RESEARCH LETTER

Mutation in a D-alanine–D-alanine ligase of *Azospirillum brasilense* Cd results in an overproduction of exopolysaccharides and a decreased tolerance to saline stress

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Abstract

Bacteria of the genus *Azospirillum* are free-living nitrogen-fixing, rhizobacteria that are found in close association with plant roots, where they exert beneficial effects on plant growth and yield in many crops of agronomic importance. Unlike other bacteria, little is known about the genetics and biochemistry of exopolysaccharides in *Azospirillum brasilense*. In an attempt to characterize genes associated with exopolysaccharides production, we generated an *A. brasilense* Cd Tn*5* mutant that showed exopolysaccharides overproduction, decreased tolerance to saline conditions, altered cell morphology, and increased sensitivity to detergents. Genetic characterization showed that the Tn*5* was inserted within a *ddlB* gene encoding for a D-alanine–D-alanine ligase, and located upstream of the *ftsQAZ* gene cluster responsible for cell division in different bacteria. Heterologous complementation of the *ddlB* Tn*5* mutant restored the exopolysaccharides production to wild-type levels and the ability to grow in the presence of detergents, but not the morphology and growth characteristics of the wild-type bacteria, suggesting a polar effect of Tn*5* on the *fts* genes. This result and the construction of a nonpolar *ddlB* mutant provide solid evidence of the presence of transcriptional coupling between a gene associated with peptidoglycan biosynthesis and the *fts* genes required to control cell division.

Introduction

Bacteria of the genus *Azospirillum* are free-living, nitrogen-fixing rhizobacteria that are found in close association with plant roots, where they exert beneficial effects on plant growth and yield in many crops of agronomic importance. Upon inoculation, these bacteria attach to plant roots and proliferate, and some strains subsequently invade and colonize the internal plant tissues (Okon & Vanderleyden, 1997). As a result of this association, several greenhouse- and field-inoculation experiments with *Azospirillum*, carried out at different locations over the last 25 years, have demonstrated that these bacteria have beneficial effects on plant yields (Döbereiner & Pedrosa, 1987; Okon & Labandera-González, 1994; Burdman *et al.*, 2000b; Dobbelaere *et al.*, 2001, 2002; El Zemrany *et al.*, 2006). These benefits are mainly derived from morphological and physiological changes in the roots of the inoculated plant that lead to an enhancement of water and mineral uptake. Secretion of plant growth-promoting substances such as auxins, gibberellins, and cytokinins by the bacteria seems to be at least partially responsible for these effects (Steenhoudt & Vanderleyden, 2000). Although other mechanisms – such as biological nitrogen fixation and nitrate reduction by the microorganisms – have been reported (Kapulnik *et al.*, 1985; Boddey & Döbereiner, 1994; Fallik *et al.*, 1994), the contribution of these mechanisms to plant growth has been shown to be of little significance in these systems.

Even though considerable evidence has been accumulated over many years indicating the involvement of extracellular polysaccharides and proteins in both the cell-aggregation and root-attachment processes, the precise mechanisms of

these phenomena remain unexplained. The difficulty in elucidating these processes derives from their complexity because it seems that they are mediated by several different cell-surface components.

Although the *Azospirillum*–plant interaction is not yet fully understood, partially owing to the lack of an easily detectable plant phenotype following inoculation, some bacterial surface components have been demonstrated to be involved in the early stages of the interaction with the plant (Croes *et al.*, 1993; Katupitiya *et al.*, 1995; De Troch & Vanderleyden, 1996; Pereg-Gerk *et al.*, 1998, 2000; Vande Broek *et al.*, 1998; Burdman *et al.*, 2000a; Jofré *et al.*, 2004). Several authors have shown that the attachment of *Azospirillum* to plant roots involves a two-step process, starting with a rapid and reversible bacterial adsorption onto the root system, followed by an irreversible anchoring of the bacteria to the root surface (Michiels *et al.*, 1991). The adsorption of *Azospirillum brasilense* to wheat roots was shown to be highly dependent on the presence of polar flagella, whereas the anchoring (the second step) was associated with the biosynthesis of a polysaccharide-containing fibrillar material. The lipopolysaccharide produced by *A. brasilense* Cd also plays a role during early interaction with maize roots, because a mutant affected in the lipopolysaccharide core showed impaired attachment to these roots as well as diminished plant–root colonization (Jofré *et al.*, 2004).

Azospirillum brasilense Cd produces at least one exopolysaccharide, which consists of a heteropolymer containing glucose (47%), fucose (28%), galactose (11%), rhamnose (6%), mannose (3%), xylose (4%), and arabinose (1%) as the main sugars (Burdman *et al.*, 2000a; Fischer *et al.*, 2003). Two molecular forms of this exopolysaccharide have been described, one of high molecular weight and another of low molecular weight (Michiels *et al.*, 1988). The biosynthetic pathway of the *A. brasilense* exopolysaccharide remains uncharacterized and the genes encoding the enzymes involved in the synthesis of its saccharide repeating unit as well as in the polymerization and export of the molecule have yet to be determined.

In an attempt to determine the genes associated with exopolysaccharide biosynthesis and regulation, we generated an *A. brasilense* Cd Tn*5*-mutant library and searched for changes in subsequent exopolysaccharide production. One of the Tn*5* mutants displayed exopolysaccharide overproduction along with increased sensitivity to both detergents and saline conditions as well as altered cell morphology. The genetic characterization of this mutant allowed us to identify a gene cluster that includes a *ddlB*-homolog encoding for a D-alanine–D-alanine ligase as well as the *ftsQAZ* genes responsible for the septum formation in different bacterial species, suggesting that the integrity of bacterial cell envelopes is required for adaptation to saline environments.

Materials and methods

Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *Azospirillum brasilense* Cd was grown at 33 °C in Luria–Bertani (LB) medium supplemented with 2.5 mM $CaCl_2$ and 2.5 mM $MgSO_4$ (Miller, 1972) or in minimal medium lactate (MML; Dreyfus *et al.*, 1983). *Escherichia coli* strains were grown at 37 °C in LB medium. Antibiotics were added at the following concentrations when required: ampicillin, 25 μg mL^{-1}; chloramphenicol, 25 μg mL^{-1}; kanamycin (Km), 25 μg mL^{-1}; gentamicin, 20 μg mL^{-1}; and tetracycline, 5 μg mL^{-1}.

DNA manipulations

Plasmid and total DNA preparations, agarose gel electrophoresis, restriction-endonuclease digestion, and cloning were performed according to standard protocols (Sambrook *et al.*, 1989). *Escherichia coli* transformation was effected by electroporation with a Gene Pulser apparatus (Bio-Rad Laboratories Ltd). Southern hybridizations were carried out with a nonradioactive detection kit, and a chemiluminescence method was used to detect hybridization bands according to the instructions of the manufacturer (Gibco BRL, Life Technologies). The probe was obtained after EcoRI digestion of pPG1: the resulting EcoRI fragment containing Tn*5* and flanking DNA from *A. brasilense* Cd PG1 was then isolated from an agarose gel and purified by means of the Wizard® DNA Clean-Up System kit (Promega).

Mutagenesis

Random transposon Tn*5* mutagenesis of *A. brasilense* Cd was carried out using the mobilizable pGS9 suicide plasmid (Vanstockem *et al.*, 1987). The kanamycin-resistant (Km^r) transconjugants were selected on MML medium, restreaked on LB medium supplemented with calcofluor (200 μg mL^{-1}), and screened for exopolysaccharide production by fluorescence microscopy as described below.

Fluorescence microscopy

Cells grown on LB agar plates supplemented with calcofluor (Fluorescent Brightener 28, Sigma) were suspended in 0.88% (w/v) NaCl solution and then observed under an epifluorescent microscope (Zeiss) at 365 nm.

For 4′,6-diamidino-2-phenylindole (DAPI) staining, strains were grown in MML medium until an $OD_{600\,nm}$ of 0.3. Cells from 0.2 mL of culture were harvested by centrifugation at 3500 ***g*** for 4 min, suspended in 15 μL of 0.2 mg mL^{-1} DAPI, and incubated during 10 min at room temperature. DAPI was removed by centrifugation at 3500 ***g***

Table 1. Strains and plasmids used in this study

	Relevant characteristics	Reference or source
Strains		
A. brasilense Cd	ATCC 29710 wild-type.	Tarrand *et al.* (1978)
A. brasilense PG1	*ddlB*::Tn*5* mutant of *A. brasilense* Cd.	This work
A. brasilense AL1	*A. brasilense* nonpolar *ddlB* mutant	This work
E. coli DH5α	*endA1 recA1 hsdR17 supE44 thi-1 gyrA96 relA1* Δ(*lacZYA-argF*)*U169* φ80d *lacZ* Δ*M15.*	Hanahan (1983)
E. coli JM109	*recA endA1 gyrA96 thi hsdR17 supE44* Δ(*lac-proAB*) *relA1.*	Sambrook *et al.* (1989)
E. coli S17-1	*pro recA hsdR*, *thi* RP4-2-Tc::Mu-Km::Tn*7* integrated into the chromosome.	Simon *et al.* (1983)
Plasmids		
pK18mob2	pK18mob derivative with unique KpnI and SacI sites in polylinker, Km^r	Tauch *et al.* (1998)
pGS9	Cm^r, Km^r, (Tn*5*) p15A replicon N-*tra* Tn*5* donor.	Selveraj & Iyer (1983)
pSUP102	Tc^r, Cm^r, vector movilizable pACYC184 derivative.	Simon *et al.* (1989)
pBluescript SK	Cloning and sequencing vector, Ap^r, phagemid, M13 derivative, f1 origin of replication.	Stratagene, La Jolla, California
pJN105	*araC-*P_{BAD} cassette cloned in pBBR1MCS-5, Gm^r.	Newman & Fuqua (1999)
pPG1	20-kb EcoRI fragment containing Tn*5* insertion and flanking DNA from *A. brasilense* PG1 cloned into pSUP102; Km^r Tc^r.	This work
pPG2	3.8-kb EcoRI–BamHI fragment, containing flanking DNA, IS*50*L and *nptII*, from pPG1 cloned into pBluescript SK; Km^r Ap^r.	This work
pPG3	16-kb BamHI fragment containing IS*50*R and flanking DNA, from pPG1, cloned into pBluescript SK; Ap^r *lac*$^-$.	This work
pKddlB	249-bp internal fragment of *ddlB* from *A. brasilense* cloned into pK18mob2, Km^r.	This work
pJNddlB	1000-bp fragment containing *ddlB* from *S. meliloti* cloned into pJN105, Gm^r.	This work

Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Gm^r, gentamicin resistance.

for 4 min and cells were suspended in 0.88% (w/v) NaCl and observed under a Zeiss Axiophot microscope equipped with epifluorescence optic sets and the corresponding filter sets. Images were recorded with an AxioCam HRc camera (Zeiss) and processed using the Adobe PHOTOSHOP program.

Test of sensitivity to detergents

Azospirillum brasilense Cd strains were grown in MML medium until an $OD_{600\,nm}$ of 0.3. Cells from 1 mL of culture were collected by centrifugation – 2 min at 3500 ***g*** – suspended in 0.88% (w/v) NaCl solution, and streaked on MML-agar plates containing 0.01% (w/v) sodium dodecyl sulfate (SDS). Plates were incubated at 33 °C for 3 days.

Exopolysaccharides isolation and quantification

For exopolysaccharide isolation, *A. brasilense* Cd strains were grown in MML containing 10 mM NH_4Cl at 33 °C for 2 days. Exopolysaccharides were precipitated from culture supernatants with three volumes of cold ethanol. Pellets were air-dried, suspended in distilled water, and dialyzed (MWCO 12 000 Da) against distilled water for 2 days at 4 °C. Exopolysaccharides were quantified by the anthrone method (Dische, 1962).

Protein determination and growth curves

Protein determination was carried out according to Bradford (1976). The time course of cell growth in the selected Tn*5* mutant was examined in standard MML medium. Growth under saline conditions was performed in MML medium supplemented with 300 mM NaCl. Cultures were grown at 33 °C on a rotary shaker at 100 r.p.m. Bacterial growth was monitored spectrophotometrically at 600 nm, and at early-, mid-, and late-exponential-phase growth an aliquot was used to estimate the viable cell number by plate counting in MML medium.

Lipopolysaccharide isolation and analysis

Cultures were grown in LB broth al 33 °C. Lipopolysaccharides were isolated in agarose-immobilized polymyxin B

(Affi-prep polymyxin support, Bio-Rad Laboratories Ltd) according to Valverde *et al.* (1997). Lipopolysaccharides were separated by 20% (w/v) polyacrylamide-gel electrophoresis (PAGE) in an SDS-Tricine buffer system and visualized by silver staining (Tsai & Frasch, 1982).

Cloning of the Tn*5*-interrupted region

Total DNA from *A. brasilense* Cd PG1 was digested with EcoRI and ligated to pSUP102. The ligation mixture was used to transform electrocompetent *E. coli* S17-1 cells. Transformant clones were selected on LB medium containing kanamycin and tetracycline and screened for the presence of IS50 sequences by a PCR assay with the primers IS1 (5′-GCTCGATCTAGAACGTTCATGATAACTTCTGC-3′) and IS2 (5′-TCTGCGGACTGGCTTTCTAC-3′), which leads to a PCR product of 1.2 kb. One of the plasmid isolates [tetracycline-resistant (Tc^r), Km^r] was designated pPG1.

Subcloning and outward sequencing from the transposon in both directions

Plasmid pPG1 was digested with BamHI–EcoRI and BamHI and the products were ligated to pBluescript SK. The recombinant plasmids were used to transform electrocompetent *E. coli* JM109 cells. Clones were selected on LB medium containing ampicillin and kanamycin and LB medium containing ampicillin and X-gal. Plasmids were isolated and purified by means of a Wizard™ DNA Clean-Up System kit (Promega). DNA was sequenced using Taq FS DNA polymerase and fluorescent-dideoxy terminators in a cycle sequencing method and the resultant DNA fragments were electrophoresed and analyzed using an automated Applied Biosystems 377 DNA sequencer. Sequencing service was carried out by HHMI/Keck DNA Sequencing Facility. The amino acid sequences deduced from the nucleotide sequence were compared with the GenBank database, through the use of the BLAST algorithm (Altschul *et al.*, 1997).

Site-directed mutagenesis by plasmid integration

An internal 249-bp fragment of *ddlB* was amplified by PCR using *A. brasilense* Cd DNA template and primers ddlB f [5′-AAAAGGTACCTACATCGTCAAGCCGGTGGA-3′ (KpnI restriction site underlined)] and ddlB r [5′-AAAAAAGCTTATGACCGGCGCTGTATTTA-3′ (HindIII restriction site underlined)]. The PCR product was purified by means of the QIAquick®/™ PCR-purification kit (Qiagen), restricted with KpnI and HindIII, and then ligated to the corresponding sites in the pK18mob2 vector. The recombinant plasmid, designated pKddlB, was mobilized by mating from *E. coli* S17-1 to *A. brasilense*. Transconjugants were selected in MML medium supplemented with kanamycin.

Heterologous complementation

A 1000-bp fragment containing the coding region of *ddlB*, 47-bp upstream from *ddlB* start codon and 26-bp downstream from *ddlB* stop codon, was amplified by PCR using *Sinorhizobium meliloti* 2011 DNA template and primers SmddlB f [5′-AAAACTGCAGAGGTGCAAGCAAGCTTCG-3′ (PstI restriction site underlined)] and SmddlB r [5′-GCTCTAGAACGAACCCTCTTGCCCCTT-3′ (XbaI restriction site underlined)]. The PCR product was purified by means of the QIAquick®/™ PCR-purification kit (Qiagen), restricted with PstI and XbaI, and then ligated to the corresponding sites in the pJN105 vector. The recombinant plasmid, designated pJNddlB, was mobilized by mating from *E. coli* S17-1 to *A. brasilense*. The selection of transconjugants was performed on MML medium supplemented with gentamicin.

Nucleotide sequence accession number

The nucleotide sequence has been deposited in the GenBank database under accession number AF492457.

Statistical analysis

Results were analyzed by one-way ANOVA, with the means evaluated for significance through the least-significant-difference test at $P < 0.05$.

Results

Random Tn*5* mutagenesis of *A. brasilense* Cd and screening for altered exopolysaccharide production

For a better understanding of genes controlling exopolysaccharide biosynthesis in *A. brasilense*, we generated a collection of Tn*5* mutants of *A. brasilense* Cd as described in Materials and methods and screened for putative alterations in exopolysaccharide production by the fluorescence microscopy of cells growing on LB medium containing calcofluor (Fig. 1). Calcofluor is a fluorescent dye that binds predominantly to β-1,4- and β-1,3-linked glucans (Wood & Fulcher, 1978). One clone, designated PG1, out of 2800 transconjugants showed enhanced fluorescence when compared with the wild-type strain, thus indicating a possible alteration in exopolysaccharide production (Fig. 1a and b).

In order to confirm whether the enhanced fluorescence observed in the PG1 mutant is associated with increased exopolysaccharide production, we determined the amount of exopolysaccharides produced both by the mutant and by its wild-type strain. Figure 2a shows that mutant PG1 produced almost three times more exopolysaccharides than the wild-type strain. In spite of the increased exopolysaccharide production, mutant PG1 did not show any alteration in the mucoid-colony phenotype (Fig. 1d and e). Since

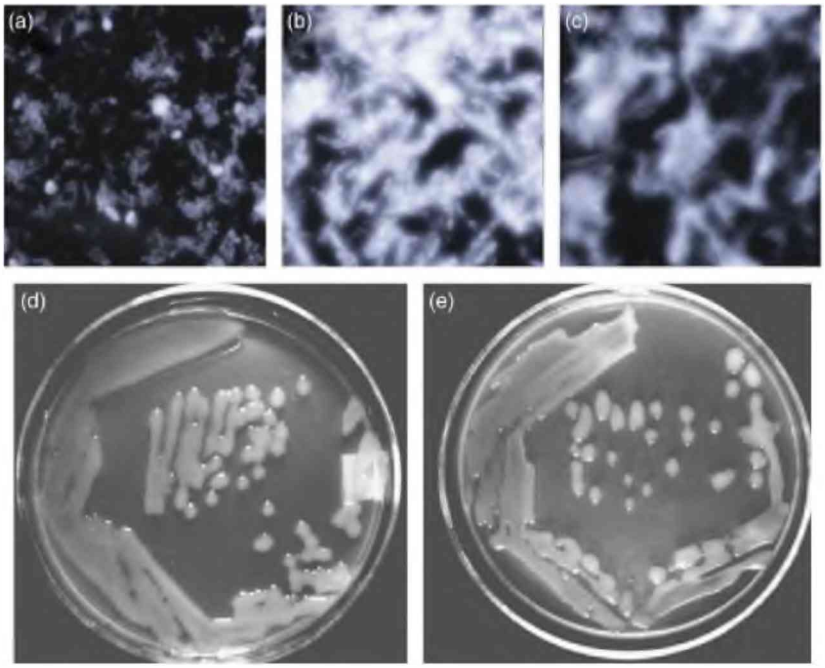

Fig. 1. (a–c) Fluorescence microscopy showing the calcofluor phenotype of *Azospirillum brasilense* cells. (a) *Azospirillum brasilense* Cd wild-type strain. (b) *Azospirillum brasilense* Cd PG1 mutant. (c) The resultant clone after the homogenotization (marker exchange) of the wild-type strain with plasmid pPG1. Cells were grown as described in Materials and methods. (d and e) Colony morphology of *A. brasilense* Cd wild type (d) and *A. brasilense* Cd PG1 mutant (e) grown on MML-agar medium.

alterations in exopolysaccharide production are often associated with changes in other polysaccharides such as the lipopolysaccharides, we performed an SDS-PAGE analysis of this latter polysaccharide in the mutant. No differences were observed, however, between the lipopolysaccharide electrophoretic profile of the mutant and that of the wild-type strain (Fig. 2c).

Bacterial adaptation to saline environments often involves changes in cell-surface components such as exopolysaccharides or lipopolysaccharide (Lloret *et al.*, 1995; Jofré *et al.*, 1998; Chowdhury *et al.*, 2007). For this reason, we examined the ability of the mutant PG1 to grow under conditions of saline stress. The growth of mutant PG1 was thus monitored by reading $OD_{600\,nm}$ as well as by estimation of the viable cell number at different growth phases (early-, mid-, and late-exponential-phase growth). When the growth was estimated by reading OD, no appreciable differences were observed between the growth of the mutant PG1 and its wild-type strain under normal conditions (Fig. 3a). However, at any OD, the viable cell number of the mutant PG1 was lower than that of the wild-type strain under normal conditions (Fig. 3b). Such a difference was more evident under saline-stress conditions (Fig. 3b).

Cloning of the Tn*5*-interrupted DNA region in the PG1 mutant

A 20-kb EcoRI Tn*5*-containing fragment isolated from mutant PG1 was cloned into vector pSUP102 to yield plasmid pPG1 as indicated in Materials and methods. The 20-kb insert was used as a probe in Southern hybridization against a total EcoRI digestion of genomic DNA from the mutant and from the wild type. Both wild-type and mutant DNA gave a single hybridization signal corresponding to DNA fragments of 14.5 and 20 kb, respectively. The 5.5 kb difference in size was consistent with the presence of a Tn*5* insertion in the mutant.

Reverse genetics

The mobilizable nonreplicative plasmid pPG1 was used to homogenotize the Tn*5* mutation into the wild-type strain *A. brasilense* Cd via double crossing-over (i.e. marker exchange). The recombinant obtained (PG2) showed a calcofluor phenotype coincident with that of the original mutant PG1 (Fig. 1c). This result confirmed that the cloned DNA region containing the Tn*5* insertion was responsible for the observed changes in the fluorescence of the mutant in the presence of calcofluor.

Genetic characterization of the Tn*5*-interrupted region present in *A. brasilense* Cd PG1

The DNA region interrupted by Tn*5* in plasmid pPG1 was subcloned and 3.8 kb were sequenced (accession number AF492457) as described in Materials and methods. The proposed genetic organization of the DNA bordering the Tn*5* is presented in Fig. 4. The Tn*5* insertion mapped at the 3′ end (18 bp upstream from the stop codon) of a 632-bp ORF whose partial deduced amino acid sequence showed significant similarity to D-alanine–D-alanine ligase (*ddlB*) from *Agrobacterium tumefaciens* (67%), *Gluconobacter oxydans* (65%), *Mesorhizobium* sp. BNC1 (66%), *Zymomonas*

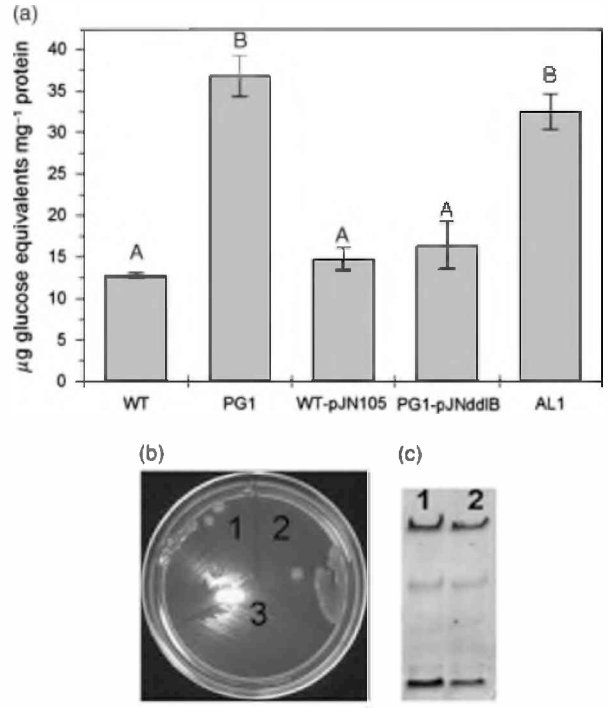

Fig. 2. (a) Production of exopolysaccharides by wild type *Azospirillum brasilense* Cd and its mutants PG1 and AL1. WT: *A. brasilense* Cd wild type; PG1: *A. brasilense* Cd PG1; AL1: *A. brasilense* Cd AL1; WT-pJN105: *A. brasilense* Cd wild type harboring the plasmid pJN105; PG1–pJNddlB: *A. brasilense* Cd PG1 complemented with plasmid pJNddlB. Data are the average of four determinations ± SE. Different letters represent significant differences (ANOVA, LSD test, $P \leq 0.05$). (b) Growth of *A. brasilense* Cd wild type and mutant PG1 on MML-agar medium supplemented with 0.01% SDS. 1, *A. brasilense* Cd wild type; 2, *A. brasilense* Cd PG1 complemented with plasmid pJNddlB; 3, *A. brasilense* Cd PG1. (c) Lipopolysaccharide profiles from *A. brasilense* Cd and its mutant PG1 after resolution by SDS-PAGE and silver staining. Lane 1, *A. brasilense* Cd wild-type; lane 2, *A. brasilense* Cd PG1.

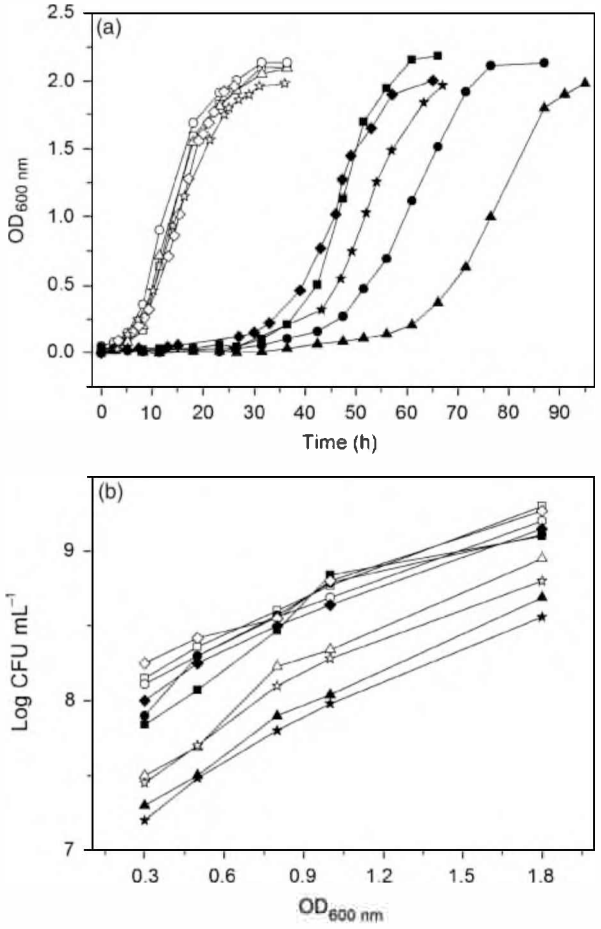

Fig. 3. Effects of saline conditions on the bacterial growth rate of wild-type *Azospirillum brasilense* and *ddlB* mutants. (a) The OD increase, while (b) shows the counts of the CFU mL^{-1}. Square: *A. brasilense* Cd wild type; triangle: *A. brasilense* Cd PG1 mutant; circle: *A. brasilense* Cd AL1 mutant; lozenge: *A. brasilense* Cd wild type harboring plasmid pJN105; star: *A. brasilense* Cd PG1 complemented with plasmid pJNddlB. Saline conditions were reached adding 300 mM NaCl (black-solid symbols) to MML medium. MML medium without NaCl was used as control (open symbols). Data are the average of three determinations.

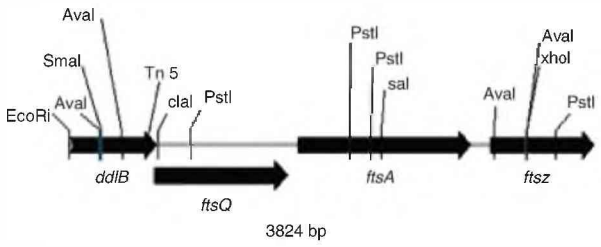

Fig. 4. Schematic representation of the gene organization of the 3.8-kb region containing the Tn*5* insertion present in PG1 mutant.

mobilis (62%), *S. meliloti* (62%), and *Bartonella bacilliformis* (61%) among others. D-Alanine–D-alanine ligase is an enzyme involved in peptidoglycan biosynthesis (Belanger *et al.*, 2000). Immediately downstream of *ddlB* and transcribed in the same direction, three complete ORFs could be identified. The translation products of these ORFs shared significant sequence similarity to FtsQ, FtsA, and FtsZ; the cell-division proteins involved in septum formation (Jensen *et al.*, 2005). The amino acid sequence similarity ranged from 47% and 54% for FtsQ, 60% and 68% for FtsA, and 82% and 85% for FtsZ.

It has been reported that mutations in *ftsA* are responsible for anomalous filamentous growth. The FtsA protein is a component of the septum which may interact with FtsZ. FtsZ is an essential protein for bacterial cell division that assembles into a dynamic ring on the inner surface of the cytoplasmic membrane at the location of cell division (Karimova *et al.*, 2005). Taking into account the genetic evidence obtained, we examined – using fluorescence microscopy – the cell morphology of the mutant strain PG1 grown in MML medium under normal and saline-stress

conditions. The obtained images showed the presence of highly pleomorphic bacilli (Fig. 5c). This abnormal cell morphology was more evident under saline-stress conditions (Fig. 5d).

Interestingly, the observed cell morphology in mutant PG1 could explain the lower viable cell number achieved in mutant PG1 compared with the wild-type strain (Fig. 3b), because cells from mutant PG1 are longer and wider than

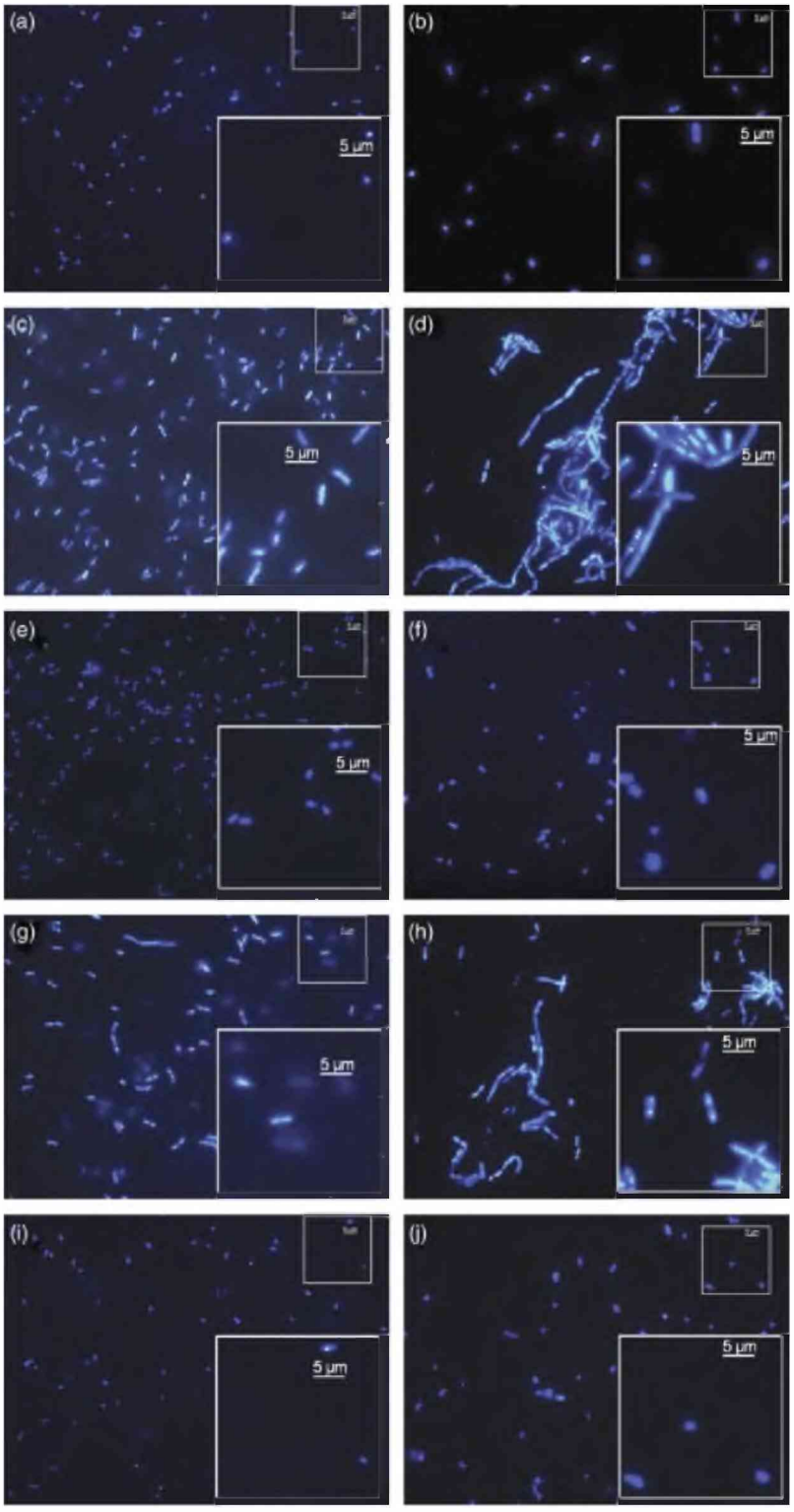

Fig. 5. Fluorescence microscopy of DAPI-stained *Azospirillum brasilense* cells showing the cell morphology of *ddlB* mutants under normal and saline-stress conditions. Cultures were grown in MML medium until an $OD_{600\,nm}$ of 0.3. Saline-stress conditions were achieved supplementing the culture medium with 300 mM NaCl. (a and b) *A. brasilense* Cd wild-type; (c and d) *A. brasilense* Cd PG1 mutant; (e and f) *A. brasilense* Cd AL1 mutant; (g and h) *A. brasilense* Cd PG1 complemented with plasmid pJNddlB; (i and j) *A. brasilense* Cd wild type harboring plasmid pJN105. (a, c, e, g, and i) Normal conditions; (b, d, f, h, and j) Saline-stress conditions. The square in the right corner indicates the magnified zone. Scale bar: 5 µm.

those from the wild-type strain (Fig. 5a–d). In addition to cell morphology, we also examined the sensitivity to SDS in the mutant PG1. The results, presented in Fig. 2b, showed that mutant PG1 was more sensitive to SDS than its wild-type strain, suggesting alterations in the integrity of bacterial cell envelopes. The observed phenotype could be attributed to the mutation in *ddlB*, because such an alteration was abolished by the introduction of plasmid pJNddlB carrying a *ddlB* ortholog from *S. meliloti* (Fig. 2b).

Generation of a nonpolar *ddlB* mutant of *A. brasilense* Cd

The observed changes in cell morphology in mutant PG1 (Fig. 5c and d) could be the consequence of a polar effect on the transcription of *ftsQAZ* genes caused by Tn*5* insertion. To ascertain whether such a hypothesis was correct, we constructed the nonpolar *ddlB* mutant *A. brasilense* Cd AL1 by a site-directed plasmid integration in which the *lac* promoter of vector pK18mob2 was reading downstream *ddlB*. Restoration of the wild-type cell morphology was subsequently observed in the nonpolar *ddlB* mutant AL1 (Fig. 5e and f), thus suggesting that the morphologic alteration in the PG1 mutant may have been caused by a polar effect of Tn*5* insertion. Exopolysaccharide production in mutant AL1 was found to be increased to the same extent as that in mutant PG1 (Fig. 2a).

Heterologous complementation of the *A. brasilense* Cd PG1 mutant with *S. meliloti* DdlB

To make our assumption of a polar effect of Tn*5* insertion in mutant PG1 stronger, we performed heterologous complementation of mutant PG1 with a *ddlB* ortholog from *S. meliloti* and evaluated the exopolysaccharide production, cell morphology, and growth of the complemented strain compared with mutant PG1.

The introduction of plasmid pJNddlB into *A. brasilense* Cd PG1 restored exopolysaccharide production to wild-type levels as well as the ability to grow in the presence of SDS, indicating functional complementation (Fig. 2a and b). As expected, normal exopolysaccharide production was also observed when plasmid pJNddlB was introduced into mutant AL1 (not shown). The effect of high concentrations of sodium chloride on the complemented PG1 mutant was still observed (Fig. 3b, CFU counts), suggesting that changes in cell division – and not exopolysaccharide overproduction – were mainly responsible for mutant growth behavior under salt stress. Similarly, cell morphology was shown to be still impaired in the complemented mutant PG1 as compared with the wild type (Fig. 5g and h). The results are fully consistent with our assumption for a polar effect of Tn*5* insertion in the mutant PG1. As a control, plasmid pJN105 was introduced in the wild-type strain to evaluate any possible alterations in exopolysaccharide production and/or cell morphology caused by the presence of plasmid pJN105. No significant differences were observed with the wild type either in the amount of exopolysaccharides (Fig. 2a) or in cell morphology (Fig. 5i and j).

Discussion

In this work, we have characterized an *A. brasilense* Cd Tn*5* mutant displaying an enhanced fluorescence phenotype on medium containing calcofluor. This phenotype was shown to be associated with increased exopolysaccharide production. Furthermore, the mutant was found to be more sensitive to SDS and salinity – a frequent phenotype, in turn, associated with mutants altered in surface components in *Azospirillum*.

Genetic characterization of the EcoRI DNA fragment that contains Tn*5* in mutant PG1 led to the recognition of four ORFs transcribed in the same direction and with sequence similarity to *ddlB*, *ftsQ*, *ftsA*, and *ftsZ* from *A. tumefaciens*, *G. oxydans*, *Mesorhizobium* sp. BNC1, *Z. mobilis*, *S. meliloti*, and *B. bacilliformis* among others. The genomic organization of the *A. brasilense ddlB–ftsQ–ftsA–ftsZ* cluster presents perfect synteny with these same six bacterial species. In *A. brasilense* Cd the *ddlB* and *fstQ* ORFs were found to be overlapped by 16 bp, indicating possible transcriptional coupling.

Bacterial peptidoglycan is an important biopolymer layer that protects the bacterial cell from environmental stresses. Its biosynthesis begins with an initial cytoplasmic stage in which an UDP-*N*-acetyl muramic acid (UDP-MurNAc)-pentapeptide precursor is synthesized. In a second membrane-associated stage, the MurNAc-pentapeptide is transferred to a lipid carrier, derivatized with a *N*-acetyl glucosamine (GlcNAc) residue, and translocated across the membrane. During a third stage, these lipid-linked precursors are incorporated into the membrane through transglycosylation and transpeptidation reactions (Bugg & Walsh, 1992). The *ddlB* gene encodes the D-alanine–D-alanine ligase that catalyzes the condensation of two D-alanine residues to form the D-alanine–D-alanine dipeptide that is subsequently ligated to UDP-*N*-acetylmuramoyl tripeptide during the first cytoplasmic stage of peptidoglycan biosynthesis.

In constrast, FtsQ, FtsA, and FtsZ are proteins involved in the cell-division process. In *E. coli*, for example, at least 15 proteins have been implicated in cell division. All of these proteins localize to a ring-like structure, at midcell, commonly referred to as the divisome (Buddelmeijer & Beckwith, 2002; Pichoff & Lutkenhaus, 2002, 2005). The structural basis of this divisome is the Z ring, whose structure is the result of the GTP-dependent polymerization of the bacterial-tubulin homolog FtsZ. The cytoplasmic protein FtsA is another divisome-essential protein that is

required for the stabilization of the Z ring and its anchoring to the membrane. Once the Z ring is formed, the remaining divisome component proteins are recruited. FtsQ appears to play a central role in the assembly of the divisome. FtsQ is a bitopic membrane protein consisting of a short cytoplasmic domain, a transmembrane segment, and a periplasmic domain (Scheffers *et al.*, 2007). Two-hybrid analysis has suggested that FtsQ interacts with itself as well as with FtsA, FtsK, FtsX, FtsL, FtsB, FtsW, FtsI, FtsN, and YmgF (Di Lallo *et al.*, 2003; Karimova *et al.*, 2005; D'Ulisse *et al.*, 2007). The interaction of FtsQ with FtsA and FtsK requires its N-terminal domain, whereas all other interactions require only its periplasmic domain (Karimova *et al.*, 2005; D'Ulisse *et al.*, 2007). In addition, the FtsQ transmembrane segment plays a role in FtsQ placement at the division site (Scheffers *et al.*, 2007). Several promoters have been identified within the *ddlB–ftsQA* coding region in *E. coli* and *B. bacilliformis*. In *E. coli*, the high ratio of FtsZ to FtsA and FtsQ is created by the presence of six strong promoters and one weak promoter in the sequence upstream of the *ftsZ* gene, while in *B. bacilliformis* three promoters have been identified in the *ddlB–ftsQA* coding region (Fiskus *et al.*, 2003).

Considering that the translation product of *ddlB* is associated with the biosynthesis of peptidoglycan, the increased exopolysaccharide production in the PG1 mutant is most likely the result of surface-compensation effects accompanying changes in the integrity of the bacterial envelope. Recent results reported by Nagarajan *et al.* (2007) showed an increased expression of genes related to exopolysaccharides and lipopolysaccharide under saline stress in *A. brasilense*. In our case, however, the increase in exopolysaccharide production in the nonpolar mutant affected in *ddlB* did not result in an improved tolerance to salinity.

The *A. brasilense* Cd PG1 mutant showed changes in its cell morphology that were attributed to a polar effect of Tn*5* insertion on the transcription of *ftsQAZ* genes. Strong evidence for this assumption arose from the fact that the nonpolar mutant AL1 recovered a nearly normal bacterial morphology being still altered in exopolysaccharide production. In addition, complementation of the Tn5 (polar) mutant PG1 with a *ddlB* ortholog from *S. meliloti* abolished overproduction of exopolysaccharides, restored wild-type tolerance to SDS, but could not correct the filamentous cell morphology. Likely, Tn*5* insertion interrupts the transcription of *ftsQAZ* from promoters located upstream/within *ddlB*. Several authors have demonstrated that FtsA and FtsZ are required in an adequate ratio for assembling into a ring. Such a ratio of protein species is maintained from the transcription of different promoters located within the *ddlB–ftsQAZ* coding region. Alterations of the FtsZ/FtsA ratio are well known to lead *E. coli* cells to grow in long filamentous chains (Dai & Lutkenhaus, 1992). In our PG1 mutant, the Tn*5* mutation that abolishes promoter activities originated from upstream/within *ddlB* is expected to result in an imbalance in the FtsA : FtsZ ratio and, as a consequence, in altered cell morphology.

The participation of enzymes related to cell-envelope biosynthesis in the adaptation to saline environments has already been demonstrated by Fulda *et al.* (2000) and Nagarajan *et al.* (2007). These authors postulated that alterations in the external cell layers probably establish an enhanced diffusion barrier that reduces the influx of inorganic ions into the periplasm. In *S. meliloti* 1021, Vriezen (2005) identified a chromosomal transcriptional fusion Tn*5luxAB* resulting in a rhizobial mutant that was sensitive to desiccation. The Tn*5luxAB* from this mutant was inserted into an NaCl-inducible ORF encoding a putative D-alanine–D-alanine ligase, supporting a likely active metabolism of peptidoglycan during adaptation of *S. meliloti* to saline environments. Although we do not yet know how *ddlB* transcription under saline stress is modulated, in *A. brasilense* Cd the mutation of the D-alanine–D-alanine ligase in the nonpolar mutant AL1 did not result in changes in the strain tolerance to salinity. The impaired growth under high salt environment in mutant PG1 has thus to be attributed to a deficient expression of *fts* genes and not directly to the *ddlB* mutation.

The results presented described a genetic region associated with the envelope structure in *A. brasilense*, and showed transcriptional coupling between an enzyme of the peptidoglycan biosynthesis (DdlB) and proteins that control cell division (Fts). We have shown that alteration in these activities results in changes in the amount of exopolysaccharides produced, in the bacterial tolerance to saline stress, and in the cell morphology, depending on the kind of genes affected. New studies will be required to understand the molecular basis on which exopolysaccharide production is upregulated in the *ddlB* mutant, as well as the kind of Fts protein whose expression is dependent on upstream *dllB* transcription.

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