC-MS)

For GLC-MS analysis, LPSs were extracted as described by Ridley et al. [27] with some modification. Following growth for 48 h in MML medium, bacterial cultures were centrifuged at 8000 rpm for 20 min at 4°C. Cells were collected, washed three times in phosphate–saline buffer, and resuspended in 5 ml of 0.25 M EDTA, titrated with triethylamine to reach pH 7.0. After 1 h under stirring, the suspension was centrifuged at 12 000 rpm for 10 min and cells were discarded. Supernatant was dialyzed against twice-distilled water for 48 h and then lyophilized. LPSs were hydrolyzed with 2 N HCl at 100°C for 3 h. The resultant hydrolysate was washed twice with distilled water. Samples were treated with chloroform in order to eliminate lipids. The sugar content of the hydrolyzed LPSs was quantified by the anthrone method using glucose as standard [28] and the monosaccharide composition of the isolated LPSs was analyzed by GLC-MS. The hydrolyzed LPSs were reduced with $NaBH_4$, acetylated and alditol acetates were analyzed by GLC-MS with a SP2330 capillary column (Supelco) in a Hewlett-Packard 5890 chromatograph. The temperature employed was 210°C and then increased at 1°C min^{-1} to 250°C.

2.10. EPS isolation and analysis

A. brasilense strains were grown in MML at 33°C for 2 days. EPSs were precipitated from culture supernatants with three volumes of cold ethanol. Pellets were suspended and dialyzed against distilled water. EPSs were quantified by the anthrone method [28]. Cells grown on LB agar plates supplemented with 200 μg calcofluor ml^{-1} (Fluorescent Brightener 28, Sigma) were observed under an epifluorescent microscope (Zeiss) at 365 nm. Calcofluor is a fluorescent dye that binds to β-linked polysaccharides [13].

2.11. Protein determination

Protein determination was carried out according to Bradford [29].

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2.12. Cultivation of maize seedlings

Seeds of *Zea mays* cv. 'Funk's Tronador G422T' were surface-sterilized and germinated as previously described [30]. Individual seedlings from sterile seeds were aseptically transferred to cotton-plugged 200×22-mm glass tubes containing 30 ml of hydroponic solution MPCL [31]. The tubes were placed in a growth chamber with a 16-h light:8-h dark cycle, at a constant temperature of 23°C and relative humidity of 80%.

2.13. Attachment of A. brasilense to maize roots

Two-day-old maize seedlings were inoculated with 3 ml of MPCL medium [11] containing 2×10^6 CFU ml^{-1} of *A. brasilense*. After 2 h of incubation, at 33°C and 50 rpm, root pieces of 2 cm in length were cut, and washed two times by immersion in sterile NaCl 0.88%. Roots were vortexed during 20 s in 1 ml of sterile NaCl 0.88%. The supernatant was used to determine the number of bacteria attached by plate counting in MML medium.

2.14. Colonization of maize roots by A. brasilense

One-week-old *Z. mays* seedlings growing in nitrogen-free MPCL medium were inoculated with approximately 2×10^6 CFU ml^{-1} of *A. brasilense*. Two weeks after inoculation, root pieces 5 cm in length were taken from seedlings, washed two times by immersion in sterile NaCl 0.88% and ground in 1 ml of NaCl 0.88%. Enumeration was carried out by plate counting in MML medium.

2.15. Statistical analysis

Results were analyzed using one-way analysis of variance, with the means evaluated for significance using the least significant difference test at $P<0.05$.

3. Results

3.1. Generation and characterization of the Tn5 LPS mutant A. brasilense EJ1

A collection of Tn*5* mutants of *A. brasilense* Cd were generated as outlined in Section 2 and screened for putative alterations in LPSs using MML medium containing 0.01% SDS. Usually, changes in the saccharidic moiety of LPSs increase cell surface hydrophobicity and make the bacteria more sensitive to detergent agents. One clone, designed EJ1, out of 2800 mutants was unable to grow in minimal medium supplemented with 0.01% SDS. The colonies of the mutant were non-mucoid and smaller than the wild-type strain ones (Fig. 1A,B), indicating the possible presence of LPS modifications (Fig. 1C, lane 2). The analysis of LPSs from mutant EJ1 by SDS–PAGE showed that all LPS molecular forms had a higher mobility than the homologous components present in the wild-type LPS (Fig. 1C, lane 1). The modifications in the electrophoretic pattern of the LPSs are compatible with changes either in lipid A and/or in the core region of the molecule since all LPS forms presented a mobility shift. The EJ1 mutant and the wild-type strain exhibited a comparable fluorescence on LB-calcofluor plates suggesting that the genetic defect did not eliminate the production of calcofluor-binding polysaccharides (Fig. 1D). Interestingly, when EPSs were analyzed, an unexpected higher production was detected in the EJ1 mutant (22.34±1.05 μg glucose equivalents mg protein^{-1}) compared to the wild-type strain (10.96±0.92 μg glucose equivalents mg protein^{-1}). No differences in the growth rate were observed between the wild-type and mutant strain in MML medium.

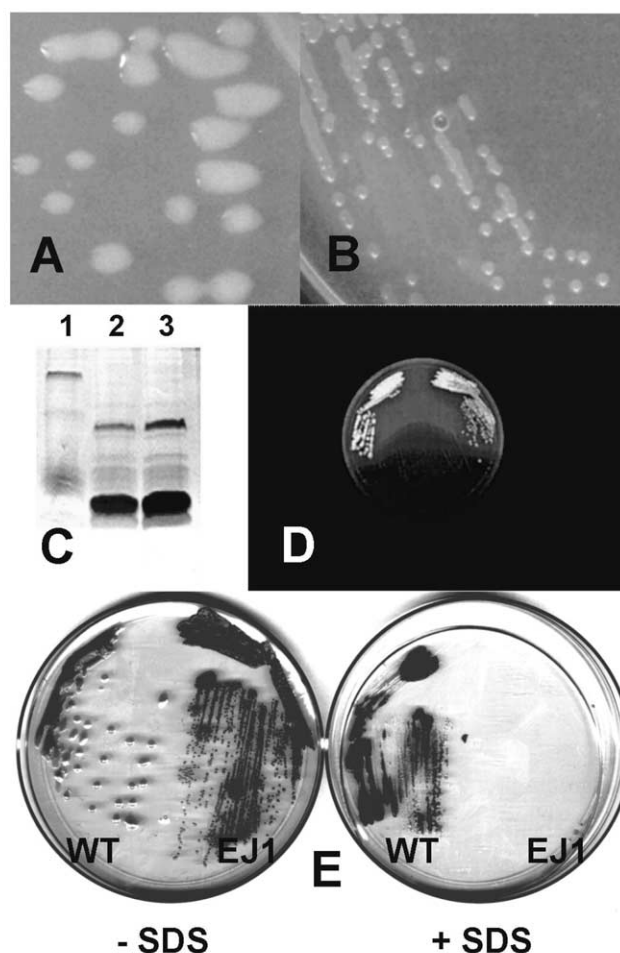

Fig. 1. Phenotypic characterization of the mutant EJ1. A,B: Colony morphology of the wild-type strain (A) and the mutant EJ1 (B). C: Electrophoretic analysis by SDS–PAGE showing the LPS pattern of the wild-type strain (lane 1), the mutant EJ1 (lane 2), and the resulting clone after homogenotization (marker exchange) of the wild-type strain with the plasmid pEJ1 (lane 3). D: Fluorescence phenotype on LB-calcofluor plates. E: Growth of the wild-type strain (WT) and EJ1 mutant (EJ1) in MML agar plates without (−SDS) and supplemented with SDS 0.01% (+SDS).

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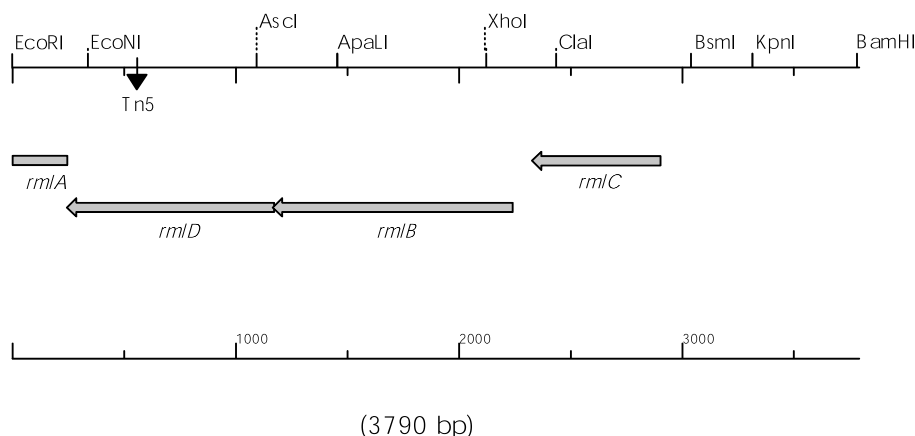

Fig. 2. Genetic structure and restriction map of a 3.8-kb region that contains the Tn*5* insertion present in the mutant EJ1. *rmlC*: dTDP-6-deoxy-D-glucose 3,5-epimerase, *rmlB*: dTDP-D-glucose-4,6-dehydratase, *rmlD*: dTDP-4-dehydrorhamnose reductase, *rmlA*: glucose-1-phosphate thymidylyltransferase.

3.2. Cloning of the Tn5-interrupted region in the LPS mutant EJ1. Reverse genetics

A 15-kb *Eco*RI Tn*5*-containing fragment isolated from mutant EJ1 was cloned into vector pSUP102 to yield plasmid pEJ1. Then, total *Eco*RI-digested DNA from the wild-type strain and its mutant EJ1 were hybridized against a biotinylated probe generated with the 15-kb cloned insert. Wild-type and mutant DNA gave a single hybridization signal corresponding to 9.5 kb and 15 kb respectively. The 5.5-kb difference in size was consistent with the presence of a Tn*5* insertion in the mutant. When the mobilizable non-replicative plasmid pEJ1 was used to homogenotize the Tn*5* mutation into the wild-type strain *A. brasilense* Cd via double crossing-over (marker exchange), the obtained recombinants showed an LPS phenotype that was coincident with that of strain EJ1. The result confirmed that the cloned Tn*5* insertion was responsible for the observed LPS changes (Fig. 1C, lane 3).

3.3. Genetic characterization of the Tn5 mutated region present in A. brasilense EJ1

The proposed genetic structure within the 3.8-kb *Eco*RI-*Bam*HI region (accession number AF349575) and the position of the Tn*5* insertion is shown in Fig. 2. The deduced

Table 2
Sequence similarities between the proposed rhamnose biosynthetic Rml products from *A. brasilense* and different proteins from other organisms

A. brasilense Rml protein	Related proteins from other organisms	Identity/similarity[a] (%)	Proposed function	Reference
RmlC (RfbC)	Rrub3491 *Rhodospirillum rubrum*	53/72	Probable dTDP-4-dehydrorhamnose-3,5-epimerase	NZ_AAAG01000018[b]
	RfbC *Mesorhizobium loti*	53/71	dTDP-6-deoxy-D-glucose-3,5-epimerase	[49]
	RfbC *Agrobacterium tumefaciens*	51/69	dTDP-rhamnose-3,5-epimerase	AAG35059[b]
	RfbC *Rhizobium* sp. NGR234	50/69	Probable dTDP-4-dehydrorhamnose-3,5-epimerase	[50]
	RfbC *Shigella flexneri*	48/65	dTDP-4-dehydrorhamnose-3,5-epimerase	[51]
RmlB (RfbB)	Rrub3480 *Rhodospirillum rubrum*	66/78	Probable dTDP-glucose-4,6-dehydratase	NZ_AAAG01000018[b]
	RfbB *Escherichia coli* O157:H7	63/79	dTDP-glucose-4,6-dehydratase	[52]
	RfbB *Neisseria meningitidis*	63/77	dTDP-glucose-4,6-dehydratase	[53]
	RfbB *Agrobacterium tumefaciens*	61/73	dTDP-glucose-4,6-dehydratase	AF314183[b]
	RfbB *Shigella flexneri*	60/74	dTDP-glucose-4,6-dehydratase	[54]
RmlD (RfbD)	OAC2 *Azorhizobium caulinodans*	54/68	OAC2 protein	[55]
	RmlD *Xanthomonas campestris* pv. *citri* 306	52/60	dTDP-4-keto-L-rhamnose reductase	NP_643889[b]
	RfbD *Rhizobium* sp. NGR234	42/53	Probable dTDP-4-dehydrorhamnose reductase	[50]
RmlA (RfbA)	RmlA *Salmonella enterica*	80/89	Glucose-1-phosphate thymidylyltransferase	[41]
	RfbA *Shigella flexneri*	79/90	Glucose-1-phosphate thymidylyltransferase	[54]
	RmlA *Escherichia coli*	79/90	Glucose-1-phosphate thymidylyltransferase	[56]
	RfbA *Rhizobium* sp. NGR234	76/86	Probable glucose-1-phosphate thymidylyltransferase	[50]

[a]Alignments of protein sequences were carried out using the PHI- and PSI-BLAST algorithm.
[b]SwissProt database accession number.

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gene structure was obtained with the aid of the ORF-finder software (available in the GenBank database), and the BLAST algorithm to compare nucleotide sequences and their translation products against the GenBank database [32]. Three complete open reading frames (ORFs) were recognized and designated *rmlC*, *rmlB*, and *rmlD* according to their sequence similarity (about 50–60%) to rhamnose biosynthetic genes (*rml*) from different bacteria (Table 2). Downstream of *rmlD* the presence of a 246-bp sequence with higher sequence similarity (about 75–80%) to several *rmlA* genes, was recognized. The position of Tn*5* insertion mapped 615 nucleotides downstream of the *rmlD* start codon. Genetic analysis indicated that mutant *A. brasilense* EJ1 should be apparently defective in rhamnose biosynthesis since no other functional *rml* genes were present in the genome of *A. brasilense*.

3.4. *Sugar analysis of the LPSs produced by* A. brasilense *Cd and the EJ1 mutant*

GLC-MS analysis of *A. brasilense* Cd LPS showed the presence of fucose, rhamnose, glucose, and galactose as the main sugars. Xylose and glucosamine were also found as minor sugars. Additionally, LPSs produced by the EJ1 mutant had a different composition of the sugars compared to the wild-type LPSs, glucose being the quantitatively predominant sugar (85.7%) (Table 3). As expected, rhamnose was significantly diminished, although its synthesis was not completely abolished, indicating that other functional genes involved in the biosynthesis of this sugar could be found in *A. brasilense*. Glucosamine was not detected and other sugars like fucose, rhamnose, xylose and galactose were detected in smaller amounts.

3.5. *Colonization phenotype of maize roots by* A. brasilense *and the EJ1 mutant*

In order to investigate if the changes observed in EJ1 LPS could affect the colonization of maize roots, the colonization phenotype at early and late stages was analyzed. Early colonization was evaluated in a short-term assay: roots and bacteria were incubated together for 2 h, in order to assess the attachment. Late colonization was studied 15 days post inoculation. Significant differences were observed in the number of bacteria, at both early and late stages, indicating that the changes observed in the LPSs from EJ1 did affect colonization events. Values obtained for early colonization were $2.42\times10^4\pm0.30$ CFU per root for the wild-type strain and $0.35\times10^4\pm0.10$ for the EJ1 mutant. The wild-type strain values of root colonization were $1.23\times10^5\pm0.11$ CFU per root while the EJ1 mutant ones were $0.5\times10^5\pm0.04$ CFU per root.

Table 3
Monosaccharide composition of the LPSs produced by *A. brasilense* Cd (WT) and its EJ1 mutant

Monosaccharide	WT	EJ1
Fucose	25.9	2.2
Rhamnose	26.2	3.4
Xylose	3.5	5.8
Galactose	14.8	2.9
Glucose	24.8	85.7
Glucosamine	4.8	ND

Data of GLC-MS are expressed as relative percentage, compared to standard peaks. Values are the mean of three determinations.
ND: not detected.

4. Discussion

Bacterial colonization in the plant rhizosphere is a process of selective enrichment of the best-adapted microorganisms. In the *A. brasilense*–plant association in different environmental conditions, several molecules appear to be involved (a 100-kDa outer membrane protein, EPS, capsular polysaccharides, glucans and LPS) [30].

Major LPS components of the outer membrane of Gram-negative bacteria are divided into two groups: LPS-1, a complex macromolecule which carries a variable number of repeating oligosaccharides, termed the O-antigen; and the simpler LPS-2, which consists of the core oligosaccharide and lipid A and shows a higher electrophoretic mobility [33,34]. Changes in the structure of LPSs have been related to an adaptation to different environmental situations, such as pH, oxygen concentration, osmotic pressure and salinity [35,30].

LPSs seem to be essential for the virulence of *Erwinia chrysanthemi*, which induces soft rot in many tropical and subtropical plant species [36]. They are required for a functional *Rhizobium*–legume symbiosis [18] and for efficient colonization of potato roots by *P. putida* [19].

The role of LPSs in the *Azospirillum*–plant association had not been described and only one report about the construction of a mutant of *A. brasilense* Sp245 deficient in O-antigen could be found in the literature [37]. However, the plant phenotype of this mutant was not characterized and the interrupted region was not sequenced. We report on the isolation of a Tn*5* mutant of *A. brasilense* Cd with apparently modified LPS designated EJ1. Phenotypic characterization of this mutant demonstrated an altered LPS electrophoretic pattern with changes in the LPS core mobility. The EJ1 colonies were non-mucoid and smaller than the wild-type ones indicating the possibility that these changes could also be related to alterations in EPS synthesis. When EPS production was analyzed in the EJ1 mutant a higher production than in the wild-type strain was detected.

We have characterized a 3.8-kb DNA fragment which contains four ORFs designated *rmlC*, *rmlB*, *rmlD*, and *rmlA*. The *rmlCBDA* cluster (formerly *rfbCBDA*) from *Azospirillum* showed to some extent homology to the *rmlBDAC* cluster from *Shigella flexneri*, *Shigella dysenter-*

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iae, and *Salmonella enterica* involved in the LPS biosynthesis.

In Gram-negative bacteria, L-rhamnose residues are often present in LPSs, dTDP-rhamnose being the nucleotide product. In all the bacteria studied, dTDP-rhamnose functions as the rhamnose donor [38]. The *rmlABCD* gene cluster encodes the enzymes glucose-1-phosphate thymidylyltransferase, dTDP-D-glucose-4,6-dehydratase, dTDP-6-deoxy-D-glucose 3,5-epimerase, and dTDP-4-dehydrorhamnose reductase, respectively, which are responsible for the four-step biosynthesis of dTDP-rhamnose from glucose 1-phosphate and TTP [39]. The genes encoding these four biosynthetic transformations (known as *rfbA*, *rfbB*, *rfbC*, and *rfbD*) have been cloned and sequenced in many organisms. They have invariably been found in a biosynthetic operon with the genes usually in the order *rmlBDAC* [40], although the gene order may vary from species to species [41].

The four genes identified were closely linked and in fact overlapped in the case of *rmlB* and *rmlD*, as also seen in *rmlB* and *rmlD* of *S. enterica* serotype Typhimurium, *Shigella*, and *Leptospira interrogans* serovar *copenhageni*. However, the *Azospirillum* gene order (*rmlCBDA*) was different from the *rmlBDAC* observed in *S. flexneri*, *S. dysenteriae*, and *S. enterica*. Similar results have been found in *L. interrogans* where the order of the genes was *rmlCDBA* [42].

The *Azospirillum rmlCBDA* gene cluster encodes a set of enzymes involved in the biosynthesis of dTDP-rhamnose, which is one of the sugar components of LPS core and O-antigen. GLC-MS analysis of *A. brasilense* LPS preparations showed the presence of glucose, galactose, xylose, rhamnose, fucose, and glucosamine as sugar constituents of LPS. The mutant EJ1 LPSs have a decreased proportion of rhamnose, fucose, galactose, and glucosamine compared to wild-type strain.

It has been demonstrated that rhamnose is a common sugar found in other polysaccharides in *A. brasilense*. For instance glucose, galactose, fucose, and rhamnose are constituents of EPSs produced by *A. brasilense* Cd [10,43]. Because of that, it is not surprising that the changes observed in the mutant EJ1 could be affecting the biosynthesis of other polysaccharides. Thus the increased synthesis of EPSs observed in the mutant EJ1 could be explained as a consequence of the modifications in the LPS structure and moreover in the hydrophobicity and charge of the cell surface. Similar results have been obtained in *Azorhizobium caulinodans* where a mutation in the *rmlD* gene changed the LPS pattern and affected the extracellular polysaccharide production leading to an ineffective symbiosis with *Sesbania rostrata* [44].

In many symbiotic or associative plant–bacteria interactions the biosynthesis of rhamnose seems to have a key role in the establishment of a successful plant–bacteria association. The EJ1 mutant colonized maize roots to a lesser extent than the wild-type strain, probably because of the changes in the LPS core and/or in the EPS production. The first step of the colonization process (attachment), which has been shown to be highly dependent on polar flagellum [6], was also found to be diminished. We show here that the disruption of *rmlD* caused a pleiotropic phenotype with altered LPS core structure, colony morphology, increased EPS production, impaired attachment to maize roots and diminished maize root colonization. To the best of our knowledge, this is the first report about the construction, genetic and phenotypic characterization of an *A. brasilense* LPS mutant, where a role of the LPS in the *Azospirillum*–plant interaction could be assigned. In the future we are planning to elucidate the structure of the *Azospirillum* LPS and to obtain more mutants impaired in LPS and EPS synthesis.

Acknowledgements

This work was supported by the Secretaría de Ciencia y Técnica de la Universidad Nacional de Río Cuarto, the Agencia Córdoba Ciencia and the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina.

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