The *Azospirillum brasilense* Sp7 *noeJ* and *noeL* genes are involved in extracellular polysaccharide biosynthesis

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*Azospirillum brasilense* is a plant root-colonizing bacterium that exerts beneficial effects on the growth of many agricultural crops. Extracellular polysaccharides of the bacterium play an important role in its interactions with plant roots. The pRhico plasmid of *A. brasilense* Sp7, also named p90, carries several genes involved in synthesis and export of cell surface polysaccharides. We generated two Sp7 mutants impaired in two pRhico-located genes, *noeJ* and *noeL*, encoding mannose-6-phosphate isomerase and GDP-mannose 4,6-dehydratase, respectively. Our results demonstrate that in *A. brasilense* Sp7, *noeJ* and *noeL* are involved in lipopolysaccharide and exopolysaccharide synthesis. *noeJ* and *noeL* mutant strains were significantly altered in their outer membrane and cytoplasmic/periplasmic protein profiles relative to the wild-type strain. Moreover, both *noeJ* and *noeL* mutations significantly affected the bacterial responses to several stresses and antimicrobial compounds. Disruption of *noeL*, but not *noeJ*, affected the ability of the *A. brasilense* Sp7 to form biofilms. The pleiotropic alterations observed in the mutants could be due, at least partially, to their altered lipopolysaccharides and exopolysaccharides relative to the wild-type.

**INTRODUCTION**

Gram-negative bacteria possess polysaccharides on their cell surface, including lipopolysaccharides (LPS), exopolysaccharides (EPS) and capsular polysaccharides (CPS) (Vorholter et al., 2001). LPS contain three regions: the lipid A, the core oligosaccharide and the O-antigen. The lipid A anchors the molecule into the outer leaflet of the outer membrane. The core oligosaccharide, attached to the lipid A, is relatively conserved among related strains, while the O-antigen portion can vary in size and is often composed of strain-specific oligosaccharide repeating unit polymers (Cava et al., 1989; Kannenberg & Carlson, 2001). EPS are polysaccharides that are loosely bound to the cell surface, while CPS are tightly bound to the cell (Leigh & Coplin, 1992). These cell surface polysaccharides are involved in the interaction of bacteria with their surrounding environment (Whitfield & Valvano, 1993).

The genus *Azospirillum* comprises Gram-negative, free-living, nitrogen-fixing, plant growth-promoting rhizobacteria (PGPR). *Azospirilla* have been isolated from the rhizosphere of many grasses and cereals. *Azospirillum brasilense*, one of the best-studied species within this genus, has been shown to exert beneficial effects on plant growth and yield of many agricultural crops (Steenhoudt & Vanderleyden, 2000).

EPS and CPS play a major role in the interaction of *A. brasilense* with plant roots (Jofré et al., 2004; Burdman et al., 2000b). It has been proposed that *A. brasilense* cells adhere to plant roots in two steps: while the first step, likely mediated by polar flagella, is relatively rapid and reversible, in the second step, named the anchoring phase, the cells are anchored firmly and irreversibly to the plant roots, in a process mediated by EPS and CPS (Michiels et al., 1991; Moens & Vanderleyden, 1996; Steenhoudt & Vanderleyden, 2000). EPS and CPS have been proposed...
to be important for proliferation and normal colonization of A. brasilense cells on the surface of wheat roots (Katupitiya et al., 1995).

The 90 MDa pRhico plasmid (also called p90) of A. brasilense Sp7 has been sequenced and annotated (Vanblieu et al., 2004). This plasmid contains genes that are involved in chemotaxis, motility, synthesis and export of cell surface polysaccharides, and interactions with plant roots (Vanblieu et al., 2004). Two of the genes found on this plasmid are noeJ (pRhico016) and noeL (pRhico007), which encode mannose-6-phosphate isomerase (MPI; EC 5.3.1.8; also named phosphomannose isomerase) and GDP-mannose 4,6-dehydratase (GMD; EC 4.2.1.47), respectively. In Rhizobium tropici, impairment of noeJ has been shown to affect LPS biosynthesis as well as rhizosphere and root colonization (Ormeño-Orrillo et al., 2008).

MPI catalyses the interconversion of D-fructose 6-phosphate and D-mannose 6-phosphate (Wu et al., 2002). GDP-(D)-mannose, which is synthesized from D-mannose 6-phosphate (MPI; EC 5.3.1.8; also named phosphomannose isomerase) and GDP-mannose 4,6-dehydratase (GMD; EC 4.2.1.47), respectively. In Rhizobium tropici, impairment of noeJ has been shown to affect LPS biosynthesis as well as rhizosphere and root colonization (Ormeño-Orrillo et al., 2008).

Bacterial strains, plasmids and media.

METHODS

Bacterial strains, plasmids and media. Strains and plasmids used in this study are listed in Table 1. A. brasilense strains were grown at 30 °C in Luria–Bertani medium (LB; Difco) or in fructose high or low carbon-to-nitrogen ratio (C:N) media (37 mM C:4 mM N or 37 mM C:18 mM N, respectively) (Burdman et al., 1998). LB was used for growth of Escherichia coli strains at 37 °C. Triparental mating was performed on D-medium (Revers et al., 2000) as described by Vanstockem et al. (1987), and A. brasilense transconjugants were selected on minimal medium for A. brasilense (MMAB) (Vanstockem et al., 1987).

DNA manipulations and sequence analysis. Cloning, transformation procedures and Southern blot analyses were performed as described previously (Lerner et al., 2009). Restriction enzymes and DNA ligase were purchased from New England BioLabs. Taq polymerase and dNTPs were purchased from Promega. Oligonucleotide primers were purchased from Hy Laboratories. Sequence analyses were performed using the BLAST network service (Altschul et al., 1997) and InterProScan (EMBL-EBI; http://www.ebi.ac.uk/tools/InterProScan/). Sequences were analysed for protein localization using CELLO v.2.5 (Subcellular Localization Predictor; Molecular Bioinformatics Center; National Chiao Tung University; http://cello.life.nctu.edu.tw). The Expsy plI/Mw tool (http://www.expsy.ch/tools/pi_tool.html) was used for protein molecular mass prediction.

Construction of A. brasilense Sp7 noeJ::Km and noeL::Km mutants. Internal fragments of the noeJ and noeL coding regions of 1360 and 910 bp, respectively, were PCR-amplified using primers noeJ (5′-CACCTCGATCAGGTTCAG-3′) and noeJ-1428r (5′-CAGACACCGGATACAC3′) for the former, and noeL (5′-CAGCTAGGCGCAATG-3′) and noeL-R (5′-AGCCACCGACCATG-3′) for the latter. The primers were designed based on the pRhico sequence (Vanblieu et al., 2004; GenBank AY529373.1). PCR mixtures (25 μl) contained 0.3 μl Taq polymerase, 2.5 μl 10 × buffer, 3.75 mM MgCl₂, 0.8 μM of each primer, 0.2 mM of each dNTP, 0.01 mg BSA (Sigma) and 1 μl Sp7 DNA (about 30 ng) as template. Amplifications were performed in a Mastercycler Gradient (Eppendorf) with an initial denaturation cycle of 3 min at 95 °C, followed by 35 cycles of denaturation for 30 s at 94 °C, annealing for 45 s at 58.5 °C for noeJ, or 30 s at 55 °C for noeL, and elongation at 72 °C for 45 s. A final extension step was performed at 72 °C for 5 min. The PCR products were purified and cloned into pGEM-T Easy (Promega), to generate plasmids pNOEJ and pNOEL, which were transformed into E. coli DH15a. Following sequencing to confirm the identity of the cloned fragments, a 1.2 kb HinCl kanamycin-resistance (Km) cassette from pUC800 was inserted into the noeJ Stul site in pNOEJ to generate pNOEJ-Km, and the Tn5-derived 1.8 kb BamHI Km cassette from pUC18-2 was inserted into the BglII site of noeL in pNOEL to generate pNOEL-Km (Fig. 1). The 2.5 kb SalI/SphI noeL::Km and the 2.7 kb EcoRI noeL::Km fragments were excised from pNOEJ-Km and pNOEL-Km, respectively, and cloned into the suicide vector pSUP202, previously cut with the same enzymes, to yield pSUP-NOEJ-Km and pSUP-NOEL-Km, respectively. In pSUP-NOEJ-Km, the noeL::Km fragment was introduced inside the tet gene that encodes tetracycline (Tc) resistance, while in pSUP-NOEL-Km, the noeL::Km fragment was introduced into the cat gene that confers chloramphenicol (Cm) resistance. These plasmids were transformed into E. coli S17.1, which was used to mobilize them to A. brasilense Sp7 through triparental mating, using E. coli HB101 with the helper vector pRK2073. A. brasilense transconjugants were selected on MMAB supplemented with appropriate antibiotics. For selection of noeJ mutants, kanamycin (Km, 25 μg ml⁻¹), Cm (25 μg ml⁻¹), trimetoprim (Tr, 25 μg ml⁻¹) and ampicillin (Ap, 100 μg ml⁻¹) were used. Tr was used to avoid growth of donor cells (E. coli is susceptible to this antibiotic while A. brasilense is resistant), and Ap was used as an additional selection method as A. brasilense cells are resistant to this antibiotic. Selection of noeJ mutants was done using Km, Tr, Ap (at concentrations as described above) and Tc (10 μg ml⁻¹), Km/’Cm’ and Km/’Tc’ clones, for noeJ and noeL, respectively, were verified by PCR and Southern blotting. One each of the confirmed noeJ::Km and noeL::Km mutants (hereafter, noeJ and noeL mutants) were used for further analysis.
Extraction of EPS and LPS and colony staining. EPS composition and concentration were determined as described by Lerner et al. (2009). LPS extraction was performed using two methods. In one procedure, LPS were extracted and analyzed by SDS-PAGE as previously described (Lerner et al., 2009). In a second procedure, LPS were extracted with the LPS extraction kit (Intron Biotechnology) according to the manufacturer’s instructions and samples were run on a sodium deoxycholate (DOC)-PAGE gel. Table 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Reference or source</th>
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<tr>
<td>A. brasilense strains</td>
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<td>Sp7 (ATCC 29145)</td>
<td>Wild-type strain</td>
<td>Tarrand et al. (1978)</td>
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<td>noel::km</td>
<td>Sp7 transconjugant carrying a disruptional insertion of a Km cassette in the Stul site of the noel gene</td>
<td>This study</td>
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<td>HB101</td>
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<td>Invitrogen</td>
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<td>S17.1</td>
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<td>Simon et al. (1983)</td>
</tr>
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<td>Plasmids</td>
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<td>Phage f1 region, lacZ, cloning vector, Ap′</td>
<td>Promega</td>
</tr>
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<td>pSUP202</td>
<td>ColE1 replicon, mobilizable plasmid, suicide vector suitable for A. brasilense, Ap′ Tc′ Cm′</td>
<td>Simon et al. (1983)</td>
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<td>pUC18-2</td>
<td>pUC18 with a 3.4 kb HindIII fragment carrying the Km′ cassette from Tn5 (excised from pSUP2021)</td>
<td>Croes et al. (1991)</td>
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<td>pUC800</td>
<td>pUC8 with a Km′ cassette from pUC4K</td>
<td>Moens &amp; Vanderleyden (1996)</td>
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<td>pRK2073</td>
<td>pRK2013::Tn5, Km′ Sm′</td>
<td>Figurski &amp; Helinski (1979)</td>
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<td>This study</td>
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<td>This study</td>
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<td>This study</td>
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![Fig. 1. Physical location of the noeL (pRhico007) and noeJ (pRhico016) genes in the pRhico plasmid of A. brasilense Sp7 (Vanbleu et al., 2004). These genes are located in a cluster of genes involved in transport and biosynthesis of extracellular polysaccharides (Vanbleu et al., 2004; Lerner et al., 2009). Genes around noeL and noeJ are also shown: pRhico006, ATP-transporter permease (wzm); pRhico008–9, annotated as noeL; pRhico015, hypothetical protein; pRhico017, glycosyltransferase protein. Arrows indicate directions of transcription. Insertions of Km′ cassettes in the BgII and Stul sites of noeL and noeJ, respectively, for generation of knockout mutants, are indicated.](image-url)
according to the method of Reuhs et al. (1998). LPS staining was performed according to Tsai & Frasch (1982). Congo red and Calcofluor white staining was performed as described by Rodriguez Caceres (1982) and Groes et al. (1991), respectively.

**Extraction of cytoplasmic/periplasmic (CP) and outer membrane (OM) proteins.** Proteins were extracted from wild-type and noeJ/noeL mutants grown for 24 h in LB as described previously (Burdman et al., 1998). This procedure does not discriminate between non-membrane (cytoplasmic or periplasmic) proteins. Protein concentrations were determined using the Bio-Rad Protein kit. Proteins were separated by SDS-PAGE (12% acrylamide) for 2.5 h at 20 mA, and stained with Coomassie brilliant blue by standard methods (Laemmli, 1970).

**Analysis of mannose-6-phosphate isomerase (MPI) activity.** The noeJ mutant and the wild-type were grown in 250 ml fructose high C:N medium for 72 h. Cells were harvested by centrifugation at 16300 × g for 15 min at 4°C, washed with 50 mM phosphate buffer (pH 7.0) and resuspended in 10 ml of the same buffer. Crude extracts from harvested cells were obtained after 10 min sonication, followed by centrifugation at 17200 × g for 30 min. The supernatants were collected and assessed in MPI enzyme assays according to Kornmann et al. (2003) with a few modifications. Essentially, the activity was determined at 25°C by recording the reduction of NADP⁺ at 340 nm (molar absorption coefficient 6200 M⁻¹ cm⁻¹). Reaction mixtures (1 ml) were buffered with 100 μM Tris/HCl (pH 7.5) and contained 100 μM MgCl₂, 10 μM NADP⁺, 10 μM 250 μM glucose-6-phosphate dehydrogenase (G6PDH; Sigma) and 50 μM 250 mM mannose-6-phosphate. Protein concentrations were determined using the Bio-Rad Protein kit, with BSA as a standard protein.

**Assessment of survival to stress and sensitivity to antimicrobial compounds.** These experiments were performed as described previously (Lerner et al., 2009). Briefly, to assess bacterial survival after heat exposure, 10 ml bacterial cells was exposed to 55°C for 60 min, with their viability being determined every 15 min. In UV radiation experiments, the cells were exposed to UV for 120 s, and cell viability was determined every 30 s. Viable cells (c.f.u. ml⁻¹) were determined by dilution plating. Each experiment was conducted three times. The mutants were also compared with the wild-type strain for their sensitivity to several antibiotics, hydrogen peroxide and SDS, as described previously (Lerner et al., 2009). The noeJ mutant was also assessed for its response to saline stress in comparison with the wild-type strain. The noeJ mutant and wild-type were grown overnight in 5 ml LB. Samples (30 μl) of these cultures (at 6×10⁶ c.f.u. ml⁻¹) were transferred to 100 ml Erlenmeyer flasks with 30 ml fructose high C:N medium containing NaCl at 1.7 (controls), 300, 400 or 500 mM. Cultures were grown for 48 h at 30°C with shaking (200 r.p.m.), and OD₆₀₀ was measured. This experiment was conducted three times.

**Biofilm assays.** Biofilm formation was tested on glass surfaces by the procedure of Uhlich et al. (2006) with minor modifications. Briefly, glass microscope slides [3×1 inch (75×25 mm), Marienfeld Laboratory Glassware] were degreased with acetone, submerged in ethanol, rinsed with distilled water, and autoclaved. The slides were then placed in disposable, 50 ml polypropylene (Miniplast Ein-Semer), screw-topped tubes, containing 20 ml fructose high C:N or low-C:N medium (Burdman et al., 1998). Wild-type and mutant strains were grown for 48 h at 30°C without agitation. Then, cultures were poured out and the slides were heat-fixed at 80°C for 30 min. The biofilms were stained with 0.1% crystal violet for 20 min and washed with PBS. Quantitative analysis was done following solubilization of the stained biofilms with 95% ethanol for 15 min and by measurement of the A₅₃₀ of the stained suspension in a spectrophotometer. The experiments were carried out three times with six replicates per strain in each experiment.

**Statistics.** Data were subjected to one-way ANOVA using the JMP IN v3.2.1 software (SAS Institute).

**RESULTS**

**Sequence analysis of noeJ and noeL**

noeJ (pRhico016; annotated as mannose-6-phosphate isomerase; MPI) and noeL (pRhico007; annotated as GDP-mannose 4,6-dehydratase; GMD) possess coding regions of 1443 and 969 bp, respectively, and are located on the A. brasilense Sp7 pRhico plasmid, in a region containing genes that are involved in the transport and biosynthesis of extracellular polysaccharides (Fig. 1; Vanbleu et al., 2004, 2005; Lerner et al., 2009).

noeJ is the only gene in pRhico that is annotated as encoding an MPI. Recently, the draft genome of another A. brasilense strain, Sp245, became available (I. Zhulin; unpublished results) to some research groups, including ours. The Sp7 noeJ gene aligned with only one gene from the Sp245 draft genome sequence (results not shown). The identity percentages were 71% (at the DNA level, alignment of 1400 bp) and 60% (at the protein level, complete sequences). Southern blot analyses of Sp7 DNA treated with different restriction enzymes, following hybridization with noeJ probes, consistently yielded a single band (results not shown). Altogether, these data suggest that noeJ is present as a single-copy gene in A. brasilense Sp7.

In addition to noeL (pRhico007), another gene, pRhico008–009 (1062 bp), encodes a protein with predicted GMD activity. These genes showed 65% identity at the DNA level, for a partial alignment of 570 bp. BLAST analysis suggested that two genes encoding GMD activity are also present in Sp245. The Sp7 noeJ matched, with both genes at the protein level (63 and 46% identity), but matched with only one of them at the DNA level (71% identity for a partial alignment of 940 bp) (results not shown). Southern blot analyses of Sp7 DNA treated with different restriction enzymes, following hybridization with noeL probes, consistently yielded a single band (results not shown), which likely reflects the differences between pRhico007 and pRhico008–009.

**Analyses of LPS and EPS of noeJ and noeL mutants**

To assess whether noeJ or noeL plays a role in the synthesis of LPS and/or EPS of A. brasilense Sp7, we generated knockout mutants of these genes. LPS of the mutants and wild-type were extracted (Lerner et al., 2009) and compared by SDS-PAGE. Clear differences in LPS profiles were found between the three strains. While the wild-type pattern showed both high- and low-molecular-weight
bands (HMW and LMW, respectively) typical of this strain (Katz et al., 1998; Vanbleu et al., 2005; Konnova et al., 2008; Lerner et al., 2009), only an LMW band was detected in the LPS pattern of the noel mutant (Fig. 2). Intriguingly, no band could be detected from LPS extractions of the noeL mutant following various extractions. Similar results were obtained following LPS extraction of the noeL mutant with the Intron LPS extraction kit and DOC-PAGE analysis. These results indicate that impairment of noeJ or noeL affects LPS synthesis in *A. brasilense* Sp7.

LPS structure is known to influence colony roughness and morphology (Darveau & Hancock, 1983). Indeed, on solid fructose high C:N medium, the colonies of the two mutant strains were smoother and less regular than those formed by the wild-type strain, although these differences were not very pronounced (results not shown).

We further assessed EPS production in noeJ and noeL mutants. Three experiments were carried out to determine the EPS concentration and composition of the mutants relative to the wild-type strain. No differences in EPS concentrations [mg EPS (g bacterial dry weight)⁻¹] were found between wild-type and mutant strains (results not shown). Similarly, no differences among strains were found among wild-type and mutants in Congo red and Calcofluor white staining after growth for 48 h (results not shown). However, clear differences were observed in EPS composition between the strains. The EPS of the wild-type and the noeJ mutant were primarily composed of glucose and galactose (Table 2). In contrast, the EPS of the noeL mutant contained less galactose and glucose than the wild-type, although these differences were not very pronounced (results not shown).

*ND, Not detected.

<table>
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<th>Sugar</th>
<th>Sp7</th>
<th>noeJ mutant</th>
<th>noeL mutant</th>
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<td>Glucose + galactose</td>
<td>79.9 ± 1.6</td>
<td>90.6 ± 7.4</td>
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<td>Mannose</td>
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<td>7.8 ± 6.1</td>
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<tr>
<td>Rhamnose</td>
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<td>1.2 ± 0.9</td>
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<tr>
<td>Fucose</td>
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<td>0.06 ± 0.03</td>
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<tr>
<td>Ribose</td>
<td>1.3 ± 0.3</td>
<td>ND*</td>
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<td>Xylose</td>
<td>0.6 ± 0.2</td>
<td>0.3 ± 0.1</td>
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<td>Arabinose</td>
<td>0.7 ± 0.02</td>
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<td>5.1 ± 0.5</td>
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</table>

of this sugar as a result of reduced/abolished GMD activity. Arabinose and ribose could not be detected in the EPS of the noeJ mutant, and as expected, the noeJ EPS showed reduced concentrations of mannose and fucose relative to the wild-type EPS (Table 2).

**CP and OM protein analysis**

The predicted molecular masses of NoeJ and NoeL are 52.6 and 35.8 kDa, respectively. Both proteins were predicted to localize in the bacterial cytoplasm by CELLO v.2.5, with CELLO prediction scores of 3.472 and 4.231 for NoeJ and NoeL, respectively. SDS-PAGE of CP proteins revealed several differences in band patterns among wild-type and mutants (Fig. 3a). Arrows 1 (~52.4 kDa) and 2 (~35.9 kDa), marking the expected positions for NoeJ and NoeL, show that these bands are apparently absent in the CP protein profiles of the corresponding mutants. While the CP protein profile of the noeJ mutant was less distinct than that of the wild-type, the protein profile of the noeL mutant showed more pronounced alterations (Fig. 3a).

Modifications in LPS structure have been associated with changes in assembly and composition of OM proteins (Ames et al., 1974; Ried et al., 1990; de Cock et al., 1999), in *A. brasilense* Sp7 also (Lerner et al., 2009). Therefore, we examined whether the mutant strains differed in their OM protein profiles relative to the wild-type. Indeed, several differences were found. As for the CP protein profiles, the noeJ mutant showed pronounced differences in its OM protein composition relative to the wild-type (Fig. 3b). The typical 38.7 kDa major OM protein of Sp7, OmaA (Burdman et al., 2000a), was also dominant in the OM protein profile of the noeL mutant, while it was detected at

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**Fig. 2.** SDS-PAGE analysis of LPS extracted from *A. brasilense* Sp7 and noeJ and noeL mutant strains grown in LB medium. Arrows indicate the position of the typical high- and low-molecular-weight (HMW and LMW, respectively) bands of Sp7 LPS.
and the wild-type following exposure to heat (55 °C) and UV radiation under tested conditions (data not shown). In contrast, when strains were exposed to 0.01 % SDS on solid fructose high C:N medium, no growth of the noeJ mutant was observed, while the noeL mutant grew to a lesser extent than the wild-type strain (results not shown).

Both mutants were also more sensitive to 1.5 % hydrogen peroxide than the wild-type, as inhibition haloes caused by this treatment were significantly \((P=0.05)\) larger in the mutants than in the wild-type. Results from representative experiments (among three with similar results for each mutant) are shown in Fig. 4(a, b). In addition, differences in sensitivity to several antibiotics were observed between the mutants and the wild-type. Fig. 4(c, d) shows data from one representative experiment for each strain (of three with similar results). A consistent trend was observed by which the mutants were more sensitive than the wild-type to all tested antibiotics. However, significant differences \((P=0.05)\) were observed only for chloramphenicol, tetracycline and sulfafurazole for the noeJ mutant, and chloramphenicol, streptomycin and tetracycline for the noeL mutant.

Salinity may cause detrimental effects on soil microorganisms and on the productivity of crop plants. In some bacteria, MPIs have been shown to be involved in salt tolerance (Nogales et al., 2002; Zuleta et al., 2003; Wei et al., 2004). Therefore, we evaluated the effect of different NaCl concentrations on the A. brasilense Sp7 noeJ mutant. Both the noeJ mutant and the wild-type were unable to grow in the presence of 400 or 500 mM NaCl (not shown). However, while the wild-type strain was able to grow in the presence of 300 mM NaCl, the growth of the noeJ mutant under these conditions was significantly compromised (Fig. 5), supporting the hypothesis that noeJ contributes to salt stress tolerance in A. brasilense.

**Biofilm formation**

LPS and EPS have been reported to be involved in biofilm formation (Wai et al., 1998; Davey & O’Toole, 2000; Jackson et al., 2004). Herein, we evaluated whether the A. brasilense Sp7 noeJ and noeL mutants are affected in biofilm formation relative to the wild-type. During growth for 48 h in fructose media at two different C:N ratios, the noeL mutant formed significantly \((P=0.05)\) less biofilm than the other strains (Fig. 6). No significant differences in biofilm formation were found between the noeJ mutant and the wild-type strain under the conditions tested (Fig. 6).

**DISCUSSION**

Here, we characterized A. brasilense Sp7 mutants affected in noeJ (pRhico016) and noeL (pRhico007) genes, which encode MPI and GMD, respectively. These genes are located in the pRhico plasmid, which contains several genes involved in the biosynthesis of surface polysacchar-

[Image of the graph showing the protein profiles of A. brasilense Sp7, noeJ and noeL mutants by SDS-PAGE. Arrows indicate bands that are referred to in the text.]
Such polysaccharides as EPS and LPS (Vanbleu et al., 2004; Lerner et al., 2009).

MPI catalyses the reversible conversion of fructose 6-phosphate to mannose 6-phosphate (Schmidt et al., 1992). While there are four types of MPIs, sequence analysis revealed that the Sp7 noeJ encodes a type II MPI. Type II MPIs include bifunctional enzymes with MPI and guanosine diphospho-D-mannose pyrophosphorylase (GMP) activities, which are found, for instance, in Pseudomonas aeruginosa and Xanthomonas campestris. These MPIs are involved in a variety of microbial pathways, including D-mannose metabolism and synthesis of surface polysaccharides, including LPS (Dunwell et al., 2000, 2004).

In bacteria, fucose is found in complex polysaccharides in the cell wall and has been found to be associated with pathogenicity and nodulation (Somoza et al., 2000). The primary biosynthetic route to GDP-fucose is from GDP-D-mannose. This reaction is catalysed by two enzymes, GMD and GFS. GMD catalyses the conversion of GDP-D-mannose to the intermediate product GDP-4-keto-6-deoxy-D-mannose. This intermediate is then converted by GFS into GDP-L-fucose, which can be converted by GDP-4-keto-6-deoxy-D-mannose reductase (RMD) into GDP-D-rhamnose (Maki & Renkonen, 2004). Kneidinger et al. (2001) found a bifunctional enzyme with both GMD and RMD activities in Aneurinibacillus thermoaerophilus. This enzyme shows 45 and 55% identity with pRhico007 and pRhico008–009 products, respectively. Sequence analyses of these pRhico genes suggest that the former possesses GMD activity, while the latter possesses the GMD/RMD bifunctional activities (results not shown).

The involvement of noeJ and noeL in LPS and EPS synthesis has already been studied in other bacteria such as R. tropici (Ormeño-Orrillo et al., 2008), E. coli (Stevenson et al., 1996; Shao et al., 2003), Vibrio cholerae and Vibrio anguillarum (Stroher et al., 1998), and P. aeruginosa (Sa-Correia et al., 1987). Impairment of noeJ or noeL in A. brasilense Sp7 indeed affected its EPS composition and LPS structure. Some of the changes observed in the EPS composition of the mutants were expected based on the nature of the mutated genes: for example, disruption of noeL caused accumulation of mannose, while the noeJ mutation led to a decreased concentration of this sugar in the EPS.

**Fig. 4.** Inhibition haloes of A. brasilense noeJ and noeL mutants relative to the wild-type after exposure to 1.5% hydrogen peroxide [(a) and (b), respectively] and to several antibiotics [(c) and (d), respectively]. Data represent mean ± SD of three representative experiments with similar results. Different letters indicate significant (P<0.05) differences between strains for each treatment. Antibiotics: Na, nalidixic acid at 50 mg ml⁻¹; Tc, tetracycline at 10 μg ml⁻¹; S, streptomycin at 25 μg ml⁻¹; Sf, sulfafurazole at 100 μg ml⁻¹; Cm, chloramphenicol at 25 μg ml⁻¹.
However, mannose is found in small traces in the LPS of A. brasilense Sp7 (Vanbleu et al., 2005). The LPS pattern of the noeJ mutant was characterized by the presence of a single low-molecular-weight rough LPS (R-LPS) band. This result suggests that noeJ is involved in the synthesis of the O-antigen but not of the core oligosaccharide. It is possible that the inability of the noeJ mutant to convert fructose into mannose not only affects the incorporation of mannose into the LPS, but also affects fucose availability, as mannose is a precursor of the fucose biosynthesis pathway. Sequence and Southern blot analyses support the suggestion that noeJ is present in a single copy in the genomes of strains Sp7 and Sp245. In agreement with these results, enzyme activity assays revealed that MPI activity was likely abolished in the noeJ mutant.

The alterations in LPS structure caused by disruption of noeJ and noeL led to slight differences in roughness and colony morphology between the mutants and the wild-type. Changes in LPS structure are not always accompanied by pronounced alterations in colony morphology. Scupham & Triplett (1997) reported that in Brucella abortus, mutation of galE (or exoB), which is involved in LPS and EPS production, does not cause visible changes in cell morphology. Dharmapuri et al. (2001) found that Xanthomonas oryzae pv. oryzae mutants that are deficient in EPS and LPS production do not differ from the wild-type in colony roughness. In our study, noeJ and noeL mutants did not differ from the wild-type in their EPS concentration, or in Congo red and Calcofluor white staining. In V. cholerae, Deleya marina and X. campestris pv. campestris, rough appearance has been linked to EPS production (Hotte et al., 1990; Shea et al., 1991; Wai et al., 1998).

noeJ and noeL disruptions affected the composition of CP and OM proteins. Intact LPS is necessary for the proper expression, processing and insertion of certain OM proteins as well as for leakage of periplasmic enzymes (Hall & Silhavy, 1981; Michel et al., 2000). Each of the LPS components is involved in stabilization of the outer leaflet of the cell. For example, Salmonella typhimurium and E. coli mutants that have lost much of their LPS-core oligosaccharide show decreased amounts of several OM proteins (Sen & Nikaido, 1991). The lipid A moiety in E. coli has been found to be necessary for OmpA folding (Dornmair et al., 1990). Recently, we have shown that in A. brasilense Sp7, disruption of wzm, encoding a component of an ATP binding cassette (ABC) transporter involved in LPS synthesis, leads to pronounced alterations in the composition of OM proteins (Lerner et al., 2009).

The OM of Gram-negative bacteria forms an effective barrier to hydrophobic compounds. Alterations of the LPS structure in Rhizobium leguminosarum bv. trifoli, Shigella flexneri, E. coli and other bacteria cause increased susceptibility to SDS (Fralick & Burns-Kelihier, 1994; Janczarek et al., 2001; Edwards-Jones et al., 2004). In agreement with these findings, the Sp7 noeL mutant was
more susceptible than the wild-type to SDS, although the mutant was still able to grow in the presence of this detergent. Ormeño-Orrillo et al. (2008) showed that impairment of noeJ leads to an alteration of the LPS structure and an increased sensitivity to SDS in R. tropici. Indeed, the Sp7 noeJ mutant was clearly more sensitive to SDS than the wild-type strain. We also observed that the Sp7 noeJ mutant showed more pronounced changes in OM protein composition relative to the wild-type than the noeL mutant. The enhanced sensitivity of the noeJ mutant to SDS could be associated with the pronounced effects of this mutation on OM protein composition.

Alterations in the OM caused by changes in LPS may influence the susceptibility of bacteria to antibiotics (Nikaido, 2003). Here, we showed that mutations in noeJ and noeL increase the susceptibility of A. brasilense Sp7 to some antibiotics. One of the mechanisms by which hydrophilic antibiotics such as chloramphenicol and tetracycline penetrate into bacterial cells is via OM porins (Peterson et al., 1985; Nikaido, 1989, 2003). Indeed, the noeL and noeJ mutants showed increased susceptibility to these antibiotics, while the noeJ mutant also showed increased susceptibility to the hydrophobic antibiotic sulfafurazole.

EPS play a role in the response of bacteria to different stresses, such as heat and UV radiation (Wang et al., 2007; Davies & Walker, 2007). Under the conditions tested, the noeJ and noeL mutants showed similar responses to the wild-type to these stresses. The lack of differences in EPS concentration between the mutants and the wild-type could explain these findings. In Sinorhizobium meliloti, R. tropici and Azorhizobium caulinodans, the response to hydrogen peroxide has been shown to be associated with EPS rather than with LPS (D’Haeze et al., 2004; Davies & Walker, 2007; Ormeño-Orrillo et al., 2008). Here, we showed that the mutants were more sensitive to this stress than the wild-type, which might be the result of an LPS deficiency and/or EPS composition changes. We also found that the Sp7 noeJ mutant was compromised in its ability to grow in the presence of 300 mM NaCl. This finding is in agreement with results obtained with R. tropici, S. meliloti and Caulobacter crescentus, in which MPIs were found to be involved in salt tolerance (Nogales et al., 2002; Zuleta et al., 2003; Wei et al., 2004). The A. brasilense Sp7 wzm mutant, also affected in its LPS structure, has also been shown to be more sensitive to salt stress than the wild-type strain (Lerner et al., 2009).

LPS play a role in the initial attachment of bacteria to surfaces (Davey & O’Toole, 2000). LPS-defective mutants of Salmonella enterica and Pseudomonas fluorescens have been shown to differ from their parental strains in their ability to adhere to inert surfaces (Williams & Fletcher, 1996; Prouty & Gunn, 2003). Here, while the noeJ mutant was able to adhere to and form biofilm on glass similarly to the wild-type, the noeL mutant was defective in its biofilm formation ability on glass. While differences in LPS structure between the two mutants could be related to the observed differences in biofilm formation, we cannot exclude the possibility that differences in EPS composition between these strains are involved in the determination of this phenotype, as EPS are known to be associated with biofilm formation (Wai et al., 1998; Davey & O’Toole, 2000; Jackson et al., 2004).

To summarize, here we showed that noeJ and noeL are involved in LPS and EPS synthesis of A. brasilense Sp7. Disruption of either of these genes leads to a pleiotropic phenotype, which is associated with substantial changes in the protein set of the bacterium, and with altered responses to some stresses and antimicrobial compounds. As mentioned above, in R. tropici, a noeJ mutant has been shown to be affected in rhizosphere and root colonization (Ormeño-Orrillo et al., 2008). Further studies should evaluate whether the noeJ and noeL mutations affect the fitness of A. brasilense in natural niches, such as soil and rhizosphere, as well as plant growth promotion ability.

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Azospirillum brasilense noeJ and noeL


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