

The *wzm* gene located on the pRhico plasmid of *Azospirillum brasilense* Sp7 is involved in lipopolysaccharide synthesis

Anat Lerner, Yaacov Okon and Saul Burdman

Correspondence

Saul Burdman
saulb@agri.huji.ac.il

Department of Plant Pathology and Microbiology and The Otto Warburg Center for Agricultural Biotechnology, The Robert H. Smith Faculty of Agriculture, Food and Environment, The Hebrew University of Jerusalem, Rehovot 76100, Israel

Several genes involved in the interaction between *Azospirillum brasilense* Sp7 and plants are located on the pRhico plasmid. Here we report the characterization of an Sp7 mutant strain with impairment of the pRhico-located gene *wzm*. This gene encodes an inner-membrane component of an ATP-binding cassette (ABC) transporter with similarity to transporters involved in surface polysaccharide export. Indeed, SDS-PAGE revealed that LPS synthesis is affected in the *wzm* mutant. No significant differences were observed between wild-type and mutant strains in exopolysaccharide (EPS) amount; however, several differences were observed between them in EPS monosaccharide composition, and only wild-type colonies stained positively with Congo red. Microscopy revealed that *wzm* mutant cells are longer and thinner, and exhibit several differences in their cell surface relative to the wild-type. The *wzm* mutant was more resistant to oxidative stress, starvation, desiccation, heat and osmotic shock than the wild-type. In contrast, the mutant was more susceptible than the wild-type to UV radiation and saline stress. The strains also differed in their susceptibility to different antibiotics. Differences between the strains were also observed in their outer-membrane protein composition. No differences were observed between strains in their ability to attach to sweet corn roots and seeds, and to promote growth under the tested conditions. As LPS plays an important role in cell envelope structural integrity, we propose that the pleiotropic phenotypic changes observed in the *wzm* mutant are due to its altered LPS relative to the wild-type.

Received 30 June 2008

Revised 11 November 2008

Accepted 14 November 2008

INTRODUCTION

The *Azospirillum* genus belongs to the alpha-proteobacteria and comprises free-living, nitrogen-fixing, vibrio- or spirillum-shaped rods that exert beneficial effects on plant growth and yield of many crops of agronomic importance (Dobbelaere *et al.*, 2001). Plant growth promotion by *Azospirillum* is attributed to morphological and physiological changes in inoculated plant roots, which enhance water and mineral uptake. Plant growth substances, such as auxins, cytokinins and gibberellins (Steenhoudt & Vanderleyden, 2000; Dobbelaere *et al.*, 2001), as well as nitric oxide (Creus *et al.*, 2005) produced by the bacteria are at least partially responsible for the plant growth promotion.

Within the *Azospirillum* genus, one of the most studied species is *Azospirillum brasilense* (Tarrand *et al.*, 1978). *A. brasilense* cells are surrounded by a dense, tightly cell-bound layer of capsular polysaccharides (CPSs), and by outer exopolysaccharides (EPSs), which are loosely bound to the cell and easily detached by centrifugation (Burdman *et al.*, 2000c). EPSs and CPSs, as well as extracellular proteins, have been shown to play important roles in bacterial aggregation and in the establishment of the bacterium–plant association (Burdman *et al.*, 1999, 2000c; Steenhoudt & Vanderleyden, 2000). It has been suggested that EPSs and CPSs are involved in anchoring the bacteria to the surface of wheat roots (Michiels *et al.*, 1991), and in the proliferation of azospirilla in the root (Katupitiya *et al.*, 1995).

In media characterized by a low carbon-to-nitrogen (C:N) ratio, *A. brasilense* cells tend to grow in a dispersive form, whereas in a high C:N medium, the cells tend to aggregate and flocculate (del Gallo *et al.*, 1989; Burdman *et al.*, 2000b). Cell aggregation in *A. brasilense* has been shown to correlate positively with the amount of EPS produced (Burdman *et al.*, 2000b). Moreover, EPS mutants of *A.*

Abbreviations: ABC, ATP-binding cassette; CPS, capsular polysaccharide; d.a.s., days after sowing; DOC, deoxycholic acid; EPS, exopolysaccharide; OM, outer membrane; OMP, outer-membrane protein; SEM, scanning electron microscopy.

A supplementary table listing bacterial strains and plasmids used in this study, with supplementary references, is available with the online version of this paper.

brasile that have lost the ability to bind calcofluor are not able to aggregate and anchor to wheat roots, thus supporting the involvement of extracellular β 1-3 and/or β 1-4 polysaccharides in aggregation and root attachment (del Gallo *et al.*, 1989; de Troch, 1993). Several studies have been performed on *A. brasile* LPS, and a few LPS-defective mutants are available (Katzy *et al.*, 1998). However, little is known about the involvement of LPS in the interaction of *A. brasile* with plant roots, or about the role played by LPS in the response of the bacterium to the environment.

A. brasile strains carry large plasmids (Holguin *et al.*, 1999). For instance, *A. brasile* Sp7 contains five large plasmids, three with molecular masses of 46, 90 and 115 MDa, and two with molecular masses greater than 300 MDa (Vanstockem *et al.*, 1987). The 90 MDa plasmid, termed pRhico or p90, is widespread among *A. brasile* strains, and contains genes involved in their interaction with plant roots, synthesis of surface polysaccharides, motility and growth on minimal medium (Croes *et al.*, 1991; Vanbleu *et al.*, 2004). Recent release of the Sp7 pRhico sequence (Vanbleu *et al.*, 2004) has revealed that this plasmid contains two genes, *wzm* (pRhico062) and *wzt* (pRhico064), which encode components of transport systems belonging to the ATP-binding cassette (ABC) superfamily. ABC transport systems are composed of a hydrophobic integral membrane protein, which spans the membrane multiple times in an α -helical conformation, and a hydrophilic, membrane-associated ATP-binding protein, which is exposed to the cytoplasm (Davidson, 2002).

In Gram-negative bacteria, extracellular polysaccharides such as LPS, CPS and EPS are exported across the two membranes via ABC transporters (Schneider & Hunke, 1998; Silver *et al.*, 2001). Sequence analysis of the Sp7 *wzm* and *wzt* products suggests that they could be involved in translocation of extracellular polysaccharides. To deepen our understanding of the biosynthesis of extracellular polysaccharides in *A. brasile* Sp7 and of their role in the plant-bacterium association, we generated a *wzm* knockout mutant. Here we report the characterization of this mutant, which was assessed for its LPS pattern, EPS composition, response to several stresses and morphological properties. We show that the *wzm* mutation affects LPS synthesis and is responsible for many pleiotropic phenotypic changes in strain Sp7.

METHODS

Bacterial strains, plasmids and media. The strains and plasmids used in this study are listed in Supplementary Table S1. For short-term maintenance and preparation of starter cultures, *A. brasile* strains were grown at 30 °C in Luria-Bertani medium (LB; Difco). Experiments were performed in high C:N ratio medium, with 37 mM fructose as the carbon source (Burdman *et al.*, 1999) unless otherwise stated. LB was used to cultivate *Escherichia coli* strains at 37 °C. Triparental mating was performed on D-plates (8 g Bacto

nutrient broth l^{-1} , 0.25 g $MgSO_4 \cdot 7H_2O$ l^{-1} , 1.0 g KCl l^{-1} , 0.01 g $MnCl_2$, 2 % agar) and *A. brasile* transconjugants were selected on minimal medium for *A. brasile* (MMAB) as described by Vanstockem *et al.* (1987), supplemented with kanamycin (Km; 25 μ g ml^{-1}) and trimethoprim (Tr; 25 μ g ml^{-1}).

DNA manipulations and sequence analyses. Cloning and transformation procedures were performed according to standard methods (Sambrook *et al.*, 1989). Total DNA was isolated using the Wizard Genomic DNA Purification kit (Promega). For Southern blotting, DNA was digested with restriction enzymes purchased from New England BioLabs, electrophoresed, and blotted onto MSI nylon transfer membranes (Roche Diagnostics) by standard methods (Sambrook *et al.*, 1989). Detection was performed using the DIG DNA Labeling kit (Roche Diagnostics). PCR amplifications were performed on an automated Eppendorf Mastercycler. Sequence analyses were performed using the BLAST network service (Altschul *et al.*, 1997) and Pfam (<http://pfam.sanger.ac.uk/>). Oligonucleotide primers were synthesized using the Primer3 program (<http://www.es.emblnet.org/cgi-bin/primer3-www.cgi>) and purchased from Hy Laboratories. A hydropathy plot of the *wzm* product was obtained by the method of Kyte & Doolittle (1982).

Construction of an *A. brasile* Sp7 *wzm::Km* mutant. A 734 bp internal fragment of the *wzm* (pRhico062) coding region was PCR-amplified using primers *abcT*-L (5'-GAGATCGAAGCCC-GATAC-3') and *abcT*-R (5'-ATGTCGGCAAATCCAGTA-3'). These primers were designed based on the pRhico sequence (Vanbleu *et al.*, 2004). The PCR mixtures (25 μ l) contained 0.3 μ l *Taq* polymerase (Promega), 2.5 μ l 10 \times buffer (Promega), 3.75 mM $MgCl_2$, 0.8 μ M of each primer, 0.2 mM of each dNTP (Promega), 0.4 mg BSA ml^{-1} (Sigma), and 1 μ l template DNA. Amplifications were performed 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 30 s at 58.5 °C, and elongation for 45 s at 72 °C. A final extension step was performed at 72 °C for 5 min. The PCR product was purified and cloned into pGEM-T Easy Vector (Promega) to generate plasmid pABC, which was transformed into *E. coli* DH5 α . Following sequencing to confirm the identity of the cloned fragment, the 1.2 kb *HincII* Km-resistance cassette from pUCA800 was inserted into the unique *EcoNI* site of *wzm* (Fig. 1) in pABC to yield pABC-Km. Then, the 2 kb *wzm::Km* fragment from pABC-Km was excised from this plasmid using *SalI* and *SphI*, and cloned into the suicide vector pSUP202 that was pretreated with the same enzymes, to give pSUP-ABC-Km. This vector was subsequently transformed into *E. coli* S17.1, which was further used to mobilize it to *A. brasile* Sp7 through triparental mating, using *E. coli* HB101 carrying the helper vector pRK2073 (Vanstockem *et al.*, 1987). *A. brasile* transconjugants were selected on MMAB supplemented with Km and Tr, and Km-resistant clones were verified by Southern blotting and PCR. One of the confirmed *wzm::Km* mutants (hereafter, *wzm* mutant) was selected for further analysis.

Extraction of LPS. LPSs were extracted from wild-type and *wzm* mutant cells grown in LB and in high C:N medium, using published procedures (Cava *et al.*, 1989; Brink *et al.*, 1990; Tao *et al.*, 1992). Briefly, 1 ml of overnight-grown cultures were centrifuged (13 000 g, 1 min, twice), and the resulting pellets were resuspended in 130 μ l SDS sample buffer. The samples were then boiled for 5 min at 100 °C, and following removal of cell debris, proteinase K was added to a final concentration of 0.5 μ g μ l $^{-1}$. The samples were incubated at 37 °C for 1 h; afterwards, 20 μ l aliquots were loaded onto SDS-PAGE gels containing 3 and 15 % polyacrylamide concentrations in the stacking and resolving gels, respectively. The samples were electrophoresed at 15 mA for 3.5 h, and following electroblotting (70 V, 1 h) to a nylon membrane (Rosch) to remove residual proteins, the gels were silver-stained according to Tsai & Frasch (1982). In other experiments, LPSs

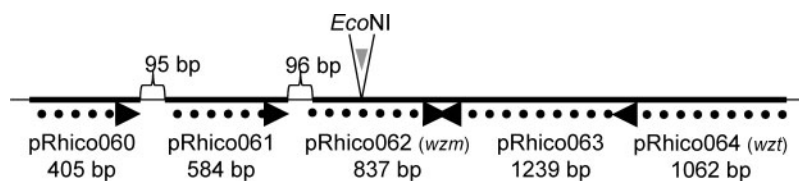


Fig. 1. Location of the *wzm* gene (pRhico062) in the pRhico plasmid of *Azospirillum brasilense* Sp7 (Vanbleu *et al.*, 2004). Insertion of the Km cassette in the *EcoNI* site for generation of the knockout strain is indicated.

were extracted with an LPS extraction kit (Intron Biotechnology), and samples were run on a deoxycholic acid (DOC)-polyacrylamide gel according to Reuhs *et al.* (1998).

Extraction of EPS. A 6 ml aliquot of overnight cultures of *A. brasilense* Sp7 or *wzm* mutant in high C:N medium at 6×10^8 c.f.u. ml^{-1} was used to inoculate 1 l of high C:N medium. After 48 h of growth at 30 °C with agitation at 250 r.p.m., when the cultures reached OD_{600} 0.8–0.9, EPSs were extracted as described by Burdman *et al.* (2000b). Sugar amount was evaluated by the anthrone method (Dische, 1962). Microbial mass was determined by measuring the dry cell mass of pelleted cells at 80 °C until a constant weight was reached. Identification of EPS monosaccharide composition was performed at the Center for Glycobiology at Ben-Gurion University of the Negev (Beer-Sheva, Israel) according to Albersheim *et al.* (1967), with minor modifications. Sugar composition of modified alditols was determined in a Hewlett Packard HP 5890 Series II gas chromatograph, equipped with a DB-225 capillary column (30 m \times 0.25 mm) from J&W Scientific and a flame-ionization detector (FID) at 250 °C. Samples (1 μl) of mixed alditols were separated at 220 °C with helium as the carrier gas. These experiments were conducted twice.

Congo red and calcofluor staining. Congo red and calcofluor staining was performed twice for each strain, as described by Rodríguez Cáceres (1982) and Croes *et al.* (1991), respectively.

Microscopy. For observation of viable bacterial cells and qualitative evaluation of cell motility, bacteria were grown for 24 h in high C:N medium, and cells were visualized in an Olympus BX 51 phase contrast microscope with an Olympus SC 35 type 12 camera. For scanning electron microscopy (SEM), cells were grown as described above and 500 μl of culture (at 10^7 c.f.u. ml^{-1}) was collected. Cells were centrifuged (4500 g, 10 min, 20 °C) and washed twice with 100 mM phosphate buffer (pH 6.8). The pellets were resuspended in fixation solution (2 % glutaraldehyde, 3 % paraformaldehyde, 0.1 M cacodylate buffer) and gently shaken for 1 h at room temperature. Following centrifugation (3620 g, 10 min, 20 °C), 0.1 M cacodylate buffer was added to the pellets. The cells were attached to silica chips coated with polylysine and kept overnight in a humid atmosphere. The samples were exposed to an additional fixation treatment with 1 % OsO_4 in 0.1 M cacodylate buffer for 1 h, and then washed twice in 0.1 M cacodylate buffer with gentle shaking for 5 min, and twice with 0.5 ml double-distilled water (DDW). Tannic acid (1 %) was added to the tubes, which were kept for 5 min in the dark. After two washes with DDW, 1 % uranyl acetate was added and the samples were shaken for 30 min in the dark. Dehydration was performed after two washes in DDW, by graded ethanol series and final drying in a critical point dryer (CPD 030, Bal-Tec). Dried bacteria were coated with gold in an Edwards S150 sputter coater and the samples were observed by SEM (Carl Zeiss, SUPRA 55VP).

Growth curves. Wild-type and mutant strains were grown overnight in 5 ml LB supplemented with antibiotics Tr and Km+Tr, respectively. Then, 30 μl of the cell suspensions (at about 6×10^8 c.f.u. ml^{-1}) were transferred to 100 ml Erlenmeyer flasks containing 30 ml high C:N medium with different carbon sources (D-fructose,

DL-malic acid, D-mannose, D-glucose, D-galactose, L-rhamnose, D-arabinose, L-arabinose and D-xylose) at 37 mM. Cultures were grown at 30 °C with agitation at 200 r.p.m., and the OD_{600} was measured every 2–3 h over the course of 48 h using a spectrophotometer (Coleman Junior II 6/20, Perkin-Elmer). The experiments were conducted twice, and in each experiment, the different media (with the different carbon sources) were inoculated with aliquots from the same pre-culture.

Starvation experiments. Aliquots (10 ml) of overnight, high C:N medium cultures of wild-type and mutant strains at approximately 6×10^8 c.f.u. ml^{-1} were washed twice by centrifugation (4000 g, 10 min). Cells were resuspended in 0.06 M potassium phosphate buffer (pH 6.8) and incubated on a shaker at 200 r.p.m., 30 °C for 12 days under starvation, as described by Kadouri *et al.* (2002). Bacterial viability was determined by dilution plating at the beginning and end of the incubation period. This experiment was repeated three times.

Stress endurance. In all experiments, 0.5 ml aliquots of LB-grown overnight cultures of wild-type and mutant strains were used to inoculate 100 ml Erlenmeyer flasks containing 30 ml high C:N medium, and grown for 24 h at 30 °C with agitation at 250 r.p.m. The resulting cultures were used to assess bacterial survival under various stresses. The percentage of viable cells was determined following dilution plating at the beginning and end of each experiment, and/or during the course of the experiment. In all experiments, the initial number of cells was 5×10^8 – 7×10^8 c.f.u. ml^{-1} . All experiments were conducted three times as described by Kadouri *et al.* (2003) with the following modifications: in the heat-resistance experiments, 10 ml of culture was incubated in a water bath at 55 °C for 60 min. Bacterial viability was determined every 15 min. In experiments performed to assess resistance to UV radiation, cells were exposed to UV radiation for 120 s, and bacterial viability was determined every 30 s. To assess survival of cells following desiccation, 1 ml cultures were washed twice by centrifugation with sterile DDW. Pelleted cells were resuspended in 100 μl DDW and transferred to 0.2 μm pore-size membrane filter papers (Whatman). The membranes were air-dried as described by Kadouri *et al.* (2003), transferred to 50 ml flasks and resuspended in 1 ml 0.06 M potassium phosphate buffer (pH 6.8). The flasks were shaken overnight at room temperature and cell viability was determined as described above. Sensitivity to osmotic pressure was determined by adding one volume of 2 or 4 M fructose to the cultures, yielding final fructose concentrations of 1 and 2 M, respectively. The suspensions were incubated at 30 °C for 24 h.

Sensitivity to hydrogen peroxide, antibiotics and SDS. Sensitivity to hydrogen peroxide and several antibiotics was measured as described by Kadouri *et al.* (2003). To assess the sensitivity of the strains to SDS, cells were grown in high C:N medium supplemented with 0.01 % SDS. All experiments were performed three times.

Sensitivity to saline stress. Wild-type and *wzm* mutant strains were grown overnight in 5 ml LB supplemented with Tr and Km+Tr, respectively. Aliquots (30 μl) of cultures (at $\sim 6 \times 10^8$ c.f.u. ml^{-1}) were

transferred to 100 ml Erlenmeyer flasks containing 30 ml high C:N medium with 300, 400 or 500 mM NaCl. Cultures were grown for 48 h at 30 °C with shaking (200 r.p.m.), and their OD₆₀₀ was measured every 2–3 h. The experiment was conducted twice.

Extraction of outer-membrane proteins (OMPs). OMP fractions were obtained as described by Burdman *et al.* (1998), from wild-type and *wzm* mutant strains grown for 48 h in high C:N medium. Protein concentrations were determined using the Bio-Rad protein assay reagent. Proteins were separated by SDS-PAGE (12% acrylamide), and stained with Coomassie brilliant blue by standard methods (Laemmli, 1970).

Plant growth promotion experiments. Seeds of sweet corn (*Zea mays* cv. Jubilee; Neta Quality Seeds) were surface-sterilized by soaking them for 5 min in absolute ethanol followed by five washes with sterile distilled water (DW). They were then placed in 50 ml Falcon tubes containing 30 ml bacterial suspension (Sp7 or *wzm* mutant) at 3×10^7 – 6×10^7 c.f.u. ml⁻¹. Controls were seeds incubated in 30 ml sterile DW. The tubes were shaken at 200 r.p.m. for 3 h at room temperature. Then the seeds were collected, air-dried and sown in pots (11.5 × 9 × 6 cm) filled with autoclaved vermiculite 3G (Agrekal), and covered with a 1 cm layer of autoclaved perlite 4 (Agrekal). The pots were maintained in a greenhouse (25 °C, 15 h light period), and were watered with 100 ml sterile DW every 4–5 days. Four experiments were carried out. In two experiments, five seeds were sown per pot, with three pots per treatment. After germination, three plants were kept in each pot, and plants were grown up to 21 days after sowing (d.a.s.). In the other two experiments, four seeds were sown per pot, with seven pots per treatment. Only one plant per pot was kept after germination and plants were grown for up to 18 d.a.s. Tested growth parameters included root and shoot wet and dry weight, and root and shoot length.

Root adhesion assays. Sweet corn seeds ('Jubilee') were surface-sterilized, treated with wild-type and mutant strains, and sown as described above for the first two growth promotion experiments. To determine the approximate number of bacterial cells that adhered to the seeds, four seeds per treatment were randomly selected. These seeds were transferred to 15 ml Falcon tubes containing 1 ml sterile DW, and were shaken at 200 r.p.m. overnight at room temperature. Then 0.5 ml of the suspensions was used for cell counting by dilution plating. To determine the approximate number of bacterial cells present on the roots, plants were removed from the pots 18 or 21 d.a.s., and the roots were cut and transferred to 50 ml Falcon flasks containing 25 ml sterile DW. The flasks were shaken at 200 r.p.m. overnight at room temperature. Then the suspensions were collected and used for cell counting by dilution plating. For dilution plating, solid high C:N medium containing Tr and cycloheximide (Ch; 25 µg ml⁻¹) was used.

Statistics. Experiments were statistically analysed by one-way analysis of variance (ANOVA) using JMP software (SAS Institute), unless otherwise stated.

RESULTS

Sequence analysis of the *A. brasilense* Sp7 *wzm* gene

The Sp7 pRhico plasmid contains a cluster of genes involved in surface polysaccharide biosynthesis (Vanbleu *et al.*, 2004). These genes can be grouped into three distinct categories based on the processes in which they are

involved: (i) synthesis of nucleotide sugar precursors; (ii) transfer of activated sugars to the growing carbohydrate chain; and (iii) export, polymerization and assembly of the oligosaccharides or polysaccharides. This cluster contains two genes, *wzm* (pRhico062, GenBank accession no. AAS83098) and *wzt* (pRhico064, GenBank accession no. AAS83100) (Fig. 1), which putatively encode a 278 aa ATP transporter permease and a 353 aa ABC transporter ATP-binding component-like protein, respectively (Vanbleu *et al.*, 2004).

In this study, we focused on *wzm*. Pfam analysis revealed that the predicted Wzm contains an ABC-2 membrane domain (PF01061) from amino acids 26 to 239. This domain is characteristic of a subfamily of ABC-type transport systems that catalyse export of drugs or carbohydrates, including surface polysaccharides (Reizer *et al.*, 1992). BLASTP confirmed that the *wzm* product is similar to inner-membrane subunits of ABC-2-type transporters. The highest similarity among reference (refseq) proteins was a *Pseudomonas fluorescens* ABC-2-type transporter protein (YP_262563), with 50% identity and 72% similarity for a large portion of these proteins (amino acids 22–278 of Wzm). Similar homology parameters (46–50% identity, and 66–70% similarity to similar portions of the predicted proteins) were found between Wzm and the inner-membrane proteins of an ABC polysaccharide efflux pump of *Burkholderia xenovorans* (YP_554653), an ABC O-antigen/LPS exporter of *Xanthomonas oryzae* pv. *oryzicola* (ZP_0224686), an ABC polysaccharide/polyol phosphate export pump of *Burkholderia* sp., and a putative LPS transporter of *Burkholderia pseudomallei* (ZP_02456953), among others. Hydropathy plot analysis of the *wzm* product supports a hydrophobic protein, which is predicted to span the membrane six times (data not shown), as is typical for inner-membrane components of ABC transporters.

Generation of an *A. brasilense* *wzm* mutant and its initial characterization

To assess whether *wzm* plays a role in the synthesis of LPSs and/or EPSs of *A. brasilense*, we generated a knockout strain for this gene in the background of strain Sp7 by marker exchange. The selected mutant was confirmed by PCR and Southern blotting (not shown). Initial observations revealed that, in contrast to the relatively smooth colonies produced by wild-type Sp7 after 48 h of growth in solid high C:N medium, colonies of the *wzm* mutant have a rough appearance (results not shown). LPSs of Gram-negative bacteria generally consist of lipid A, a polysaccharide component with an inner and outer core, and the variable O-antigen portion composed of oligosaccharide subunits. In several species, a switch from smooth to rough colonies occurs in mutants that fail to add the inner core or the O-specific chain (Godowski, 2005; Wolf & Goldberg, 2006). Thus, the observed differences in colony morphology between the wild-type and *wzm* mutant support the

involvement of *wzm* in LPS synthesis, as inferred by sequence analysis of this gene.

The *wzm* mutant colonies showed an unusual orange pigmentation when grown on solid media (results not shown). Therefore, molecular assays were performed to ensure that the mutant was indeed *A. brasilense* rather than a contaminant. These tests included PCR using an *A. brasilense* 16S rDNA primer set (Herschkovitz *et al.*, 2005) and sequencing of the obtained product, as well as denaturing gradient gel electrophoresis (DGGE) analysis using universal 16S rDNA primers (Lerner *et al.*, 2006). Results from these tests confirmed that the mutant was indeed derived from *A. brasilense* Sp7 (results not shown).

Analyses of LPSs and EPSs of the *wzm* mutant

We then compared LPS patterns of mutant and wild-type strains, following LPS extraction from boiled cells and SDS-PAGE analysis. Clear differences in LPS profiles were found between the two strains after growth in both LB and high C:N medium with fructose as the carbon source (Fig. 2). In both cases, the wild-type pattern showed a high and an intermediate molecular mass band absent in the *wzm* mutant profiles (Fig. 2, arrows 1 and 2, respectively). Similarly sized bands have been observed in other LPS studies of *A. brasilense* strains, including with strain Sp7 (Katzy *et al.*, 1998; Konnova *et al.*, 2008). These bands (arrows 1 and 2, respectively) have been shown to correspond to the smooth-LPS (containing lipid A, core oligosaccharide and O-antigen) and rough-LPS (lacking the O-antigen part) (Konnova *et al.*, 2008). In contrast to the wild-type, the *wzm* mutant pattern showed diffuse bands of lower molecular mass (Fig. 2, arrow 3), confirming that the *wzm* mutation affects the LPS structure of *A. brasilense* Sp7. Other experiments, in which LPSs were

extracted with a commercial kit and analysed by DOC-PAGE, showed similar results (not shown), supporting major LPS changes in the mutant strain relative to the wild-type.

We further assessed EPS production in the *wzm* mutant. In these and subsequent experiments, the high C:N fructose medium described by Burdman *et al.* (1999) was used, since conditions of carbon-source excess and nitrogen-source limitation induce EPS production by *A. brasilense* (del Gallo *et al.*, 1989), and because we have used this medium in various studies aimed at characterizing extracellular polysaccharide synthesis and other related features of *A. brasilense* (Burdman *et al.*, 1998, 1999, 2000b; Kadouri *et al.*, 2002, 2003; Bahat-Samet *et al.*, 2004).

Two experiments were carried out to determine the EPS concentration of the *wzm* mutant relative to the wild-type. The results of the two experiments were similar, and two-way ANOVA revealed that the effects of the experiments were not significant ($P=0.19$); therefore, data from the two experiments were pooled for one analysis. No significant differences in EPS concentration were found between wild-type and *wzm* mutant strains under the tested conditions. The EPS concentrations were 519.6 ± 28.2 and 506.2 ± 25.2 mg EPS (g bacterial dry weight)⁻¹ (mean \pm SE) for wild-type and mutant strains, respectively.

Two experiments were performed to determine the monosaccharide composition of the *wzm* mutant relative to the wild-type strain. The experiments gave similar results and overall, no dramatic differences were observed between strains in their monosaccharide composition (Table 1). Nevertheless, several differences were found; for instance, the relative concentration of fucose was two times higher in the EPS of the mutant than in that of the wild-type. In addition, the mutant EPS had slightly higher concentrations of glucose than that of the wild-type. In contrast, the EPS of the mutant strain showed galactose concentrations that were 1.9 times lower than those of the wild-type EPS (Table 1).

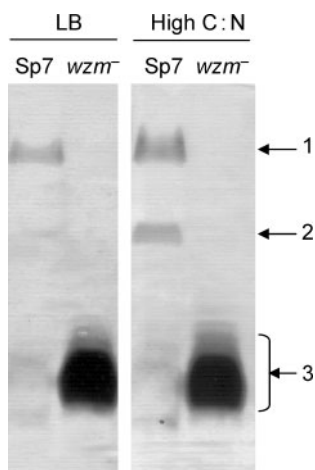


Fig. 2. SDS-PAGE of LPS extracted from *A. brasilense* Sp7 and *wzm* mutant grown in LB and high C:N fructose media. Arrows indicate positions of bands that are discussed in the text.

Table 1. Monosaccharide composition of EPSs extracted from *A. brasilense* Sp7 and *wzm* mutant strains, after 48 h of growth in high C:N fructose medium

Results represent average \pm range of the relative presence of each sugar (percentage) from two independent experiments.

Sugar	Sp7	<i>wzm</i> mutant
Rhamnose	2.1 \pm 0.6	2.4 \pm 1.9
Fucose	1.3 \pm 0.5	2.6 \pm 0.2
Ribose	2.5 \pm 0.3	1.7 \pm 1.6
Arabinose	1.4 \pm 0.2	1.1 \pm 0.1
Xylose	1.9 \pm 1.2	1.6 \pm 0.2
Mannose	28.8 \pm 1.0	25.9 \pm 4.2
Galactose	15.2 \pm 0.1	8.1 \pm 4.0
Glucose	46.8 \pm 1.3	56.7 \pm 0.6

Wild-type and mutant strains were also grown on solid high C:N medium in the presence of Congo red or calcofluor white. Both strains reacted similarly with calcofluor white (results not shown); however, only the wild-type strain showed positive staining with Congo red after 48 h of growth. Differences in Congo red staining between wild-type and mutant colonies became stronger after 72 h of growth due to stronger staining in wild-type colonies (results not shown).

Microscopic characterization of the *wzm* mutant

Observations of cells grown for 24 h in high C:N fructose medium by phase-contrast microscopy revealed substantial differences between the mutant and wild-type. While wild-type cells looked round and fat with visible polyhydroxybutyrate (PHB) granules (Fig. 3a), the cells of the *wzm* mutant looked thinner and longer than the wild-type cells, with no visible PHB granules (Fig. 3b). In addition, long chains of cells were observed in the mutant but not in the wild-type. Qualitative microscopy observations also revealed that the mutant strain appears to be affected in

swimming motility: while Sp7 cells exhibited a typical, rapid swimming motility, the *wzm* mutant cells moved slightly more slowly and in a wave-like pattern.

SEM revealed that many of the Sp7 cells were arranged in microaggregates (Fig. 3c), whereas most of the *wzm* mutant cells were individually distributed (Fig. 3d). In addition, extracellular microfibrils were produced by the wild-type (Fig. 3e; white arrows) but not by the mutant (Fig. 3f). These microfibrils have been suggested to be involved in cell aggregation (Burdman *et al.*, 1998). Finally, the *wzm* mutant cells showed different sizes and shapes as well as some variety in surface appearance (Fig. 3f), as opposed to the uniformity of the wild-type cells (Fig. 3e). Interestingly, in contrast to most wild-type cells, which lost their polar flagella during the SEM preparation procedure (Fig. 3c), most mutant cells retained their polar flagella (Fig. 3d, f). This difference could be due to alterations caused by the *wzm* mutation in cell envelope structural integrity, as it is known that LPS plays an important role in OM stabilization and in folding and assembly of bacterial OM proteins (de Cock *et al.*, 1999; Michel *et al.*, 2000).

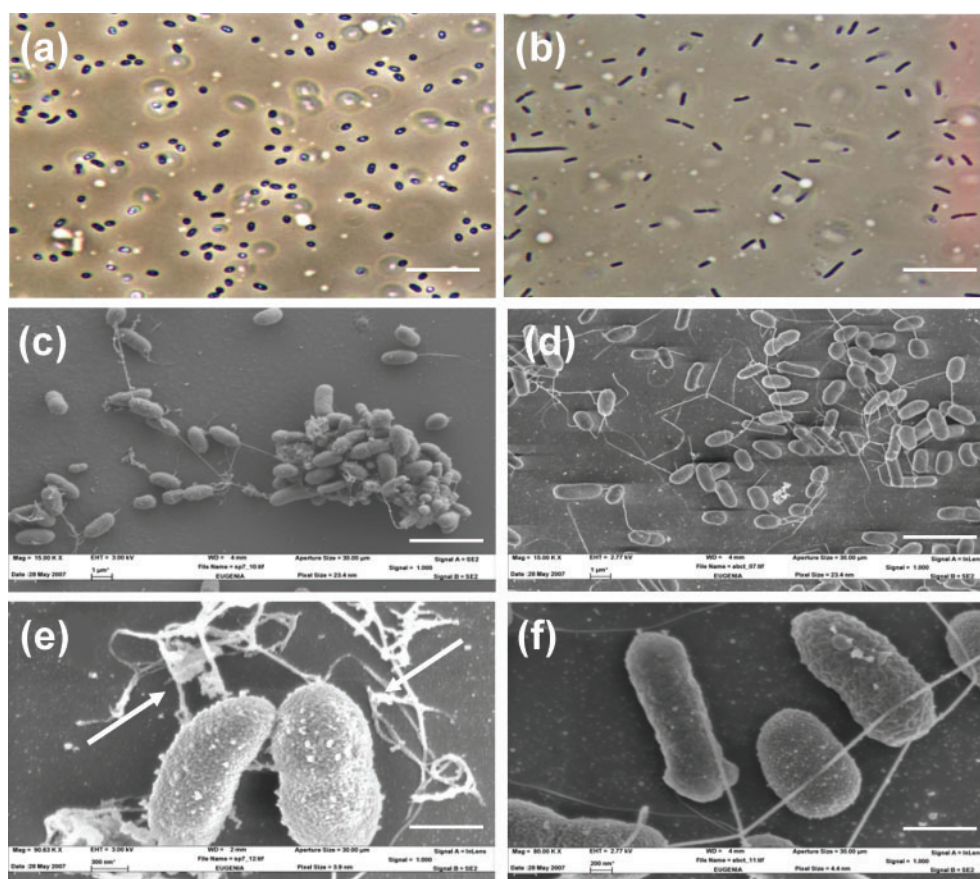


Fig. 3. Phase-contrast (a, b) and scanning electron (c–f) micrographs of *A. brasilense* Sp7 (a, c and e) and the *wzm* mutant (b, d and f) after 24 h of growth in high C:N fructose medium. White arrows in (e) indicate extracellular microfibrils produced by the wild-type. Bars: (a, b), 9.5 μ m; (c, d), 3.5 μ m; (e), 0.57 μ m; (f), 0.76 μ m.

Characterization of *wzm* mutant growth

As part of the *wzm* mutant characterization, the strain was compared with the wild-type for its ability to grow in the presence of different sugars and malic acid as sole carbon sources. In these experiments, mutant and wild-type strains were grown for 48 h in high C:N media containing the tested carbon sources at 37 mM. Differences in growth intensity were observed among the strains with most of the carbon sources tested (Table 2). Wild-type cells were able to utilize all carbon sources tested. In contrast, the mutant strain was not able to grow on L-rhamnose or D-arabinose as sole carbon sources. In addition, the mutant strain grew to a lesser extent (lower OD values) than the wild-type strain on D-fructose, D-mannose, D-glucose, D-xylose and L-arabinose (Table 2). On DL-malic acid and on D-galactose, the mutant strain grew to an OD similar to that of the wild-type. However, the lag phases of the mutant were longer than those observed for the wild-type on these and most of the other tested carbon sources. The exception was L-arabinose: although the wild-type grew to a higher OD than the mutant, it had a more prolonged lag phase (27 h compared with 18–24 h for the mutant). Representative growth curves of the *wzm* mutant and wild-type in some of the carbon sources (malic acid, D-xylose and D-arabinose) are shown in Fig. 4.

Assessment of resistance of the *wzm* mutant to different stresses

LPSs contribute to the structural integrity of the cell envelope of Gram-negative bacteria and are also involved in diverse interactions between bacterial cells and the environment (Lüderitz *et al.*, 1982). As the *wzm* mutation affected LPS in *A. brasilense* Sp7, we examined whether the

Table 2. Growth of *A. brasilense* Sp7 and *wzm* mutant strains in high C:N medium with different carbon sources at 37 mM

Cultures were grown for 48 h and results express growth level based on OD values at the end of the growth period: –, no growth; +, final OD₆₀₀=0.1–0.3 (moderate growth); ++, final OD₆₀₀ >0.3. Differences between strains in lag-phase extension are discussed in the text.

Carbon source	Sp7	<i>wzm</i> mutant
D-Fructose	++	+
DL-Malic acid	++	++
D-Mannose	++	+
D-Glucose	++	+
D-Galactose	++	++
L-Rhamnose	++	–
D-Xylose	++	+
D-Arabinose	++	–
L-Arabinose	++	+

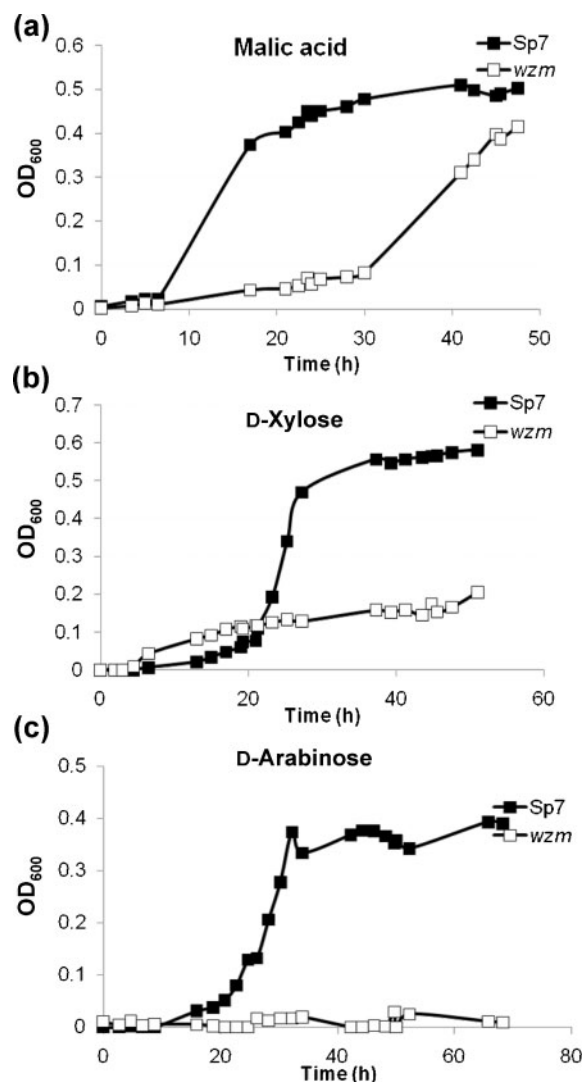


Fig. 4. Representative growth curves (of two with similar results) of *A. brasilense* Sp7 and *wzm* mutant in high C:N medium with different carbon sources: (a) malic acid; (b) D-xylose; (c) D-arabinose.

mutant strain exhibits a different response to diverse stresses relative to the wild-type. Interestingly, these experiments revealed that the mutant was more resistant than the wild-type to several stresses. For instance, following exposure of bacteria to heat (55 °C), viable cells of the wild-type declined more rapidly than those of the *wzm* mutant: after 1 h of exposure, only 0.01–0.02 % of the wild-type bacteria were still alive, whereas at the same time point, 3–6 % of the mutant cells were still alive (percentages represent ranges of survival from three different experiments with similar results; data from a representative experiment are shown in Fig. 5a). The mutant also showed significantly higher survival rates than the wild-type in response to glycerol-induced osmotic shock and to osmotic

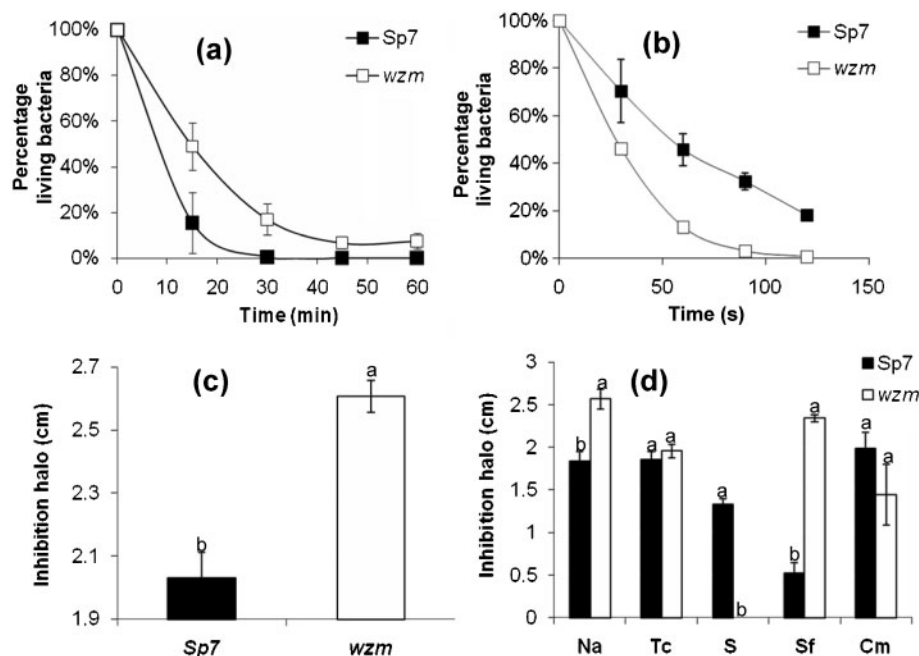


Fig. 5. Exposure of *A. brasilense* Sp7 and *wzm* mutant to different stresses and antibiotics: (a) heat (55 °C); (b) UV radiation; (c) 1.5% hydrogen peroxide; (d) antibiotics [Na, nalidixic acid (50 mg ml⁻¹); Tc, tetracycline (10 µg ml⁻¹); S, streptomycin (25 µg ml⁻¹); Sf, sulfafurazol (100 µg ml⁻¹); Cm, chloramphenicol (25 µg ml⁻¹)]. Data represent mean ± SD of representative experiments (of three with similar results). Different letters indicate significant differences ($P=0.05$) between wild-type and mutant strains.

pressure induced by high concentrations of fructose, as well as to desiccation and starvation (Table 3).

In contrast, the *wzm* mutant exhibited higher sensitivity than the wild-type strain to UV radiation: while 11–18% of the wild-type cells survived after 120 s of exposure to UV radiation, only 0.7–0.8% of the mutant cells survived under the same conditions (ranges from three different experiments with similar results; data from a representative experiment are shown in Fig. 5b). Similarly, the mutant strain exhibited significantly higher sensitivity than the wild-type to hydrogen peroxide (a representative experiment of three with similar results is shown in Fig. 5c).

The mutant was also more sensitive than the wild-type to saline stress. Neither strain was able to grow in the presence

of 400 or 500 mM NaCl. However, while the wild-type was able to grow in the presence of 300 mM NaCl ($OD_{600} \sim 0.5$ after 48 h of growth), the *wzm* mutant was not able to grow under these conditions. No differences were observed between wild-type and *wzm* mutant in their ability to grow on high C:N media supplemented with 0.01% SDS (not shown).

Assessment of the response of the *wzm* mutant to antibiotics

Alterations in the structural integrity of the cell envelope caused by defective LPS may affect the response of bacteria to antibiotics. Indeed, differences in sensitivity to antibiotics were observed between *wzm* mutant and wild-type

Table 3. Survival rates (in percent) of *A. brasilense* wild-type Sp7 and *wzm* mutant in response to exposure to osmotic shock, desiccation, starvation and osmotic pressure

Different letters indicate significant differences ($P=0.05$) between strains under the different treatments.

Strain	Osmotic shock	Desiccation	Starvation	Osmotic pressure	
				1 M fructose	2 M fructose
Sp7	$0.3 \pm 0.1a$	$4.1 \pm 2.2a$	$2.4 \pm 1.3a$	$0.004 \pm 0.001a$	$0.001 \pm 0.001b$
<i>wzm</i> mutant	$15.1 \pm 0.9b$	$15.0 \pm 2.6b$	$14.9 \pm 0.4b$	$38.2 \pm 48.6b$	$2.5 \pm 3.1b$

strains (Fig. 5d). The wild-type was significantly less susceptible than the mutant to sulfafurazol and nalidixic acid. Interestingly, in contrast to the wild-type strain, which was susceptible to streptomycin, the mutant strain was found to be resistant to this antibiotic. Under the tested conditions, no significant differences were observed between the strains in their response to tetracycline and chloramphenicol (Fig. 5d), although with the latter, reduced inhibition haloes were consistently measured for the mutant relative to the wild-type strain.

OMP profile of the *wzm* mutant

The pleiotropic phenotype of the *wzm* mutant, including its differential response under diverse stress conditions relative to the wild-type, could be due, at least in part, to changes in the composition of OMPs. To assess this possibility, we compared the OMP pattern of the mutant with that of the wild-type by SDS-PAGE. Two independent OMP extractions of wild-type and mutant strains were performed showing similar results. SDS-PAGE revealed substantial differences between the strains (Fig. 6). These included the absence or significant reduction of wild-type bands in the mutant profile, such as the band representing the typical *A. brasilense* 38.7 kDa major OMP, OmaA (Burdman *et al.*, 2000a) (Fig. 6, upper arrow). Instead of this protein, a strong band with slightly higher mobility was observed in the mutant profile (Fig. 6, lower arrow). On the other hand, several other bands were observed in the profile of the *wzm* mutant and were apparently absent or reduced in the wild-type profile. It is likely, however, that these differences were mainly due to the dominance of the OmaA protein in the OMP profile of the wild-type, but not of the mutant, which led to a significant relative reduction of other bands in the former. In support of this,

these bands had already been observed in previous studies of *A. brasilense* OMPs (Burdman *et al.*, 1999)

Plant growth promotion and adhesion to seeds and roots

We also assessed the ability of the *wzm* mutant to adhere to sweet corn seeds and roots and to induce the growth of these plants. No significant differences in adhesion to seeds and roots were observed between mutant and wild-type strains under the tested conditions. The average number of cells that could be extracted from the seeds after 3 h of incubation was 2.5×10^7 and 5×10^7 c.f.u. ml⁻¹ for wild-type and mutant, respectively, and these differences were not significant. Similarly, the strains did not differ in the number of cells extracted from inoculated roots, which varied between 10^6 and 10^7 c.f.u. (g root wet weight)⁻¹ in the different experiments.

Under the tested conditions, mutant and wild-type strains did not differ in their growth promotion-inducing ability. In the different experiments, both strains consistently induced higher weight of roots and shoots, as well as longer roots and shoots, in comparison with non-inoculated plants (results not shown), although the differences between inoculated and non-inoculated plants were not statistically significant in the different experiments.

DISCUSSION

In this study, we characterized a mutant strain of *A. brasilense* Sp7 with impairment in *wzm* (pRhico062), a gene encoding an inner-membrane protein of an ABC transporter. *wzm* is located in the pRhico plasmid, in a cluster of genes involved in the synthesis of surface polysaccharides (Vanbleu *et al.*, 2004). Indeed, sequence analyses of the predicted *wzm* product indicate that this gene belongs to the ABC-2-type transporter subfamily, which contains transporters involved in the export of surface polysaccharides (Reizer *et al.*, 1992). In agreement with this, we demonstrated that the *wzm* mutant is affected in LPS synthesis, and is altered in its EPS monosaccharide composition relative to the wild-type.

In addition to the integral inner-membrane component, ABC transporters are composed of a hydrophilic ATP-binding protein. Closely linked to *wzm*, there is a gene annotated as *wzt* (pRhico064) that encodes a typical ATP-binding protein. It is reasonable to speculate that the *wzt* product is the concomitant protein of Wzm in *A. brasilense*, although this hypothesis has yet to be experimentally validated. Homologous Wzm–Wzt systems have been shown to be involved in export of the O-antigen portion of the LPS in several Gram-negative bacterial species (Guo *et al.*, 1996; Saigi *et al.*, 1999; Feng *et al.*, 2004). In our study, the *wzm* mutant colonies had a rough appearance compared with the smoothness of those of the wild-type. This switch from smooth (S) to rough (R)

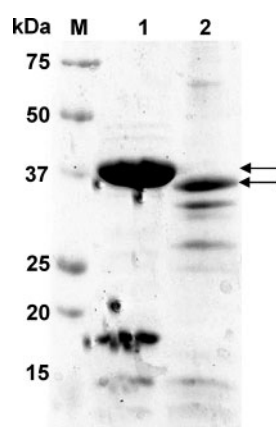


Fig. 6. SDS-PAGE of OMPs of *A. brasilense* Sp7 and *wzm* mutant (lanes 1 and 2, respectively). M, prestained marker (Precision Plus Protein Standards, Bio-Rad). Arrows indicate the position of bands discussed in the text.

colonies occurs in several Gram-negative mutants that fail to add the O-antigen portion to the LPS (Kustos *et al.*, 2000a, b; Godowski, 2005; Wolf & Goldberg, 2006). However, from our study, we cannot conclude that Wzm is involved in O-antigen export in *A. brasilense*, as we were not able to detect in the mutant the intermediate molecular mass band that is typical of the *A. brasilense* Sp7 R-LPS, consisting of lipid A and core oligosaccharide (Konnova *et al.*, 2008). Therefore, the LPS profile of the mutant strain, characterized by diffuse, low-molecular-mass bands, suggests that in *A. brasilense*, Wzm could also be involved in export of the core oligosaccharide.

Upstream of and in close proximity to *wzm* and *wzt*, there are other genes, pRhico046 to pRhico061, which are likely to be involved in LPS biosynthesis and assembly (Vanbleu *et al.*, 2004). For example, genes pRhico046 to pRhico050 are involved in the biosynthesis of dTDP-rhamnose. In *A. brasilense* Cd, rhamnose, as well as galactose and mannose, have been found to be components of the LPS (Konnova *et al.*, 2006). Fedonenko *et al.* (2002) found an O-specific pentasaccharide composed of repeating units of D-rhamnose in *A. brasilense* Sp245. Jofré *et al.* (2004) isolated an *A. brasilense* Cd mutant impaired in rhamnose production and found that it produces LPS with small traces of this specific sugar.

The *wzm* mutant and wild-type strains did not differ significantly in their EPS amount, but several differences were observed between them in EPS monosaccharide composition. Wild-type and mutant strains also differed in their ability to bind to Congo red, a property that is often related to EPS composition. Earlier findings have shown the presence of identical epitopes in *A. brasilense* LPS and EPS, leading to the hypothesis that EPS is formed by the excreted O-specific fragments of LPS (Matora *et al.*, 1995; Katzy *et al.*, 1998). Whether this phenomenon, which has also been proposed for other bacterial species (Kenne & Lindberg, 1983; Whitfield *et al.*, 1994), occurs in *A. brasilense*, and the biological relevance of the alterations in EPS by the *wzm* mutation, still need to be assessed.

The *wzm* mutant showed pleiotropic phenotypic alterations relative to the wild-type. In agreement with our findings, Bliss *et al.* (1996) and Cuthbertson *et al.* (2005) reported diverse changes in cell morphology of *E. coli* mutants defective in *kpsT* and *wzt*, which encode ATP-binding cassettes of EPS and LPS transporters, respectively. The latter is similar to the *A. brasilense* *wzt* gene (pRhico064). Microscopic observations revealed differences in cell shape and size, and in the presence or absence (in Sp7 and the mutant strain, respectively) of extracellular microfibrils connecting the cells. The occurrence of such a fibrillar matrix, likely involved in cell aggregation and root attachment, has previously been reported in *A. brasilense* (del Gallo *et al.*, 1989; Katupitiya *et al.*, 1995; Burdman *et al.*, 1998; Puente *et al.*, 1999; Galindo Blaha & Schrank, 2003). In addition to the differences in EPS composition, the differences in fibrillar matrix production between the

strains could also contribute to their differential staining with Congo red.

Using random omegon-Km mutagenesis, Katzy *et al.* (1998) isolated six LPS mutants of *A. brasilense* Sp245. Interestingly, although the exact insertion of the omegon-Km cassette was not identified, in all mutants the cassette was detected in the 120 MDa plasmid (p120). In agreement with these findings, in a further study it was shown that several fragments of this plasmid hybridize with the Sp7 pRhico plasmid (Petrova *et al.*, 2005) that, as already mentioned, contains several genes involved in the synthesis of surface polysaccharides, including LPS. In our study, the *wzm* mutant appeared to be affected in swimming motility compared with the wild-type strain. Loss of wild-type motility was also reported for one of the Sp245 LPS mutants (Katzy *et al.*, 1998).

LPSs are major components of the OM of Gram-negative bacteria, and the structural integrity of the OM may affect the response of bacteria to diverse antibiotics (Nikaido & Vaara, 1985; Sukupolvi & Vaara, 1989). Indeed, differences in sensitivity to some tested antibiotics were observed between the wild-type and mutant, with the most pronounced difference being the response to streptomycin, to which the *wzm* mutant was completely resistant under the tested conditions, in contrast to the wild-type, which was susceptible. In agreement with our findings, Bryan *et al.* (1984) reported that conversion of the smooth LPS to the rough LPS phenotype increased the resistance of *Pseudomonas aeruginosa* to aminoglycoside antibiotics, including streptomycin. In contrast, the Sp7 *wzm* mutant was more susceptible than the wild-type to nalidixic acid. Similarly, mutants of *Stenotrophomonas maltophilia* that produce less LPS and shorter O polysaccharide chains than the wild-type have been shown to be more susceptible to this antibiotic (McKay *et al.*, 2003).

While the exact mechanisms by which LPSs affect antibiotic susceptibility have not been fully discerned, they have been attributed to differences in OM permeability as a result of a disruption in the conformation of OMPs such as associated porins, which limits their ability to transport molecules (Hirai *et al.*, 1986; Moniot-Ville *et al.*, 1991; Turcotte *et al.*, 1997; Yokota & Fujii, 2007). For instance, in *P. aeruginosa*, the mechanisms that lead to an increased resistance to aminoglycoside antibiotics include impaired OM uptake and reduced active transport (El'Garch *et al.*, 2007). Our study revealed substantial differences in OMP composition between wild-type and *wzm* mutant strains, which could be involved in a differential response to antibiotics. LPS-defective mutants are often compromised in the assembly of OMPs (Zuleta *et al.*, 2003). Nurminen *et al.* (1997) reported an *E. coli* LPS-defective mutant that showed reduced levels of the OmpF porin. In agreement with our study, Rahaman *et al.* (1998) found remarkable differences between an LPS-defective mutant of *E. coli* and the wild-type in their OMP patterns: in the mutant strain, the level of OmpC was reduced, OmpF was almost absent,

and instead of OmpA, a band with higher mobility was observed. Similarly, significant differences in OMP patterns between wild-type and LPS-defective mutants have been observed in other species, such as *Burkholderia cepacia* (Rajyaguru & Muszynski, 1997) and *Proteus penneri* (Kustos *et al.*, 2000a, b).

Some OMPs function as substrate-specific porins, including some that are responsible for uptake of specific sugars (Koebnik *et al.*, 2000). Therefore, differences in OMP composition between the wild-type and the *wzm* mutant could also be responsible for the observed differences in their growth ability with diverse sugars as carbon sources (for instance, the lack of growth of the mutant on L-rhamnose and D-arabinose, in contrast to the wild-type). However, little is known about the physiological role of *A. brasilense* OMPs. Thus, the relationship between the effects of the *wzm* mutation in OMP composition and the observed pleiotropic phenotypic changes of the *wzm* mutant strain (including differences in antibiotic resistance and growth) are still to be elucidated.

LPS has been shown to contribute to bacterial survival under diverse stress conditions. For instance, Touze *et al.* (2004) used several mutants impaired in LPS biosynthesis to demonstrate an important role for O-antigen in stress adaptation of *Erwinia chrysanthemi*, and Thomsen *et al.* (2003) showed that reduced amounts of LPS affect the stress tolerance and virulence of *Salmonella enterica*. Bacteria have to adapt to fluctuations in salinity and osmolarity in the environment. To cope with this, they accumulate osmolytes such as potassium ions, glycine betaine (GB), proline and proline betaine, among others (Chowdhury *et al.*, 2007). Riou *et al.* (1991) showed the occurrence of a GB-binding protein in the periplasmic cavity of *A. brasilense* Sp7. Hartman *et al.* (1991) demonstrated the ability of some *Azospirillum* species to convert choline to GB. As already mentioned, LPSs play an essential role in the integrity of the OM; therefore, it is possible that the *wzm* mutant is not able to maintain sufficient amounts of GB in the cell, thus contributing to an enhanced susceptibility to elevated levels of NaCl in comparison with the wild-type, as observed in our study. In agreement with our findings, LPS-defective mutants of *Caulobacter crescentus* have also been found to be more susceptible to salinity than the wild-type strain (Zuleta *et al.*, 2003).

Interestingly, although likely possessing a defective OM, the *wzm* mutant cells were able to survive under certain unfavourable conditions at higher levels than the wild-type. It is known that in addition to LPSs, EPSs play a role in bacterial survival under diverse stress conditions such as heat (Mao *et al.*, 2001), UV radiation (Pattanaik *et al.* 2007; Wang *et al.*, 2007), desiccation (Roberson & Firestone, 1992; Ophir & Gutnick, 1994), starvation (Holmström & Kjelleberg, 1999; Wai *et al.*, 1999), oxidation and osmotic shock (Wai *et al.*, 1999; Chen *et al.*, 2004). As has been stated, the biological relevance (including effects on stress

response) of the differences found between the *wzm* mutant and the wild-type strain in their EPS composition still needs to be elucidated.

As *A. brasilense* is a plant growth-promoting bacterium, it was natural to ask whether the *wzm* mutation that leads to a pleiotropic phenotype also affects its ability to adhere to the root surface and to induce plant growth promotion. Plant root colonization by *Azospirillum* spp. is a process that involves extracellular proteins and polysaccharides (Burdman *et al.*, 2000b). Also, an *A. brasilense* Cd mutant affected in rhamnose production and having an altered LPS has been shown to have defective root colonization ability (Jofré *et al.*, 2004). Therefore, it was reasonable to hypothesize that the *wzm* mutant strain is compromised in its ability to adhere to plant roots. On the other hand, Arunakumari *et al.* (1992) reported that a transposon mutant of *A. brasilense* Cd, which lost the ability to aggregate, was able to efficiently colonize the plant root; and Bastarrachea *et al.* (1988) found that *A. brasilense* and *Azospirillum lipoferum* mutants that stained poorly on Congo red plates were able to anchor to and colonize plant roots.

In the experiments performed with sweet corn, under the tested conditions, there were no significant differences between mutant and wild-type strains in plant growth promotion and in adhesion to seeds and roots. It may be that in the mutant, the absence of microfibrils allows for better extraction of cells from the root surface compared with the wild-type. In addition, microaggregates formed by the wild-type cells but not by the mutant, or chains of cells formed by the *wzm* mutant but not by the wild-type, can lead to a bias of several orders of magnitude in the number of counted cells adhered to the plant root. Experiments using wild-type and mutant strains labelled with a genetic marker (such as *lacZ*) that allows direct observation of the bacteria on the roots could be used in the future to sidestep the above bias and give a more precise answer to this question.

Finally, it is important to mention that the plant growth promotion and adhesion experiments were conducted under standard, optimal conditions. Jofré *et al.* (1998) reported that the anchoring ability of *A. brasilense* to maize and wheat roots is negatively affected under saline stress, and that impairment of anchoring ability correlates with alterations in EPSs and LPSs. Therefore, we cannot exclude the possibility that differences between the wild-type and mutant strains in root adhesion and plant growth promotion might occur under other experimental conditions, including stress.

ACKNOWLEDGEMENTS

Special thanks to Y. Tekoah, Head of the Center for Glycobiology at Ben-Gurion University of the Negev (Beer-Sheva, Israel), and his team for helping with the EPS analyses. Special thanks also to O. Yeger, the manager of the Irving and Cherna Moskowitz Center for Nano and Bio-Nano Imaging at the Weizmann Institute of Science

(Rehovot, Israel), for her contribution to the electron microscopy. We also thank J. Vanderleyden and E. Vanbleu for their valuable suggestions. This research was supported by The Israel Science Foundation funded by The Academy of Sciences and Humanities (grant 253/05).

REFERENCES

- Albersheim, P., Nevins, D. J., English, P. D. & Karr, A. (1967). A method for the analysis of sugars in plant cell-wall polysaccharides by gas-liquid chromatography. *Carbohydr Res* 5, 340–345.
- Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 25, 3389–3402.
- Arunakumari, A., Lamm, R. B. & Neyra-Estens, C. A. (1992). Changes in cell surface properties of azospirilla in relation to cell pleomorphism and aggregation. *Symbiosis* 13, 291–305.
- Bahat-Samet, E., Castro-Sowinski, S. & Okon, Y. (2004). Arabinose content of extracellular polysaccharide plays a role in cell aggregation of *Azospirillum brasilense*. *FEMS Microbiol Lett* 237, 195–203.
- Bastarrachea, F., Zamudio, M. & Rivas, R. (1988). Non-encapsulated mutants of *Azospirillum brasilense* and *Azospirillum lipoferum*. *Can J Microbiol* 34, 24–29.
- Bliss, J. M., Garon, C. F. & Silver, R. P. (1996). Polysialic acid export in *Escherichia coli* K1: the role of KpsT, the ATP-binding component of an ABC transporter, in chain translocation. *Glycobiology* 6, 445–452.
- Brink, B. A., Miller, J., Carlson, R. W. & Noel, K. D. (1990). Expression of *Rhizobium leguminosarum* CFN42 genes for lipopolysaccharide in strains derived from different *R. leguminosarum* soil isolates. *J Bacteriol* 172, 548–555.
- Bryan, L. E., O'Hara, K. & Wong, S. (1984). Lipopolysaccharide changes in impermeability-type aminoglycoside resistance in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 26, 250–255.
- Burdman, S., Jurkevitch, E., Schwatsburd, B., Hampel, M. & Okon, Y. (1998). Aggregation in *Azospirillum brasilense*: effects of chemical and physical factors and involvement of extracellular components. *Microbiology* 144, 1989–1999.
- Burdman, S., Jurkevitch, E., Schwartzburd, B. & Okon, Y. (1999). Involvement of outer membrane proteins in aggregation of *Azospirillum brasilense*. *Microbiology* 145, 1145–1152.
- Burdman, S., De Mot, R., Vanderleyden, J., Okon, Y. & Jurkevitch, E. (2000a). Identification and characterization of the *omaA* gene encoding the major outer membrane protein of *Azospirillum brasilense*. *DNA Seq* 11, 225–237.
- Burdman, S., Jurkevitch, E., Soria-Diaz, M. E., Serrano, A. M. G. & Okon, Y. (2000b). Extracellular polysaccharide composition of *Azospirillum brasilense* and its relation with cell aggregation. *FEMS Microbiol Lett* 189, 259–264.
- Burdman, S., Okon, Y. & Jurkevitch, E. (2000c). Surface characteristics of *Azospirillum brasilense* in relation to cell aggregation and attachment to plant roots. *Crit Rev Microbiol* 26, 91–110.
- Cava, J. R., Elias, P. M., Turowski, L. D. A. & Noel, K. D. (1989). *Rhizobium leguminosarum* CFN42 genetic regions encoding lipopolysaccharide structures essential for complete nodule development on bean plants. *J Bacteriol* 171, 8–15.
- Chen, J., Lee, S. M. & Mao, Y. (2004). Protective effect of *Escherichia coli* O157:H7 exopolysaccharide colanic acid to osmotic shock and oxidative stress. *Int J Food Microbiol* 93, 281–286.
- Chowdhury, S. P., Nagarajan, T., Tripathi, R., Mishra, M. N., Le Rudulier, D. & Tripathi, A. K. (2007). Strain-specific salt tolerance and osmoregulatory mechanisms in *Azospirillum brasilense*. *FEMS Microbiol Lett* 267, 72–79.
- Creus, C. M., Graziano, M., Casanovas, E. M., Pereyra, M. A., Simontacchi, M., Puntarulo, S., Barassi, C. A. & Lamattina, L. (2005). Nitric oxide is involved in the *Azospirillum brasilense*-induced lateral root formation in tomato. *Planta* 221, 297–303.
- Croes, C., Van Bastelaere, E., DeClercq, E., Eysers, M., Vanderleyden, J. & Michiels, K. (1991). Identification and mapping of loci involved in motility, adsorption to wheat roots, colony morphology, and growth in minimal medium on the *Azospirillum brasilense* Sp7 90-MDa plasmid. *Plasmid* 26, 83–93.
- Cuthbertson, L., Powers, J. & Whitfield, C. (2005). The C-terminal domain of the nucleotide-binding domain protein Wzt determines substrate specificity in the ATP-binding cassette transporter for the lipopolysaccharide O-antigens in *Escherichia coli* serotypes O8 and O9a. *J Biol Chem* 280, 30310–30319.
- Davidson, A. L. (2002). Mechanism of coupling of transport to hydrolysis in bacterial ATP-binding cassette transporters. *J Bacteriol* 184, 1225–1233.
- de Cock, H., Brandenburg, K., Wiese, A., Holst, O. & Seydel, U. (1999). Non-lamellar structure and negative charges of lipopolysaccharides required for efficient folding of outer membrane protein PhoE of *Escherichia coli*. *J Biol Chem* 274, 5114–5119.
- del Gallo, M., Negi, M. & Neyra, C. A. (1989). Calcofluor- and lectin-binding exocellular polysaccharides of *Azospirillum brasilense* and *Azospirillum lipoferum*. *J Bacteriol* 171, 3504–3510.
- de Troch, P. (1993). *Bacterial surface polysaccharides in relation to plant interaction: a genetic and chemical study of Azospirillum brasilense*. PhD thesis, Katholieke University te Leuven, Faculteit Landbouwkundige en Toegepaste Biologische Wetenschappen.
- Dische, Z. (1962). General color reactions. *Methods Carbohydr Chem* 1, 477–479.
- Dobbelaere, S., Croonenborghs, A., Thys, A., Ptacek, D., Vanderleyden, J., Dutto, P., Lambandera-Gonzalez, C., Caballero-Mellado, J., Aguirre, J. F. & other authors (2001). Responses of agronomically important crops to inoculation with *Azospirillum*. *Aust J Plant Physiol* 28, 871–879.
- El'Garch, F., Jeannot, K., Hocquet, D., Llanes-Barakat, C. & Plésiat, P. (2007). Cumulative effects of several nonenzymatic mechanisms on the resistance of *Pseudomonas aeruginosa* to aminoglycosides. *Antimicrob Agents Chemother* 51, 1016–1021.
- Fedonenko, Y. P., Zatonsky, G. V., Konnova, S. A., Zdorovenko, E. L. & Ignatov, V. V. (2002). Structure of the O-specific polysaccharide of the lipopolysaccharide of *Azospirillum brasilense* Sp245. *Carbohydr Res* 337, 869–872.
- Feng, L., Senchenkova, S. N., Yang, J., Shashkov, A. S., Tao, J., Guo, H., Cheng, J., Ren, Y., Knirel, Y. A. & other authors (2004). Synthesis of the heteropolysaccharide O antigen of *Escherichia coli* O52 requires an ABC transporter: structural and genetic evidence. *J Bacteriol* 186, 4510–4519.
- Figurski, D. H. & Helinski, D. R. (1979). Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. *Proc Natl Acad Sci U S A* 76, 1648–1652.
- Galindo Blaha, C. A. & Schrank, I. S. (2003). An *Azospirillum brasilense* Tn5 mutant with modified stress response and impaired in flocculation. *Antonie Van Leeuwenhoek* 83, 35–43.
- Godowski, P. J. (2005). A smooth operator for LPS responses. *Nat Immunol* 6, 544–546.
- Guo, D., Bowden, M. G., Pershad, R. & Kaplan, H. B. (1996). The *Myxococcus xanthus* *rfaABC* operon encodes an ABC transporter homolog required for O-antigen biosynthesis and multicellular development. *J Bacteriol* 178, 1631–1639.

- Hartman, A., Prabhu, S. R. & Galinski, E. A. (1991). Osmotolerance of diazotrophic rhizosphere bacteria. *Plant Soil* **137**, 105–109.
- Herschkovitz, Y., Lerner, A., Davidov, Y., Okon, Y. & Jurkevitch, E. (2005). *Azospirillum brasilense* does not affect population structure of specific rhizobacterial communities of inoculated maize (*Zea mays*). *Environ Microbiol* **7**, 1847–1852.
- Hirai, K., Aoyama, H., Irikura, T., Iyobe, S. & Mitsuhashi, S. (1986). Differences in susceptibility to quinolones of outer membrane mutants of *Salmonella typhimurium* and *Escherichia coli*. *Antimicrob Agents Chemother* **29**, 535–538.
- Holguin, G., Patten, C. L. & Glick, B. R. (1999). Genetics and molecular biology of *Azospirillum*. *Biol Fertility Soils* **29**, 10–23.
- Holmström, C. & Kjelleberg, S. (1999). Marine *Pseudoalteromonas* species are associated with higher organisms and produce biologically active extracellular agents. *FEMS Microbiol Ecol* **30**, 285–293.
- Jofré, E., Fischer, S., Rivarola, V., Balegno, H. & Mori, G. (1998). Saline stress affects the attachment of *Azospirillum brasilense* Cd to maize and wheat roots. *Can J Microbiol* **44**, 416–422.
- Jofré, E., Lagares, A. & Mori, G. (2004). Disruption of dTDP-rhamnose biosynthesis modifies lipopolysaccharide core, exopolysaccharide production, and root colonization in *Azospirillum brasilense*. *FEMS Microbiol Lett* **231**, 267–275.
- Kadouri, D., Burdman, S., Jurkevitch, E. & Okon, Y. (2002). Identification and isolation of genes involved in poly(β -hydroxybutyrate) biosynthesis in *Azospirillum brasilense* and characterization of *phbC* mutant. *Appl Environ Microbiol* **68**, 2943–2949.
- Kadouri, D., Jurkevitch, E. & Okon, Y. (2003). Involvement of the reserve material poly- β -hydroxybutyrate in *Azospirillum brasilense* stress endurance and root colonization. *Appl Environ Microbiol* **69**, 3244–3250.
- Katupitiya, S., Millet, J., Veski, M., Vickers, L., Zeman, A., Lidong, Z., Elmerich, C. & Kennedy, I. R. (1995). A mutant of *Azospirillum brasilense* Sp7 impaired in flocculation with a modified colonization pattern and superior nitrogen fixation in association with wheat. *Appl Environ Microbiol* **61**, 1987–1995.
- Katz, E. I., Matora, L. Y., Serebrennikova, O. B. & Scheludko, A. V. (1998). Involvement of a 120-Mda plasmid of *Azospirillum brasilense* Sp245 in the production of lipopolysaccharides. *Plasmid* **40**, 73–83.
- Kenne, L. & Lindberg, B. (1983). Bacterial polysaccharides. In *The Polysaccharides*, pp. 287–365. Edited by G. O. Aspinall. Orlando, FL: Academic Press.
- Koebnik, R., Locher, K. P. & Van Gelder, P. (2000). Structure and function of bacterial outer membrane proteins: barrels in a nutshell. *Mol Microbiol* **37**, 239–257.
- Konnova, O. N., Burygin, G. L., Fedonenko, Y. P., Matora, L. Y., Pankin, K. E., Konnova, S. A. & Ignatov, V. V. (2006). Chemical composition and immunochemical characteristics of the lipopolysaccharide of nitrogen fixing rhizobacterium *Azospirillum brasilense* Cd. *Mikrobiologiya* **75**, 383–388 (in Russian).
- Konnova, O. N., Boiko, A. S., Burygin, G. L., Fedorenko, Y. P., Matora, L. Y., Konnova, S. A. & Ignatov, V. V. (2008). Chemical and serological studies of liposaccharides of bacteria of the genus *Azospirillum*. *Mikrobiologiya* **77**, 350–357.
- Kustos, I., Tóth, V., Kocsis, B., Kerepesi, I. & Emoódy, L. & Kilár, F. (2000a). Capillary electrophoretic analysis of wild type and mutant *Proteus penneri* outer membrane proteins. *Electrophoresis* **21**, 3020–3027.
- Kustos, I., Tóth, V., Kilár, F., Kocsis, B. & Emoódy, L. (2000b). Effect of spontaneous and induced mutations on outer membrane proteins and lipopolysaccharides of *Proteus penneri* strain 357. In *Genes and Proteins Underlying Microbial Urinary Tract Virulence – Basic Aspects and Applications*, pp. 177–181. Edited by L. Emoódy, T. Pál, J. Hacker & G. Blum-Oehler. New York: Springer.
- Kyte, J. & Doolittle, R. F. (1982). A simple method for displaying the hydropathic character of a protein. *J Mol Biol* **157**, 105–132.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
- Lerner, A., Herschkovitz, Y., Baudoin, E., Nazaret, S., Moenne-Loccoz, Y., Okon, Y. & Jurkevitch, E. (2006). Effect of *Azospirillum brasilense* inoculation on rhizobacterial communities analyzed by denaturing gradient gel electrophoresis and automated ribosomal intergenic spacer analysis. *Soil Biol Biochem* **38**, 1212–1218.
- Lüderitz, O., Freudenberg, M. A., Galanos, C., Lehmann, V., Rietschel, E. Th. & Shaw, D. H. (1982). Lipopolysaccharides of Gram-negative bacteria. *Curr Top Membr Transp* **17**, 79–151.
- Mao, Y., Doyle, M. P. & Chen, J. (2001). Insertion mutagenesis of *wca* reduces acid and heat tolerance of enterohemorrhagic *Escherichia coli* O157:H7. *J Bacteriol* **183**, 3811–3815.
- Matora, L. Y., Solovova, G. K., Serebrennikova, O. B., Selivanov, N. Y. & Shchyogolev, S. Y. (1995). Immunological properties of *Azospirillum* cell surface: the structure of carbohydrate antigens and evaluation of their involvement in bacteria–plant contact interactions. In *Azospirillum VI and Related Microorganisms*, pp. 377–382. Edited by I. Fendrik, M. del Gallo, M. de Zamaroczy & J. Vanderleyden. Berlin: Springer.
- McKay, G. A., Woods, D. E., MacDonald, K. L. & Poole, K. (2003). Role of phosphoglucosyltransferase of *Stenotrophomonas maltophilia* in lipopolysaccharide biosynthesis, virulence, and antibiotic resistance. *Infect Immun* **71**, 3068–3075.
- Michel, G., Ball, G., Goldberg, J. B. & Lazdunski, A. (2000). Alteration of the lipopolysaccharide structure affects the functioning of the Xcp secretory system in *Pseudomonas aeruginosa*. *J Bacteriol* **182**, 696–703.
- Michiels, K. W., Croes, C. L. & Vanderleyden, J. (1991). Two different modes of attachment of *Azospirillum brasilense* Sp7 to wheat roots. *J Gen Microbiol* **137**, 2241–2246.
- Moens, S. & Vanderleyden, J. (1996). Functions of bacterial flagella. *Crit Rev Microbiol* **22**, 67–100.
- Moniot-Ville, N., Guibert, J., Acar, J. F., Collatz, E. & Gutmann, L. (1991). Mechanisms of quinolone resistance in a clinical isolate of *Escherichia coli* highly resistant to fluoroquinolones but susceptible to nalidixic acid. *Antimicrob Agents Chemother* **35**, 519–523.
- Nikaido, H. & Vaara, M. (1985). Molecular basis of bacterial outer membrane permeability. *Microbiol Rev* **49**, 1–32.
- Nurminen, M., Hirvas, L. & Vaara, M. (1997). The outer membrane of lipid A-deficient *Escherichia coli* mutant LH530 has reduced levels of OmpF and leaks periplasmic enzymes. *Microbiology* **143**, 1533–1537.
- Ophir, T. & Gutnick, D. L. (1994). A role for exopolysaccharides in the protection of microorganisms from desiccation. *Appl Environ Microbiol* **60**, 740–745.
- Pattanaik, B., Schumann, R. & Karsten, U. (2007). Effects of ultraviolet radiation on cyanobacteria and their protective mechanisms. In *Algae and Cyanobacteria in Extreme Environments*, pp. 31–48. Edited by J. Seckbach. Dordrecht, The Netherlands: Springer.
- Petrova, L. P., Matora, L. Y., Burygin, G. L., Borisov, I. V. & Fatsy, E. I. (2005). Analysis of DNA, lipopolysaccharide structure, and some cultural and morphological properties in closely related strains of *Azospirillum brasilense*. *Mikrobiologiya* **74**, 224–230 (in Russian).
- Puente, M. E., Holguin, G., Glick, B. R. & Bashan, Y. (1999). Root-surface colonization of black mangrove seedlings by *Azospirillum halopraeferens* and *Azospirillum brasilense* in seawater. *FEMS Microbiol Ecol* **29**, 283–292.

- Rahaman, S. O., Mukherjee, J., Chakrabarti, A. & Pal, S. (1998). Decreased membrane permeability in a polymyxin B-resistant *Escherichia coli* mutant exhibiting multiple resistance to β -lactams as well as aminoglycosides. *FEMS Microbiol Lett* **161**, 249–254.
- Rajyaguru, J. M. & Muszynski, M. J. (1997). Association of resistance to trimethoprim/sulphamethoxazole, chloramphenicol and quinolones with changes in major outer membrane proteins and lipopolysaccharide in *Burkholderia cepacia*. *J Antimicrob Chemother* **40**, 803–809.
- Reizer, J., Reizer, A. & Saier, M. H., Jr (1992). A new subfamily of bacterial ABC-type transport systems catalyzing export of drugs and carbohydrates. *Protein Sci* **1**, 1326–1332.
- Reuhs, B. L., Geller, D. P., Kim, J. S., Fox, J. E., Kolli, V. S. K. & Pueppke, S. G. (1998). *Sinorhizobium fredii* and *Sinorhizobium meliloti* produce structurally conserved lipopolysaccharides and strain-specific K antigens. *Appl Environ Microbiol* **64**, 4930–4938.
- Riou, N., Poggi, M. C. & Le Rudulier, D. (1991). Characterization of an osmoregulated periplasmic glycine betaine-binding protein in *Azospirillum brasilense* Sp7. *Biochimie* **73**, 1187–1193.
- Roberson, E. B. & Firestone, M. K. (1992). Relationship between desiccation and exopolysaccharide production in a soil *Pseudomonas* sp. *Appl Environ Microbiol* **58**, 1284–1291.
- Rodríguez Cáceres, E. A. (1982). Improved medium for isolation of *Azospirillum* spp. *Appl Environ Microbiol* **44**, 990–991.
- Saigí, F., Climent, N., Piqué, N., Sanchez, C., Merino, S., Rubirés, X., Aguilar, A., Tomás, J. M. & Regué, M. (1999). Genetic analysis of the *Serratia marcescens* N28b O4 antigen gene cluster. *J Bacteriol* **181**, 1883–1891.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Schneider, E. & Hunke, S. (1998). ATP-binding-cassette (ABC) transport systems: functional and structural aspects of the ATP-hydrolyzing subunits/domains. *FEMS Microbiol Rev* **22**, 1–20.
- Silver, R. P., Prior, K., Nsahlai, C. & Wright, L. F. (2001). ABC transporters and the export of capsular polysaccharides from Gram-negative bacteria. *Res Microbiol* **152**, 357–364.
- Simon, R., Priefer, U. & Puhler, A. (1983). A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram negative bacteria. *Biotechnology* **1**, 784–791.
- Steenhoudt, O. & Vanderleyden, J. (2000). *Azospirillum*, a free-living nitrogen-fixing bacterium closely associated with grasses: genetic, biochemical and ecological aspects. *FEMS Microbiol Rev* **24**, 487–506.
- Sukupolvi, S. & Vaara, M. (1989). *Salmonella typhimurium* and *Escherichia coli* mutants with increased outer membrane permeability to hydrophobic compounds. *Biochim Biophys Acta* **988**, 377–387.
- Tao, H., Brewin, N. J. & Noel, K. D. (1992). *Rhizobium leguminosarum* CFN42 lipopolysaccharide antigenic changes induced by environmental conditions. *J Bacteriol* **174**, 2222–2229.
- Tarrand, J. J., Krieg, N. R. & Dobereiner, J. (1978). A taxonomic study of the *Spirillum lipoferum* group with the description of a new genus, *Azospirillum* gen. nov. and two species, *Azospirillum lipoferum* (Beijerinck) comb. nov. and *Azospirillum brasilense* sp. nov. *Can J Microbiol* **24**, 967–980.
- Thomsen, L. E., Chadfield, M. S., Bispham, J., Wallis, T. S., Olsen, J. E. & Ingmer, H. (2003). Reduced amounts of LPS affect both stress tolerance and virulence of *Salmonella enterica* serovar Dublin. *FEMS Microbiol Lett* **228**, 225–231.
- Touze, T., Goude, R., Georgeault, S., Blanco, C. & Bonnassie, S. (2004). *Erwinia chrysanthemi* O antigen is required for betaine osmoprotection in high-salt media. *J Bacteriol* **186**, 5547–5550.
- Tsai, C. M. & Frasch, C. E. (1982). A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. *Anal Biochem* **119**, 115–119.
- Turcotte, M. L., Martin, D., Brodeur, B. R. & Peppler, M. S. (1997). Tn5-induced lipopolysaccharide mutations in *Bordetella pertussis* that affect outer membrane function. *Microbiology* **143**, 2381–2394.
- Vanbleu, E., Marchal, K., Lambrecht, M., Mathys, J. & Vanderleyden, J. (2004). Annotation of the pRhico plasmid of *Azospirillum brasilense* reveals its role in determining the outer surface composition. *FEMS Microbiol Lett* **232**, 165–172.
- Vanstockem, M., Michiels, K., Vanderleyden, J. & Van Gool, A. P. (1987). Transposon mutagenesis of *Azospirillum brasilense* and *Azospirillum lipoferum*: physical analysis of Tn5 and Tn5-mob insertion mutants. *Appl Environ Microbiol* **53**, 410–415.
- Wai, S. N., Mizunoe, Y. & Yoshida, S. I. (1999). How *Vibrio cholerae* survive during starvation. *FEMS Microbiol Lett* **180**, 123–131.
- Wang, H., Jiang, X., Mu, H., Liang, X. & Guan, H. (2007). Structure and protective effect of exopolysaccharide from *P. agglomerans* strain KFS-9 against UV radiation. *Microbiol Res* **162**, 124–129.
- Whitfield, C., Keenleyside, W. J. & Clarke, B. R. (1994). Structure, function and synthesis of cell surface polysaccharides in *Escherichia coli*. In *Escherichia coli in Domestic Animals and Man*, pp. 437–494. Edited by C. L. Gyles. Wallingford, UK: CAB International.
- Wolf, J. K. & Goldberg, J. B. (2006). Bacterial cell walls. In *Molecular Paradigms of Infectious Disease*, pp. 176–206. Edited by C. A. Nickerson & M. J. Schurr. New York: Springer.
- Yokota, S. & Fujii, N. (2007). Contributions of the lipopolysaccharide outer core oligosaccharide region on the cell surface properties of *Pseudomonas aeruginosa*. *Comp Immunol Microbiol Infect Dis* **30**, 97–109.
- Zuleta, L. F. G., Italiani, V. C. S. & Marques, M. V. (2003). Isolation and characterization of NaCl-sensitive mutants of *Caulobacter crescentus*. *Appl Environ Microbiol* **69**, 3029–3035.

Edited by: Michael F. Hynes