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Identification of a novel ABC transporter required for desiccation tolerance, and biofilm formation in *Rhizobium leguminosarum* bv. *viciae* 3841

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Abstract

Rhizobium leguminosarum is a soil bacterium with the ability to form nitrogen-fixing nodules on the roots of leguminous plants. Soil-dwelling, free-living *R. leguminosarum* often encounters desiccation stress, which impacts its survival within the soil. The mechanisms by which soil bacteria resist the effects of desiccation stress have been described. However, the role of the cell envelope in the desiccation tolerance mechanisms of rhizobia is relatively uncharacterized. Using a transposon mutagenesis approach, a mutant of *R. leguminosarum* bv. *viciae* was isolated that was highly sensitive to desiccation. The mutation is located in the ATP-binding protein of an uncharacterized ATP-binding cassette transporter operon (RL2975–RL2977). Exopolysaccharide accumulation was significantly lower in the mutant and the decrease in desiccation tolerance was attributed to the decreased accumulation of exopolysaccharide. In addition to desiccation sensitivity, the mutant was severely impaired in biofilm formation, an important adaptation used by soil bacteria for survival. This work has identified a novel transporter required for physiological traits that are important for the survival of *R. leguminosarum* in the rhizosphere environment.

Keywords

ABC transporter; desiccation; biofilm; rhizobium; exopolysaccharide; cell envelope

Introduction

Rhizobium leguminosarum is a Gram-negative bacterium notable for its ability to fix nitrogen within root nodules of leguminous plants (recently reviewed by Gibson et al., 2008). In the absence of a host plant, *R. leguminosarum* dwells within the rhizosphere. The rhizosphere environment can be highly variable and is prone to fluctuations in oxygen concentration, decreased water availability, temperature extremes and nutrient limitations (Deaker *et al.*,

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2004). Bacteria residing in the rhizosphere frequently encounter desiccation stress and require strategies to persist during times of desiccation. The negative cellular effects of desiccation are wide ranging and include protein denaturation, DNA degradation and loss of membrane integrity (Billi & Potts, 2002). Therefore, it is not surprising that the mechanisms required for desiccation resistance are complex and require multiple genetic loci (Billi & Potts, 2002; van de Mortel & Halverson, 2004; Humann *et al.*, 2009). An increased tolerance to desiccation can enhance the survival of rhizobacteria within the soil considerably (Rokitko *et al.*, 2003). This is of particular interest with nitrogen-fixing rhizobia because desiccation is a major cause of the poor on-seed survival rates of commercial inoculants and the subsequent poor performance of rhizobial inoculants in the field (Deaker *et al.*, 2004). To further our understanding of how bacteria persist in the soil environment, and improve on-seed survival of rhizobial inoculants, a complete understanding of the mechanisms used for desiccation tolerance is necessary.

Studies with the soil bacterium *Pseudomonas* sp. have identified a variety of mechanisms used to increase their tolerance to desiccation. For instance, the production of highly hygroscopic secreted polysaccharides is increased to maintain a hydrated microenvironment relative to the surrounding environment (Roberson & Firestone, 1992; van de Mortel & Halverson, 2004). Also, fatty acid isomerization is biased in favor of the *trans*-configuration to maintain membrane fluidity during drying (van de Mortel & Halverson, 2004). In *Salmonella enterica* serovar Typhimurium, the outer membrane O antigen of lipopolysaccharide was found to be crucial for desiccation tolerance (Garmiri *et al.*, 2008). In addition, cell surface components such as fimbriae and cellulose are important in desiccation tolerance in *Salmonella* (Gibson *et al.*, 2006; White *et al.*, 2006). These results suggest that the cell envelope, which includes the inner and outer membranes, cell wall and excreted surface polysaccharides, is an important structural target for increasing desiccation tolerance in bacteria.

In rhizobia, the role of the cell envelope in desiccation tolerance has recently been described. Gilbert *et al.* (2007), isolated a *ctpA* mutant in *R. leguminosarum* bv. *viciae* with altered cell envelope integrity and a corresponding decrease in desiccation tolerance. In *Bradyrhizobium*, mild desiccation stress resulted in an increase in the degree of saturation of the fatty acids found in membrane phospholipids (Boumahdi *et al.*, 2001). Microarray data from *Bradyrhizobium japonicum* revealed that a number of exopolysaccharides and lipopolysaccharides biosynthetic and transport genes are upregulated in response to desiccation stress (Cytryn *et al.*, 2007). In addition, a *R. leguminosarum* bv. *viciae* lipopolysaccharide mutant lacking the 27-hydroxyoctacosanoatic acid of lipid A is less tolerant to desiccation stress (Vanderlinde *et al.*, 2009).

A transposon mutagenesis screen to isolate genes required for maintaining a functional cell envelope in *R. leguminosarum* bv. *viciae* 3841 identified a strain with a mutation in the ATP-binding component of a previously uncharacterized ATP-binding cassette (ABC) transporter (RL2975, RL2976 and RL2977; Young *et al.*, 2006). The level of exopolysaccharide in the cell envelope of the mutant was decreased compared with the wild type and this has been connected to a decreased tolerance to desiccation. The mutant was also defective in biofilm formation on a polystyrene support. The main findings of this study demonstrate the important role for exopolysaccharide in desiccation tolerance in *R. leguminosarum* and identify a new genetic element required for proper biofilm formation.

Materials and methods

Strains, media and growth conditions

Table 1 lists the strains, plasmids and primers used in this study. *Escherichia coli* strains were cultured using Luria–Bertani medium (Sambrook *et al.*, 1989), supplemented as necessary with the following concentrations of antibiotics (μg mL^{-1}): gentamicin, 15; ampicillin, 100;

spectinomycin, 100; and tetracycline, 10. *Rhizobium leguminosarum* cells were cultured using tryptone–yeast (TY) (Beringer, 1974) or Vincent's minimal media (VMM) (Vincent, 1970) with 10 mM mannitol as a carbon and energy source, supplemented as required with the following concentrations of antibiotics (μg mL^{-1}): gentamicin, 30; neomycin, 100; tetracycline, 5; and streptomycin, 500.

Transposon mutagenesis

Mutagenesis was performed using the mini-Tn5 derivative found on plasmid pTGN (Tang *et al.*, 1999). Biparental matings of the *E. coli* mobilizer strain S17-1 containing the pTGN vector and *R. leguminosarum* 3841 were performed at 30 °C for 24 h on VMM plates supplemented with 1 mM proline. Transconjugants were selected on VMM with streptomycin and neomycin, and subsequently screened for inability to grow on the solid complex medium TY. We have previously observed that mutants with defects in the cell envelope are unable to grow on complex solid media (Gilbert *et al.*, 2007; Vanderlinde *et al.*, 2009). Therefore, we used the lack of growth on TY as a selection to enrich for isolation of transposon mutants with defective cell envelopes. Genomic DNA was isolated from TY-negative isolates and used as a template to identify the transposon insertion site. The transposon insertion site was identified using the thermal asymmetric interlaced (TAIL)-PCR protocol and primers described by Liu & Huang (1998). Briefly, the specific primer GmTAIL1 (Table 1; SigmaGenosys Canada, Oakville, ON) that binds within the Gm cassette of the transposon and the arbitrary degenerate primer AD-1 described by Liu & Huang (1998) were used in the primary reaction, which was followed by a secondary reaction with the specific nested primer GmTAIL2 (Table 1), the arbitrary primer and 1 μL of primary reaction as template. The transposon insertion site was identified by sequencing the TAIL-PCR product and using a BLASTN search (Altschul *et al.*, 1997) and the Rhizobase database [Kazusa DNA Research Institute (http://bacteria.kazusa.or.jp/rhizobase/)].

Standard molecular techniques

Plasmid preps were performed using the alkaline lysis method (Sambrook *et al.*, 1989). Restriction endonucleases and DNA-modifying enzymes were purchased from Invitrogen (Burlington, ON) and used according to the manufacturer's instructions. When necessary, PCR products were isolated from agarose gels using reagents and protocols from the QIAEX II gel extraction kit (Qiagen, Missisuaga, ON).

Mutagenesis of RL2976

Primers 17B1F and 17B1R (Table 1) were used to PCR amplify a 1293-bp fragment of RL2975 and RL2976. The fragment contained the 3′ end of RL2975 and the 5′ start of RL2976. The genes RL2975 and RL2976 are likely part of an operon, beginning with RL2975; therefore, the fragment lacked a promoter for RL2976 and a single crossover event using this fragment resulted in mutation of RL2976. PCR reactions used 1 U of *Taq* DNA polymerase (UBI, Calgary, AB) and the following reaction conditions: 1× reaction buffer, 2 mM $MgSO_4$, 0.2 mM dNTPs and 0.2 μM of each primer. PCR amplification was performed using a Techne TC312 Thermocycler (Techne, Staffordshire, UK) at 94 °C for 5 min, followed by 30 cycles of: 95 °C for 30 s, 58 °C for 30 s and 72 °C for 75 s and a final extension at 72 °C for 5 min. PCR products were cloned into the pCR2.1 topo vector (Invitrogen) as per the manufacturer's instructions. Mutagenesis of RL2976 was accomplished by allelic exchange using pEV30 and the method described by Quandt & Hynes (1993). Briefly, the fragment of RL2975 and RL2976 was excised using SstI and XbaI restriction sites in the pCR2.1 topo vector and ligated into the same sites in the vector pJQ200SK, creating pEV30. The mobilizer strain S17-1 was transformed with pEV30, and biparental matings were performed overnight on VMM supplemented with 0.5 mM proline. Mutants were selected on the basis of gentamicin

resistance. Correct insertional disruption of RL2976 in the putative mutants was confirmed by PCR.

Sequence analysis

DNA sequencing was performed by the University of Calgary Core DNA Services (Calgary, AB). DNA sequence data were analyzed using 4PEAKS software [version 1.7.2; A. Griekspoor and Tom Groothuis (http://mekentosj.com/4peaks/)]. Primers were designed using OLIGO 4.0 software (National Biosciences, Plymouth, MN). Predictions of transmembrane domains were made using TMPRED (Hofmann & Stoffel, 1993). Analysis of conserved domains and Hidden Markov model (HMM) sequences were from the InterPro database (Mulder *et al.*, 2007). Sequence alignments were performed using CLUSTALW (Thompson *et al.*, 1994), and phylogenetic trees were constructed using NJPLOT (Perrière & Gouy, 1996).

Complementation analysis

The 371-bp fragment amplified by the primers 17BF and 17BR (Table 1) was used to probe an *R. leguminosarum* bv. *viciae* VF39SM cosmid library (Yost *et al.*, 1998). Probe labeling, hybridization and detection were performed using reagents and protocols of the digoxigenin labeling and detection system (Roche Diagnostics, Lavel, QC). Cosmids detected by the 17B probe were further confirmed for the presence of the entire ABC transporter operon by PCR and DNA sequencing. The sequences of the RL2975–RL2977 homologues in VF39SM are available from the GenBank database under accession numbers GQ183604–GQ183606.

Desiccation, detergent and osmotic stress assays

Desiccation sensitivity assays used the filtration method described by Ophir & Gutnick (1994) as modified by Gilbert *et al.* (2007). Cells were grown in TY broth or VMM broth to the late log phase and diluted in water to a total volume of 100 mL. The diluted culture was filtered in duplicate as 2 × 50 mL aliquots according to the manufacturer's specifications with a Millipore Vacuum Manifold (Millipore Inc., Bedford, MA) using Microfil Filtration Funnels and S-Pak 0.45-μm TypeHA membranes (Millipore Inc.). Membranes were placed on either TY or VMM plates and incubated for 48 h at 30 °C. Following incubation, one filter was transferred to 10 mL of water, vortexed for 5 min to remove cells and the amount of exopolysaccharide was determined using the precipitation method described below. The second filter was cut in half and transferred to either an empty Petri dish (air) or a water–agar plate (12.5 g L^{-1} agar) (water), and incubated at ambient temperature and humidity for 24 h. Following incubation, membranes were transferred to a centrifuge bottle containing 2 mL of water and vortexed vigorously for 10 min to remove cells from the membranes. The number of viable bacteria was then determined using the spread plate technique and the percent survival was calculated as the ratio of $\mathrm{CFU\ CFU\ mL_{air}^{-1}}$ to $\mathrm{CFU\ CFU\ mL_{water}^{-1}}$. For exopolysaccharide complementation experiments, exopolysaccharide was isolated from wild-type bacteria as described below, and resuspended in sterile distilled water (dH_2O), to a concentration of ~0.4 g mL^{-1}. After cells were filtered as described above, 5 mL of the exopolysaccharide solution was filtered on top. The remainder of the assay was carried out as described above.

Detergent sensitivity assays were performed as described by Gilbert *et al.* (2007). Osmotic stress assays were performed by inoculating strains into TY broth with 69.5 mM NaCl. Cultures were grown at 30 °C for 48 h and then the $OD_{600\ nm}$ was measured. Results are reported as the ratio of $OD_{600\ nm}$ with NaCl to the $OD_{600\ nm}$ without NaCl.

Quantitative determination of secreted polysaccharides

The method for total exopolysaccharide quantification was a modification of those described by Ngwai *et al.* (2006). Briefly, cells were grown to the stationary phase in 25 mL of VMM.

Cells were pelleted at 7710 ***g*** for 20 min at 4 °C, and washed with 25 mL of 1 M NaCl, 10 mM EDTA at pH 8.0. The exopolysaccharide was then precipitated from the combined culture by the addition of two volumes of ice-cold isopropanol. The precipitated polysaccharide was then spooled and dried in a sterile Petri dish at 37 °C overnight. Results are reported as the mass of exopolysaccharide produced per milligram of dry cell mass. The capsular polysaccharide (CPS) was extracted according to the method of Zevenhuizen (1984). Precipitated CPS was dried overnight at 37 °C, and results were reported as the mass of CPS produced per milligram of dry cell mass. The amount of total neutral polysaccharide was determined by combining the remaining isopropanol supernatants from the exopolysaccharide and CPS determinations and quantifying the amount of glucose using the anthrone-sulfuric acid assay as described by Laurentin & Edwards (2003).

Structural analysis of acidic exopolysaccharide

Exopolysaccharide was precipitated from culture supernatants using isopropanol as described above. Following drying, the precipitates were resuspended in 20 mL of dH_2O and dialyzed against dH_2O for 20 h using the Spectra/Por® 7 dialysis membrane with a 2000 molecular weight cut-off (Spectrum® Laboratories Inc., Rancho Dominguez, CA). The dialyzed samples were then lyophilized before further analysis.

Exopolysaccharide preparations were purified by gelfiltration chromatography using a Sephacryl S-400 HR matrix (GE Healthcare) with 50 mM ammonium formate, pH 6.8, used as the eluent. The eluting fractions were monitored using the Shimadzu Refractive Index Detector (RID-10A).

Glycosyl composition analysis was performed by the preparation and GC-MS analysis of trimethylsilyl methyl glycosides as described by York *et al.* (1985). Briefly, samples were subjected to methanolysis at 80 °C for 18 h in 1 M methanolic HCl. The resulting methyl glycosides were *N*-acetylated at 100 °C for 1 h, trimethylsilylated at 80 °C for 30 min and then analyzed on a Hewlett-Packard HP5890 gas chromatograph equipped with a mass selective detector 5970 MSD using a Supelco DB-1 fused silica capillary column (30 m × 0.25 mm ID; J & W Scientific, Folsom, CA).

Nuclear magnetic resonance (NMR) analysis was performed by exchanging the sample two times with 99.9% deuterium oxide (D_2O) and finally dissolving in 100% D_2O (Cambridge Isotope Laboratories Inc.), and 1D proton spectra were recorded using a Varian Inova 500 MHz NMR spectrometer (Varian, Palo Alto, CA) at 70 °C.

Capsule staining

Capsule stains were performed on stationary-phase cultures grown in VMM broth. Cells were negatively stained using nigrosin, followed by counter-staining with crystal violet (CV). Stained cells were then visualized under × 100 oil immersion using an Olympus BX51 light microscope (Olympus, PA).

Biofilm cultivation

Biofilms were grown either in microtiter plates, using methods adapted from O'Toole & Kolter (1998) and Fujishige *et al.* (2006), or in the Calgary Biofilm Device (CBD, commercially available as the MBEC-HTP Assay, Innovotech, Edmonton, AB, Canada), as described previously by Ceri *et al.* (1999). To begin, strains were streaked out twice on the appropriate selective medium. Colonies were collected from the second agar subculture using a sterile cotton swab and were suspended in double-distilled water (ddH_2O) to match a 1.0 McFarland standard. This standardized bacterial suspension was then diluted 1 in 15 into VMM that contained 1% mannitol and 500 μg mL^{-1} streptomycin, which, when verified by viable cell

counting, provided a starting inoculum of approximately 1×10^7 CFU mL^{-1}. For microtiter plate biofilms, 150 μL of this standardized inoculum was added to each well of a flat-bottom, cell culture-treated Nunc® brand 96-well plate (VWR International Ltd, Mississauga, ON, Canada). These plates were sealed with Parafilm™ and incubated at 30 °C for 48 h on a gyrorotary shaker set to 50 r.p.m. Alternatively, 22 mL of the standardized inoculum was added to the trough of the CBD. These devices were also sealed with Parafilm™ and then incubated at 30 °C for 72 h on a rocking table set to 3.5 rocks min^{-} as described previously (Ceri *et al.*, 1999).

CBD biofilms were rinsed by placing the peg lid into a microtiter plate that contained 200 μL of ddH_2O in each well. For viable cell counts, the peg lids were transferred into a second microtiter plate, also containing 200 μL of ddH_2O in each well, into which biofilms were disrupted using an ultrasonic cleaner as described previously (Ceri *et al.*, 1999). Cells from the disrupted biofilms were serially diluted 10-fold and plated for viable cell counting on VMM +1% mannitol agar. Spot plates were grown for 72 h before enumeration.

Tetrazolium reduction assays

Microtiter plate biofilms were rinsed once with 200 μL of ddH_2O to remove loosely adherent biomass and then immersed in fresh medium containing a metabolic indicator. To do this, 150 μL of ddH_2O, 25 μL of VMM+1% mannitol and 25 μL of CellTiter 96 AQueous One solution (Promega Corporation, Madison, WI) were added to each well of the rinsed microtiter plate. AQueous One solution contains the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS), which is presumably reduced to a colored formazan product by NADH and NADPH from metabolically active cells (Berridge & Tan, 1993). These plates were wrapped in an aluminum foil and incubated at 30 °C for 18 h. Biofilm metabolic activity was assessed by reading the $OD_{490\ nm}$ of these plates on a Thermomax microtiter plate reader with SOFTMAX PRO DATA analysis software (Molecular Devices, Sunnyvale, CA) as described previously (Harrison *et al.*, 2007).

CV staining for biomass

Microtiter plate biofilms were rinsed once with 200 μL of ddH_2O to remove loosely adherent biomass and then stained with CV using a method adapted from Fujishige *et al.* (2006). To do this, 200 μL of 0.4% CV (VWR International Ltd) was added to each well and these plates were incubated for 15 min. The CV was then aspirated and the wells were then rinsed three times with 200 μL of ddH_2O for 1 min. The CV was solubilized by adding 200 μL of 95% ethanol to each well. The amount of CV bound to the biomass was quantified by reading the $OD_{550\ nm}$ on a microtiter plate reader (as described above).

Confocal laser scanning microscopy (CLSM)

Pegs were broken from the lid of the CBD using a pair of flamed needle nose pliers. Biofilms that had been grown on the surface of the peg, were stained with acridine orange or with the Live/Dead® bacterial cell viability kit as described previously by Harrison *et al.* (2006, 2007). Acridine orange, which fluoresces green, is a nucleic acid intercalator that stains biofilm cells as well as extracelluar nucleic acids, and may thus be used as a biomass indicator. In contrast, the Live/Dead® kit contains the membrane-permeable DNA intercalator Syto-9, which fluoresces green, and the membrane-impermeable DNA intercalator propidium iodide, which fluoresces red. In principle, cells with compromised membrane integrity are dead and appear red, whereas live cells, which exclude propidium iodide from the cytoplasm, appear green.

Fluorescently labeled biofilms were placed in two drops of ddH_2O on the surface of a glass coverslip. These pegs were examined using a Leica DM IRE2 spectral confocal and

multiphoton microscope with a Leica TCS SP2 acoustic optical beam splitter (Leica Microsystems, Richmond Hill, ON, Canada) as described previously (Harrison *et al.*, 2007). For acridine orange-stained samples, biofilms were scanned using 476 nm excitation and fluorescence was collected in the green region of the spectrum. For Live/Dead® staining, samples were sequentially scanned, frame by frame, first at 488 nm (Syto-9) and then at 543 nm (propidium iodide). Fluorescence emission was then sequentially collected in the green and red regions of the spectrum, respectively. A ×63 water-immersion objective was used in all imaging experiments. Image capture and two-dimensional (2D) reconstruction of z-stacks were performed using LEICA CONFOCAL software (Leica Microsystems).

Results

Mutagenesis and bioinformatic analysis of a novel ABC transporter operon

A *R. leguminosarum* 3841 mutant (17B) was isolated during a transposon (Tn5) mutagenesis screen for mutants with defective cell envelopes. The insertion site of the transposon was mapped to base pair 750 of a gene encoding a predicted ATP-binding component (RL2975) of a previously uncharacterized ABC transporter operon (Fig. 1). DNA sequence analysis suggests that the operon is comprised of three genes: RL2975 (1001 bp), RL2976 (800 bp) and RL2977 (848 bp), encoding for the ATP-binding protein and two transmembrane proteins, respectively. The start and stop codons of the three genes overlap, which may suggest transcriptional and translational coupling. Therefore, the transposon mutagenesis likely resulted in a polar mutation. The mutant 38EV30 is an RL2976 mutant constructed by homologous recombination as described in Materials and methods. This mutation disrupts RL2976 and RL2977; however, the gene encoding the ATP-binding protein, RL2975, should still be expressed.

The proteins encoded by RL2975, RL2976 and RL2977 are predicted to contain 1, 5 and 6 transmembrane domains, respectively. Figures 1 and 2 illustrate that, with the exception of *Rhizobium etli* and *R. leguminosarum* bv. *trifolii*, this particular ABC transporter operon is not present in the genomes of other *Rhizobiales* sequenced to date. In fact, the next closest matches are to ABC transporters of unknown function in soil-dwelling Gram-positive bacteria such as *Clostridium* sp. and *Paenibacillus* sp. (Figs 1 and 2).

The two transmembrane proteins, encoded by RL2976 and RL2977, contain a conserved domain of unknown function (DUF990), which shares homology to a subgroup of ABC transporters classified as ABC-2 transporters (Reizer *et al.*, 1992). The ABC-2 transporters are involved in the export of diverse substrates from the cell, such as sodium ions, signaling molecules and polysaccharides (Reizer *et al.*, 1992). A few of these transporters have been well characterized and include the KpsMT, BexABC, CtrABC and RkpRST transporters, which are involved in exopolysaccharide export in E. coli, Haemophilus influenzae, Neisseria meningitidis and *Sinorhizobium meliloti*, respectively (Kroll *et al.*, 1990; Smith *et al.*, 1990; Frosch *et al.*, 1991; Pavelka *et al.*, 1991; Reizer *et al.*, 1992; Kiss *et al.*, 2001).

A CLUSTALW alignment was performed with RL2977, the HMM sequence of the DUF990 domain and the HMM sequence of ABC-2 transporters (data not shown). An alignment comparing RL2976 with the DUF990 and ABC-2 HMM sequences yielded results similar to the RL2977 alignment. Based on the alignment results, both the proteins have amino acid sequences that are related to the DUF990 sequence with amino acid similarities of 53.4% and 55.7%, respectively, and have a weak similarity to the HMM ABC-2 transporter sequence, with amino acid similarities of 14.9% and 27.9%, respectively. A neighbor-joining phylogenetic tree with previously characterized members of the ABC-2 group and DUF990 proteins separated the previously characterized ABC-2 transporter sequences from the RL2976 and Rl2977 amino

acid sequences and all of the other DUF990-containing proteins, suggesting that DUF990 proteins may represent a new uncharacterized subclass of ABC-2 transporters (Fig. 2).

Quantification and partial characterization of secreted polysaccharides in the 17B and 38EV30 mutants

Three types of polysaccharides constitute the majority of surface polysaccharides (SPS) in *R. leguminosarum*: exopolysaccharide, CPS and neutral polysaccharides (Skorupska *et al.*, 2006). The exopolysaccharides and CPS have a similar or an identical basic structure, and are distinguished based on their location, and the distribution of noncarbohydrate residues such as pyruvate, 3-hydroxybutyrate and *O*-acetate (Skorupska *et al.*, 2006). To determine the role of RL2975–RL2977 in SPS export, the amounts of exported exopolysaccharide, CPS and neutral polysaccharide from the mutant strains 17B and 38EV30 grown in VMM broth were quantified and compared with the wild-type strain. The amounts of exopolysaccharide produced by the 17B and 38EV30 mutants were 2.28±0.163 and 1.96±0.472 mg exopolysaccharide mg^{-1} dry cell weight, respectively, whereas the wild type, 3841, produced 6.20±0.849 mg exopolysaccharide mg^{-1} dry cell weight. The amount of CPS produced by wild type, 17B and 38EV30 was found to be 0.138±0.018, 0.092±0.010 and 0.082±0.038 mg CPS mg^{-1} dry cell weight, respectively. Finally, quantification of neutral polysaccharides determined that the wild type produced 0.054±0.0008 mg glucose mg^{-1} dry cell weight, 17B produced 0.059±0.004 mg glucose mg^{-1} dry cell weight and 38EV30 produced 0.044 mg glucose mg^{-1} dry cell weight. From these data, it is clear that while the mutants produce threefold lower amounts of exopolysaccharide in VMM broth, the amounts of CPS and neutral polysaccharides are not altered.

Gel-permeation chromatography was used to isolate the high molecular weight (HMW) acidic exopolysaccharide from both the wild type and the 17B mutant. Compositional analysis (Fig. 3) and NMR structural analysis (Fig. 4) indicated that the HMW acidic exopolysaccharide from the 17B mutant resembled that of the wild type and that the NMR spectrum was consistent with the published structure of *R. leguminosarum* HMW acidic exopolysaccharide (Amemura *et al.*, 1983;O'Neill *et al.*, 1991). These results suggest that loss of transporter function does not affect the structural composition of the HMW-secreted exopolysaccharide. Visualization of a capsule stain indicated that the transporter mutant still produced a capsule; however, the capsules produced by the mutant strains have a visibly altered structure (Fig. 5).

Desiccation, detergent and osmotic sensitivity of the mutants 17B and 38EV30

A desiccation sensitivity assay was used to demonstrate the importance of the ABC transporter for desiccation tolerance. The 17B and 38EV30 mutants were significantly reduced in desiccation tolerance when compared with the wild-type strain (Table 2). The mutants were not significantly sensitive to hyperosmotic stress, indicating that the sensitivity to decreased water availability is specific to desiccation stress. The mutants were as resistant as the wild type to the detergents deoxycholate or sarcosyl, suggesting that defects in outer membrane integrity are not responsible for the decrease in desiccation tolerance. Additionally, sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis of lipopolysaccharide isolated from the 17B mutant indicated no obvious difference in the O-antigen structure compared with the wild type (data not shown).

Exopolysaccharide production is positively correlated with desiccation tolerance in *R. leguminosarum*

The importance of exopolysaccharide for desiccation tolerance has been reported in soil-dwelling bacteria such as *Pseudomonas* sp. (Roberson & Firestone, 1992; van de Mortel & Halverson, 2004); however, the relationship between exopolysaccharide production and desiccation tolerance has never been demonstrated for *R. leguminosarum.*To demonstrate that

exopolysaccharide may play a role in desiccation tolerance in *R. leguminosarum*, the desiccation sensitivities of wild-type cells grown under conditions that produce low levels of exopolysaccharide (TY medium) and conditions that promote exopolysaccharide excretion (VMM medium) were compared (Table 3). The results confirm that conditions that promote exopolysaccharide secretion also significantly improve desiccation tolerance in *R. leguminosarum*.

To refine further the relationship between desiccation sensitivity and exopolysaccharide production in 17B and 38EV30, the strains were grown on TY and VMM and exopolysaccharide was quantified and desiccation tolerance was measured (Table 3). Both mutant strains produce considerably less exopolysaccharide than the wild type and both strains have a significantly increased sensitivity to drying, suggesting that the decrease in desiccation tolerance in these mutants may be related to a decrease in exopolysaccharide. To support this hypothesis, exogenous exopolysaccharide was added to the mutants as described in Materials and methods. The additional exopolysaccharide restored the desiccation tolerance in these mutants to wild-type levels, confirming that the desiccation sensitivity in the 17B and 38EV30 mutants is likely related to a decrease in surface exopolysaccharide.

Biofilm formation

The transporter mutants have a propensity to flocculate heavily when grown in liquid VMM (data not shown). Changes in cell–cell adhesion can alter biofilm formation dynamics (Reisner *et al*., 2003). The ability of the 17B mutant to form biofilms on polystyrene microplates was investigated in order to determine whether the transporter mutation affected attachment of *R. leguminosarum* to solid surfaces. Biofilms of both the 17B mutant and *R. leguminosarum* 3841 were grown in microtiter plates and stained using CV (Table 4). These assays indicated that the surface-adherent biomass produced by the 17B mutant was significantly less than that produced by wild-type 3841. Similar results were obtained for the 38EV30 mutant (data not shown). Staining by CV is nonspecific and stains secreted polysaccharides as well as cellular biomass; therefore, metabolic staining with a tetrazolium salt (MTS) was used to assess the number of bacterial cells that had adhered to the surfaces of microtiter plates. In agreement with the CV assays, the total metabolic activity of the wild-type biofilm was, on average, fourfold higher than the 17B biofilm (Table 4).

We further investigated the biofilm formation of the 17B mutant by growing biofilms of the mutant and wild-type strains in the CBD. The number of cells in the CBD biofilms was assessed by viable cell counting and CLSM using acridine orange and Live/Dead™ staining. The mean number of viable cells in the 17B biofilm was 52-fold lower in comparison with wild-type 3841 (Table 4). CLSM corroborated that the 17B mutant was impaired in biofilm formation. Both acridine orange and Live/Dead™ staining revealed that the wild-type strain 3841 could form micro-colonies at the air–liquid–surface interface of the CBD pegs (Fig. 6a and c). By contrast, only scattered cells of the 17B mutant had adhered to the pegs (Fig. 6b and d). Collectively, these data suggest that a functional RL2975–RL2977 transporter is essential for normal biofilm formation and subsequent maturation.

Notably, when CBD biofilms were fixed before staining, a small number of very large cell aggregates remained attached to the peg surface (data not shown). These results, in addition to the flocculation observed in VMM broth culture, suggest that the mutants, while impaired in their ability to adhere (or remain attached) to solid substrata, are likely not impaired in their ability to form cell–cell contacts, and in fact may be prone to cell–cell adhesion relative to the wild type.

Attempted complementation of the 17B and 38EV30 mutants

Attempts to restore wild-type phenotypes to the 17B and 38EV30 mutants with a cosmid (pCos879) from an *R. leguminosarum* VF39SM genomic library (Yost *et al.*, 1998) were unsuccessful. *Rhizobium leguminosarum* VF39SM is closely related to *R. leguminosarum* 3841, and DNA sequencing confirmed that the genes homologous to RL2975, RL2976 and RL2977, found on the VF39 cosmid (pCos879), coded for proteins that are 99%, 100% and 99% identical to the 3841 amino acid sequence, respectively. Therefore, DNA sequencing suggests that the genes should be functional in a 3841 background and the lack of complementation was attributed to a dominant-negative effect. Dominant-negative mutants of other ABC transporters have been reported (Bliss *et al.*, 1996; Miyamoto *et al.*, 2002). Mutations in the C-terminus of the ATP-binding protein KpsT of an *E. coli* ABC-2 transporter resulted in a dominant-negative mutation (Bliss *et al.*, 1996). Because the transposon insertion site in the 17B mutant is located in the C-terminal portion of the ATP-binding component, it is possible that the lack of complementation is caused by a dominant-negative mutation. To circumvent the dominant-negative effect, we attempted to create a mutant where the genes RL2975–RL2977 are deleted; however, attempts have been unsuccessful to date. Although the phenotypes observed for the mutants were not restored by complementation, the two mutants, 17B and 38EV30, were isolated independently, and so it is unlikely that the observed defective phenotypes are due to either a secondary site mutation or an artifact of the transposon insertion.

Discussion

Reduced desiccation tolerance and defective biofilm formation have been reported previously for mutants with alterations to secreted polysaccharides in several bacterial species (Roberson & Firestone, 1992; Ophir & Gutnick, 1994; van de Mortel & Halverson, 2004; Russo *et al.*, 2006; Balestrino *et al.*, 2008). Therefore, we hypothesize that the threefold reduction of exopolysaccharide observed in the transporter mutants is at least partially responsible for the reported phenotypes of these mutants. This has been confirmed for the desiccation phenotype by restoring desiccation tolerance to wild-type levels with the addition of exogenous exopolysaccharide. The importance of exopolysaccharide for desiccation tolerance in soil-dwelling bacteria has been reported in *Pseudomonas* (Roberson & Firestone, 1992; van de Mortel & Halverson, 2004). Our results suggest that exopolysaccharide is also important for desiccation tolerance in *Rhizobium*. The ability of exopolysaccharide to absorb large amounts of water, creating a hydrating environment surrounding a cell, has been suggested as a possible mechanism for enhancing desiccation tolerance (Roberson & Firestone, 1992). Desiccation stress affects many cellular components (Billi & Potts, 2002) and adaptation is highly complex, likely requiring numerous genetic pathways. Humman *et al.* (2009) recently demonstrated that desiccation tolerance in the related *S. meliloti* requires genes involved in DNA repair and in the regulation of stress-induced pathways. Therefore, the ABC transporter characterized in this study is likely only one component to desiccation tolerance, and other genes involved in desiccation tolerance remain to be identified in *R. leguminosarum*.

Structural analysis of the secreted exopolysaccharide from the mutants was performed to help elucidate the possible role for the transporter in exopolysaccharide secretion. The structures of the HMW exopolysaccharide were identical between the mutants and the wild type, suggesting that the transporter does not transport a substrate that plays a role in proper HMW exopolysaccharide assembly. Mutation of pssC, a gene involved in exopolysaccharide biosynthesis, results in a 50% reduction in the amount of exopolysaccharide synthesized (van Workum *et al.*, 1997). It is possible that the ABC transporter's substrate may be involved in regulating the expression of pssC, or other exopolysaccharide assembly-related genes. Future studies will attempt to identify the substrate of the RL2975–RL2977 transporter and its linkage to the regulation of exopolysaccharide production. These studies may also help elucidate the

functions and substrates of transporters classified within the DUF990 subfamily of ABC-2-type transporters.

The 17B and 38EV30 mutants flocculate extensively in VMM broth. In *Klebsiella pneumoniae*, exopolysaccharide has been attributed to preventing cell-to-cell aggregation by shielding the intercellular binding of adhesin proteins (Schembri *et al.*, 2004; Balestrino *et al.*, 2008). In *R. leguminosarum*, cell–cell aggregation in exopolysaccharidedeficient mutants is thought to be due to the cell-to-cell binding of exposed cellulose microfibrils (Napoli *et al.*, 1975; Laus *et al.*, 2005). Furthermore, a mutant strain of *R. leguminosarum* that overproduces cellulose has been observed to flocculate heavily (Ausmees *et al.*, 1999). The genome of *R. leguminosarum* 3841 contains genes that putatively code for cellulose synthesis (Young *et al.*, 2006). It is possible that intercellular shielding of the cellulose microfibrils is absent in the 17B and 38EV30 mutants as a result of the decreased level of exopolysaccharide, contributing to increased cell-to-cell aggregation.

Russo *et al.* (2006) have shown that exopolysaccharide plays an important role in the biofilm formation in *R. leguminosarum*. Biofilm formation is severely impaired in the 17B and 38EV30 mutants. Future experiments will attempt to determine whether the decreased exopolysaccharide production in 17B and 38EV30 directly contributes to the defective biofilm phenotype. Observations made from CLSM imaging of fixed and nonfixed biofilms suggested that the mutant is specifically deficient in surface adhesion. These results are similar to those found by Balestrino *et al.* (2008), who reported that exopolysaccharide-deficient mutants of *K. pneumoniae* were also impaired in the initial attachment phase of biofilm formation. In *K. pneumoniae*, attachment to the solid substrate occurred at the poles of the cells, and so it is possible that the alterations in the capsule structure at the poles of the 17B and 38EV30 mutants (Fig. 5) are interfering with surface adhesion. Biofilm formation can occur during plant root colonization by rhizobacteria (Fujishige *et al.*, 2006; Danhorn & Fuqua, 2007; Santaella *et al.*, 2008). Given the defective biofilm phenotype of 17B and 38EV30, future experiments will determine the importance of the transporter encoded by RL2975–RL2977 for root colonization of host legume plants.

Acknowledgments

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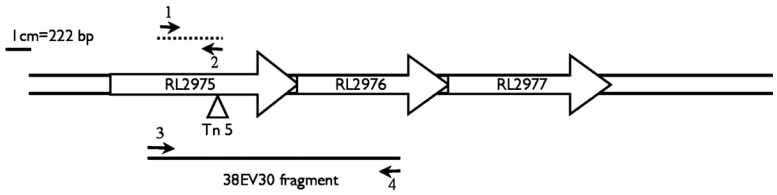

Gene	Closest homologs	AA similarity (*E*-value)	Conserved domains (*E*-value)	TM pred	Predicted function
RL2975	*Rhizobium etli* CFN 42 (YP_468684.1)	303/330 (9e-174)	Walker A/B ATP binding motif (2.7e-37)	1 TM domain	ATP binding component of ABC transporter
	Symbiobacterium thermophilum (YP_076493.1)	165/327 (9e-90)			
RL2976	*Rhizobium etli* CFN 42 (YP_468683.1)	233/266 (4e-120)	DUF990* (8e-06)	5 TM domains	Transmembrane component of ABC transporter
	Clostridium cellulolyticum H10 (ZP_01574145.1)	82/267 (6e-31)			
RL2977	*Rhizobium etli* CFN 42 (YP_468682.1)	227/267 (7e-129)	DUF990* (4.6e-22)	6 TM domains	Transmembrane component of ABC transporter
	Caldicellulosiruptor saccharolyticus DSM 8903 (YP_001179931.1)	70/253 (2e-21)			

Fig. 1.

Schematic of the RL2975—2977 ABC transporter operon. Small arrows indicate the positions of primers 17BF and 17BR (1 and 2) and 17B1F and 17B1R (3 and 4). Refer to Materials and methods for the primer sequences and amplification conditions used. The triangle indicates the transposon insertion site. The dashed line indicates the 17B probe fragment. Inset table summarizes the BLASTP analysis and predicted function of the three proteins encoded by the operon. *DUF990-domain of unknown function 990 is associated with ABC-2 transporters (Reizer *et al.*, 1992). AA, amino acid; TM, transmembrane.

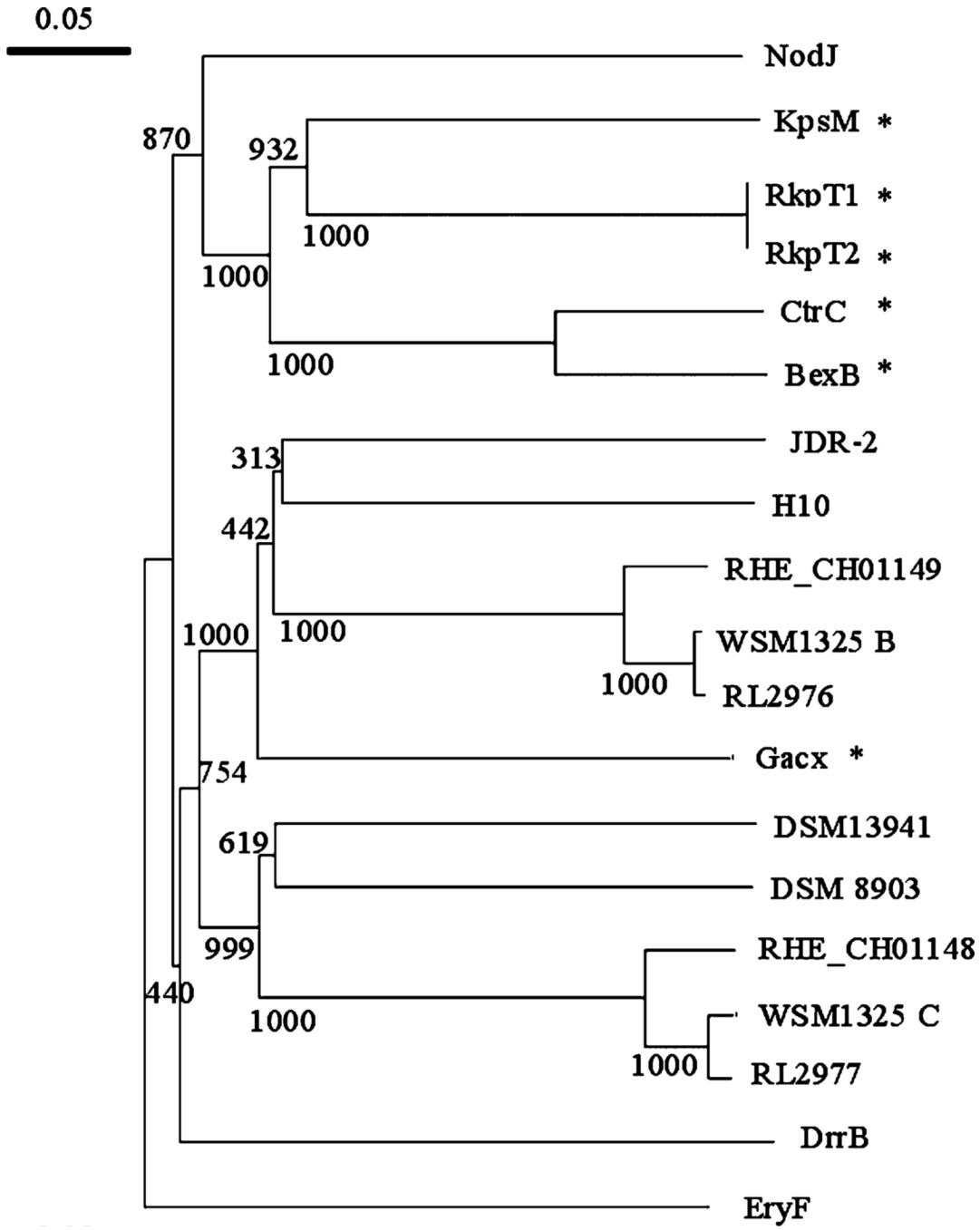

Fig. 2.

Neighbor-joining tree of RL2976 and RL2977 with the transmembrane components of well-characterized ABC-2 transporters and DUF990 proteins. Bootstrap analysis was performed with 1000 replicates, and values (out of 1000) are as displayed on tree. RHE_CH01148, *Rhizobium etli* CFN 42 (YP_468682.1); RL2977, *Rhizobium leguminosarum* bv. *viciae* 3841 (YP_768561.1); DrrB, *Mycobacterium avium* ssp. *paratuberculosis* K-10 (NP_960171.1); NodJ, *R. leguminosarum* bv. *viciae* 3841 (YP_770467.1); CtrC, *Neisseria meningitidis* Z2491 (NP_283043.1); BexB, *Haemophilus influenzae* (P22235); KpsM, *Escherichia coli* (AAC38078.1); RHE_CH01149, *R. etli* CFN 42 (YP_468683.1); RL2976, *R. leguminosarum* bv. *viciae* 3841 (YP_768560.1); RkpT1, *Sinorhizobium meliloti* 1021

(NP_437116); RkpT2, *S. meliloti* 1021 (NP_437102); JDR-2, *Paenibacillus* sp. JDR-2 (ZP_02846839); H10, *Clostridium cellulolyticum* H10 (ZP_01574145.1); WSM1325C, *R. leguminosarum* bv. *trifolii* WSM1325 (ZP_02293733); DSM 13941, *Roseiflexus castenholzii* DSM 13941 (YP_001431939); DSM 8903, *Caldicellulosiruptor saccharolyticus* DSM 8903 (YP_001179931.1); WSM1325B, *R. leguminosarum* bv. *trifolii* WSM1325 (ZP_02293734); EryF, *R. leguminosarum* bv. *viciae* 3841 (YP_764713); GacX, *Streptomyces glaucescens* (CAL64857.1). *Represents proteins where experimental data exists on their cellular function.

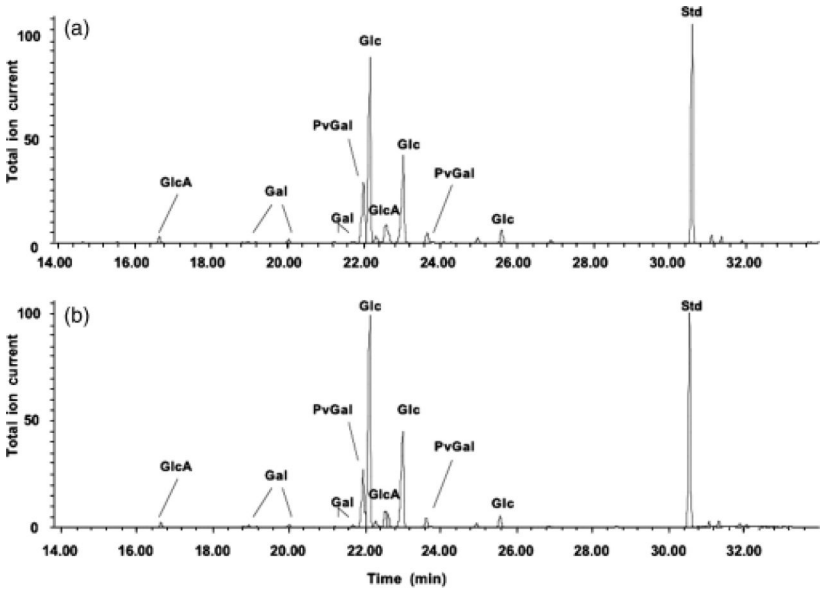

Fig. 3.

Compositional analysis of exopolysaccharides purified from *Rhizobium leguminosarum* 3841, wt (a) and 17B, mutant (b). Gas–liquid chromatography analysis of exopolysaccharide indicated a similar composition characteristics for HMW acidic exopolysaccharide. Abbreviations: GlcA, glucuronic acid; Gal, galactose; PvGal, 4,6-pyruvate acetal of galactose; Glc, glucose; Std, inositol used as a standard.

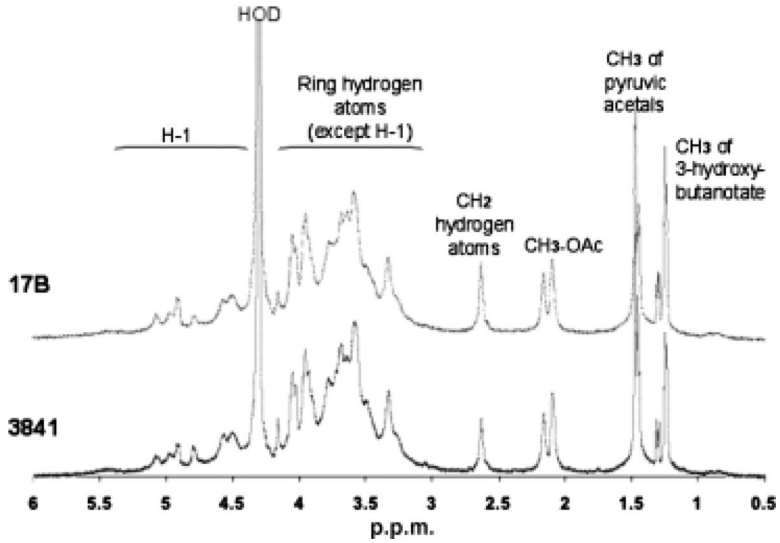

Fig. 4.
A500MHz ^{1}H-NMR spectrometry comparative analysis of *Rhizobium leguminosarum* 3841, wild type and 17B, mutant exopolysaccharide.

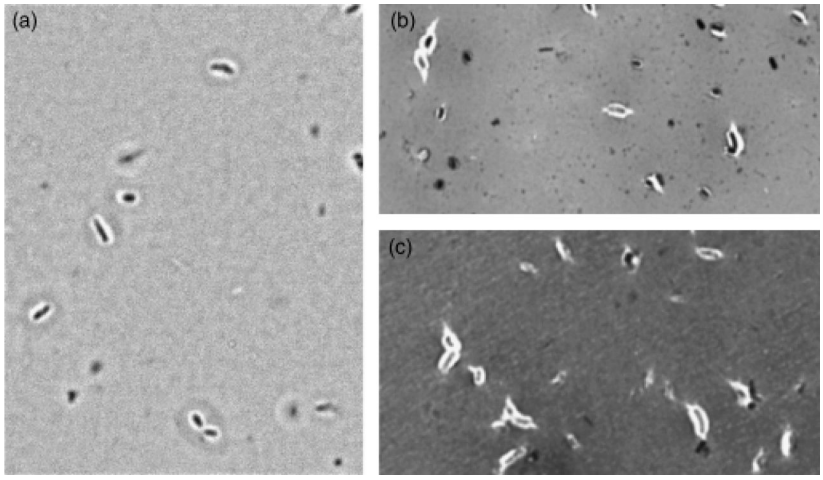

Fig. 5.
Capsule stain of RL2975-77 transporter mutants grown in VMM broth. Cells are shown at ×1000 magnification. (a) wild-type 3841; (b) 17B; (c) 38EV30.

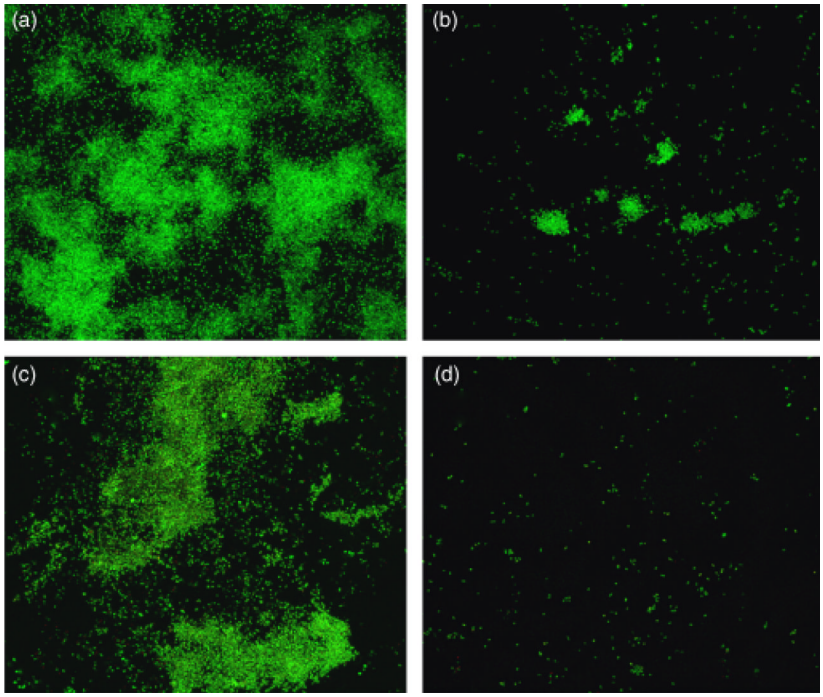

Fig. 6.
CLSM of biofilms produced by wild-type *Rhizobium leguminosarum* bv. *viciae* 3841 (a and c) and the 17B mutant (b and d). Biofilms of the wild type and mutant strains were stained with acridine orange (a and b) or using the Live/Dead™ cell viability kit (c and d).

Table 1

Strains, plasmids and primers used in this study

Strain	Relevant characteristics	Source or reference
E. coli		
TOP 10	F⁻ *mcr*AD(*mrr0hsd*RMS0*mcr*BC) f80*lac*ZDM15D*lac*X74 *rec*A1 *ara*D139D(*ara0leu*)7697 *gal*U *gal*K *rps*L (Sm^r) *end*A1 *nup*G	Invitrogen
S17-1	RP4 *tra* region, mobilizer strain *recA* derivative of MM294A with RP4-2 (Tc::Mu::Km::Tn7) integrated into the chromosome	Simon *et al*. (1983)
DH5α	*end*A1,*hsd*R17,*sup*E44,*thi*-1,*rec*A1,*gyr*A96,*rel*A1,D(*arg*F–*lac*ZYA),D*lac*U169,f80*lac*ZDM15	Invitrogen
R. leguminosarum		
3841	Spontaneous streptomycin-resistant derivative of *R. leguminosarum* bv. *viciae* strain 300	Glenn *et al*. (1980), Johnston & Beringer (1975)
17B	3841 ABC transporter, RL2975–RL2977 mutant, Tn5 TGN mutant, Sm^r, Nm^r, Gm^r	This study
38EV30	3841 ABC transporter, RL2976–RL2977 mutant, Sm^r, Gm^r	
Plasmids		
PTGN	Tn5 derivative, Gm^r, Amp^r promoter-less *nptII*, *gfp*	Tang *et al*. (1999)
pCR2.1 topo	Topo TA cloning vector, 4.0 kb, Km^r Ap^r	Invitrogen
pJQ200SK+	Suicide vector, P15a *ori*, *mob*, *sacB*, Gm^r	Quandt & Hynes (1993)
pCos879	*R. leguminosarum* VF39SM cosmid with RL2975–RL2977	Yost *et al*. (1998)
pEV25	pCR2.1 TOPO with a 1.3-kb PCR fragment containing the internal fragment of RL2975 and RL2976 Amp^r, Km^r	This study
pEV30	1.3-kb *reeA* and *reeB* fragment cloned from pEV25 into pJQ200SK using SstI/XbaI Gm^r	This study
Primers		
GmTAIL-1	5′-CAACGCGCTTGGTGCTAATGTGAT-3′	This study
GmTAIL2	5′-AGTTGGGCATACGGGAAGAAGTGA-3′	This study
17B1F	5′-AGCTCGAGCATCCTGGTGCCGA-3′	This study
17B1R	5′-AGCTCGAGTCCAGTCCAGCGAGAAAC-3′	This study
17BF	5′-TCTGCGGCGGATGGACAA-3′	This study
17BR	5′-GTGAGGATGATGGTGACG-3′	This study
AD-1	5′-AGWGNAGWANCAWAGG-3′	Liu & Huang (1998)

Table 2

Sensitivity to desiccation, osmotic stress and detergents

Strains	Percent survival*			
	Desiccation tolerance	Osmotic stress†	VMM+DOC‡	VMM+SARC‡
3841	42±1.8	79.4±1.42	125±4.45	123±3.42
17B	1.9±0.96**	69.3±4.66	94.5±7.94	79.5±5.59
38EV30	6.45±2.6**	79.7±6.07	101±20.6	117±23.6

*All data presented are the average (±SD) percent survival of three independent trials.

†Strains were grown in TY broth or TY supplemented with 69.5 mM NaCl for 2 days. ODs were measured at 600 nm.

‡Strains were grown in VMM broth or VMM supplemented with 75 μg mL^{-1} deoxycholate (DOC) or 50 μg mL^{-1} sarcosyl (SARC) for 2 days. ODs were measured at 600 nm.

**Difference in percent growth between the wild type and the mutant is statistically significant at a *P*-value < 0.001 (Student's *t*-test).

Table 3

Exopolysaccharide (EPS) production contributes to desiccation tolerance and restores tolerance to the transporter mutants

Strain	VMM		TY	
	Amount EPS† produced (μg EPS mg^{-1} cell dry weight)	Desiccation tolerance‡ (percent survival)	Amount EPS† produced (μg EPS mg^{-1} cell dry weight)	Desiccation tolerance‡ (percent survival)
3841	4.1±1.38	122±20.7	0.504±0.150*	28±11.4*
17B	0.056±0.015**	15±9.4**	0.021±0.002**	1.13±0.184**
38EV30	0.115±0.035**	26.8±7.21**	0.029±0.007**	1.20±0.516**
17B+EPS	1.80±0.593	95.9±23.8	0.97±0.478	35.1±6.41
38EV30+EPS	2.75±0.565	104±34.0	0.74±0.175	47.2±4.72

† Data presented are the average (±SD) of at least three independent trials.

‡ Data presented are the average (±SD) percent survival of three independent trials.

* Difference between 3841 (TY) and 3841 (VMM) is statistically significantly at a *P*-value < 0.002 (Student's *t* test).

** Difference between the mutant and the wild type is statistically significant at a *P*-value < 0.003.

Table 4

Quantification of biofilm formation by the RL2975 (17B) mutant

Strain	Crystal violet staining* (OD_{550nm})	Viable cell count* (log_{10} CFU peg^{-1})	MTS reduction assay* (OD_{490nm})
3841	0.157±0.113	4.87±0.34	1.92±1.03
17B	0.036±0.012**	3.15±0.78**	0.381±0.099**

* Results presented are the average (±SD) of 24 replicates (48 replicates for the viable cell count).

** Difference between the wild type and the mutant strains is statistically significant at a *P*-value < 0.001 (Student's *t*-test).