Extended phenotype of an *mreB*-like mutant in *Azospirillum brasilense*

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Tn*5* mutagenesis was used to generate an *Azospirillum brasilense* SPF94 mutant. Genetic analysis of this mutant revealed that a homologue of the *mreB* gene, which controls cell shape in *Bacillus subtilis* and *Escherichia coli*, was inactivated. The cell-surface properties of the mutant were different from those of the parental strain. The mutant colonies were highly fluorescent when grown on plates containing Calcofluor White. Light and electron microscopy revealed that the mutant cells were round and had thicker capsules than the spiral parental strain. The mutants contained up to ten times more capsule protein than the parental strain, but lacked a 40 kDa protein that is abundant in the parental strain. The phenotype of the isolated mutant resembled that of the cyst-like differentiated forms of *Azospirillum*, suggesting that the *mreB* homologue could be involved in differentiation.

INTRODUCTION

In rod-shaped bacteria, cell morphogenesis takes place during the cell cycle. Several genes are involved in the maintenance of shape in *Escherichia coli* and *Bacillus subtilis* and mutations of these genes result in a spherical shape. *mreB* is the first gene of the *murein (mre)* gene cluster, mapping at position 73·2 of the *E. coli* chromosome. *mreB* encodes a 37 kDa protein that shows significant sequence similarity with members of the Hsp70 superfamily (Bork *et al.*, 1992). MreB polymerizes to form helical filamentous structures, which are thought to be homologous to eukaryotic actin filaments (Jones *et al.*, 2001; van den Ent *et al.*, 2001). Inactivation of MreB results in the formation of spherical cells. The mutation of *mreB* in *E. coli* results in an increase in FtsI activity. This in turn increases the concentration of PBP3, a penicillin-binding protein, leading to hyperseptation (Wachi & Matsuhashi, 1989). Recently, in *Caulobacter crescentus*, it has been hypothesized that MreB filament structure serves as an organizer for the complex of PB2-peptidoglycan synthesis (Figge *et al.*, 2004). *mreB* homologues have been found in many prokaryotes, including Archaea (Costa & Anton, 1993; Abhayawardhane & Stewart, 1995; Burger *et al.*, 2000), but are absent in constitutively round bacteria (Jones *et al.*, 2001).

Members of the genus *Azospirillum* are capable of nitrogen fixation under microaerophilic conditions in association with the roots of several grasses (Döbereiner, 1991). The vibroid form of *Azospirillum brasilense* has a polar flagellum and is highly motile (Döbereiner & Day, 1976; Tarrand *et al.*, 1978). However, under certain environmental conditions, particularly when inside plant tissue, *Azospirillum* cells become round and non-motile, and are referred to as encapsulated or C-forms (Becking, 1985; Berg *et al.*, 1979; Döbereiner & Day, 1976), or as cysts (Sadasivan & Neyra, 1985, 1987). The cellular envelopes of these forms are thicker than those of vegetative cells (Murray & Moyles, 1987). Polymorphic forms of *Azospirillum* appear in response to different factors, such as medium composition (C/N ratio), pH, age of the culture, polysaccharide production and plant colonization (Bashan *et al.*, 1991; Becking, 1985; Berg *et al.*, 1980; Burdman *et al.*, 2000, 2001; Sadasivan & Neyra, 1985, 1987; Tal & Okon, 1985). *Azospirillum* appears to form several different types of cyst-like cell: pleomorphic cyst-like forms associated with cultured sugarcane-callus tissue and with root colonization

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Abbreviations: CFW, Calcofluor White; PS, polysaccharide; SEM, scanning electron microscopy.

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(Bashan *et al.*, 1991; Berg *et al.*, 1979, 1980; Whallon *et al.*, 1985). Unlike true cysts, these forms are not resting and are able to divide and to fix nitrogen.

Capsular and exocellular polysaccharides (PS) are responsible for the binding of the fluorescent dye Calcofluor White (CFW) to *Azospirillum* cells (Del Gallo *et al.*, 1989; Michiels *et al.*, 1991). These PS and other capsular material are also involved in the interaction between *Azospirillum* and roots (Michiels *et al.*, 1990; Burdman *et al.*, 2000). This process involves the adsorption of bacteria to the root surface and the colonization of the root. A number of bacterial cell-surface proteins and carbohydrates are involved in the attachment to plant surfaces and may be involved in the *Azospirillum*–root interaction. Burdman *et al.* (2001), in particular, isolated a major outer-membrane protein involved in root adhesion and adsorption.

We used Tn5 mutagenesis to create *A. brasilense* mutants with altered CFW binding. We then used these mutants to study the role of exocellular PS in cell behaviour and in morphological differentiation. These mutants had a round morphotype and altered production of capsular components. Cloning and sequencing revealed that the gene responsible for this phenotype was homologous to *mreB*, which is involved in the maintenance of bacterial shape.

METHODS

Bacterial strains, culture conditions and isolation of Tn5 mutants. *A. brasilense* SPF94 (Fani *et al.*, 1988), a spontaneous mutant resistant to rifampicin, was used as the parental strain. This strain and derived mutants were grown in LB (Sambrook *et al.*, 1989), NB (Difco), PY (Antibiotic Medium no. 3; Difco), SMM (PY plus 10% sucrose) or MM (Martinez-Dretz *et al.*, 1984) at 33 °C. *Escherichia coli* HB101 (Boyer & Roulland-Dussoix, 1969), carrying the suicide vector pGS9 (Salvaraj & Iyer, 1983), was grown in LB at 37 °C. Tn5 mutagenesis was carried out as described by Singh & Klingmüller (1986), selecting for resistance to kanamycin. Rifampicin and kanamycin were used at 20 and 40 μg ml^{-1}, respectively; CFW was used at 0·02%. *E. coli* HB101 was also used as control in plant adsorption experiments.

DNA manipulations. *Azospirillum* DNA was extracted by adding SDS and proteinase K, as previously described (Giovannetti *et al.*, 1990). Southern blotting was performed by standard protocols (Sambrook *et al.*, 1989), using as probe the 4·6 kb *Not*I fragment of Tn5 and the *A. brasilense mreB* fragments, labelled with digoxigenin according to the supplier's instructions (Roche).

The 18 kb *Eco*RI fragment of strain SPFE6, containing Tn5, was cloned into pUC18, and kanamycin-resistant (40 μg ml^{-1}) transformants were selected. The resulting construct was named pAFE61. The two *A. brasilense* DNA fragments surrounding Tn5 were subcloned from pAFE61 that had been digested with *Sal*I. The construct containing the 3′ side of Tn5 together with *A. brasilense* DNA (4800 bp) was named pAFE62, and the construct containing the 5′ side of Tn5 together with *A. brasilense* DNA (4800 bp) was named pAFE63. These plasmids were then used to transform competent *E. coli* DH5α. We then sequenced pAFE62 and pAFE63 by use of the M13 forward and reverse primers and four more primers, designed to anneal to the new sequence. The two sequences were assembled. We ensured that they corresponded to the *Azospirillum* genome sequence by amplifying DNA from strain

SPF94 with primers p*mre*Bfor (5′-CGAAGGGGCATTCGTCTAT-3′) and p*mre*Brev (5′-ATCAGTAGGCGCTGACCAAG-3′), which spanned the entire gene, and sequencing the amplified fragment. The sequence of the *mreB* gene was analysed with the MacVector 4.0 software (Life Science Products). Homology analysis was performed using the BLAST database (Altschul *et al.*, 1990, 1997) from the National Center for Biotechnology Information (NCBI). Sequence alignments were obtained with MultAlin (Corpet, 1988), available from the INRA Toulouse website.

To confirm the phenotype of the *mreB* mutant, a second independent *mreB* mutant was constructed by inserting a kanamycin-resistance cassette. The first 500 bp of the *mreB* gene were amplified using p*mre*B(a)for (5′-GAGAGCATGCTGCTTTCCAAACTCCTC-3′) and p*mre*B(a)rev (5′-GAGACTGCAGCCGATGTCCACGACC-3′). The second half was amplified using p*mre*B(b)for (5′-GAGACTGCA-GGGCATCGTCTATTCCC-3′) and p*mre*B(b)rev (5′-GAGAGGAT-CCCGTTTCGCAGGTCTTC-3′). These two fragments were restricted with appropriate enzymes and ligated outside of the kanamycin-resistance cassette (GenBlock; Pharmacia). The whole construction was cloned into pUC18 and used to transform competent *E. coli* DH5α cells. Ampicillin- (100 g ml^{-1}) and kanamycin- (40 μg ml^{-1}) resistant colonies were selected, and the plasmid named pUC*mreB*5. The *mreB* mutagenesis cassette was then transferred into the conjugative plasmid, pSUP202 (Simon *et al.*, 1983), yielding pSUP*mreB*6 (Cm^r, Amp^r, Kan^r, Tet^s). pSUP*mreB*6 was used to transform streptomycin-resistant *E. coli* S17.1 cells (Simon *et al.*, 1983). Finally, pSUPmreB6 was conjugated to *A. brasilense* SPF94 (ampicillin and chloramphenicol sensitive), as described by Singh & Klingmüller (1986). Double crossing-over events generated kanamycin-resistant, tetracycline-sensitive mutants. We used Southern blotting and PCR to check that the kanamycin cassette was correctly inserted within the *mreB* gene.

Cell-surface characterization

Cell aggregation. Cells were grown in LB to stationary phase (OD$_{600}$=1·6), and then observed under a light microscope. The number of microaggregates (more than eight cells) present in 10 microscopical fields (400× magnification) was counted 5 and 10 minutes after laying a drop of the culture on the microscope slide.

Cell dispersion. Cells were grown in NB to mid-log (OD$_{600}$=0·8) or to stationary phase (OD$_{600}$=1·6), and then harvested by centrifugation. We measured the vortexing time necessary to resuspend the resulting pellet in PBS buffer (Del Gallo *et al.*, 1989), as described by Arunakumari *et al.* (1992). Dispersion was monitored by measuring OD$_{600}$ in a spectrophotometer.

Cohesiveness and autoagglutination. Cells were grown in NB to late-log (OD$_{600}$=1·2) or to stationary phase (OD$_{600}$=1·6). The cells were harvested and resuspended in PBS. The sedimentation rate of the cells was measured by monitoring changes in OD$_{600}$ (Arunakumari *et al.*, 1992). The net electrical charge of cells was determined by electrophoresis, as described by Sakai (1986). The effect of differences in cell-surface electrical charges was assayed by measuring the sedimentation rate of stationary-phase cells that had been resuspended in 0·1 M phosphate buffer at pH 4 and at pH 10 (Sakai, 1986).

Hydrophobicity. Hydrophobicity was determined by measuring the adhesion of a cell pellet to tetradecane (Rosemberg, 1984).

Staining. Cells were stained with Alcian Blue and with Congo Red, as previously described (Del Gallo *et al.*, 1989; Forni *et al.*, 1992). Both stains are known to bind exocellular PS: Alcian Blue stains acidic PS, Congo Red all neutral PS.

Epifluorescence microscopy. Cells were grown for 24 and 48 h on MM-fructose or MM-succinate (both 5 g l^{-1}). After staining with CFW, they were observed under an epifluorescence microscope (Leitz Dialux 20, equipped with an excitation filter A2, 270–380 nm).

Electron microscopy. Transmission electron microscopy (TEM) samples were prepared as follows: bacterial cells were grown up to mid-exponential phase (OD$_{600}$ = 0·8) in LB, collected by centrifugation, embedded in 4 % agar, fixed for 1 h with 4 % glutaraldehyde in 0·2 M phosphate buffer (pH 7·2), washed with the same buffer, and post-fixed for 2 h in 2 % OsO$_4$. The samples were then dehydrated in ethanol and embedded in Epon-Araldite (Fluka), according to standard procedures. Sections were made with an LKB IV ultramicrotome. The sections were stained with uranyl acetate and lead citrate, and observed with a Philips 201-C electron microscope working at 80 kV.

Scanning electron microscopy (SEM) samples were prepared as follows: a drop of cell culture grown in LB up to mid-exponential phase (OD$_{600}$ = 0·8) was spread on polycarbonate membranes (0·6 μm pore size; Millipore), fixed for 1 h with 4 % glutaraldehyde in 0·2 M phosphate buffer (pH 7·2), washed with the same buffer and dehydrated through an alcohol series, finishing with absolute alcohol. The samples were then critical-point-dried with a Blazer CPD 030, mounted on stubs, coated with carbon and gold, and examined with a Philips 515 scanning electron microscope.

Capsule extraction and analysis. Cells were grown for 48 h in MM, then washed and resuspended in one-tenth the volume of PBS and stored at 4 °C for 3 days until the capsule had dissolved (Del Gallo & Haegi, 1990). The cells were centrifuged at 8000 *g* for 10 min. The supernatant, which contained the dissolved capsular material, was collected, and total PS content was determined by the Anthrone method, using glucose as a standard (Dische, 1962). The protein concentration was determined either by the method of Lowry *et al.* (1951) or by a derived protein assay (DC Protein Assay; Bio-Rad). Protease inhibitors, 3 mM Pefabloc (Roche) and 1·3 mM EDTA, were added to the capsular material.

Capsular material was dialysed against HPLC-grade water and analysed by HPLC after PS hydrolysis, as described previously (Del Gallo & Haegi, 1990). Quantitative analyses were carried out by use of the 'two-point absolute titration curve' described in the Shimatzu Chromatopac C-3A manual, with Bio-Rad Aminex HPX87C and Aminex HPX87H columns.

SDS-PAGE was carried out as described by Laemmli (1970) with a 4 % stacking gel and a 10 % resolving gel. The separated proteins were either stained with Coomassie Brilliant Blue R250 or electroblotted onto PVDF membranes (Immobilon; Millipore) by use of a semi-dry electroblotting apparatus (Hoefler; Pharmacia). Molecular mass markers were purchased from Sigma.

The blots were stained with Coomassie Brilliant Blue R250. Proteins of interest were excised and subjected to sequence analysis by automated Edman degradation, using a Perkin Elmer AB476 gas-phase sequencer.

Glycoproteins were detected either on Alcian Blue-stained (1 % ethanol : water) SDS-PAGE gels or on blots stained with specific DIG-labelled lectins (DIG Glycan Detection Kit; Roche).

Effect of lysozyme and temperature

Lysozyme. Bacteria were grown to the end of the exponential phase (OD$_{600}$ = 1·2) in MM supplemented with 10 mM NH$_4$Cl. They were then washed in 50 mM Tris/HCl, pH 7·5, and incubated in the same buffer plus 0·5 % Triton X-100 for 30 min. Cells were centrifuged and resuspended in SMM, pH 5·5, with 1 μg lysozyme ml^{-1} and 4 % EDTA. Aliquots were taken at different time points. The

aliquots were diluted in SMM (isotonic) or water (hypotonic) and plated out on PY agar plates. Viable cells were counted after incubation for 2 days at 33 °C.

Temperature. Cells grown in MM or NB were treated up to 60 °C, following the procedure described by Matsuzawa *et al.* (1972).

Nitrogen metabolism. Nitrogenase activity was assayed by the acetylene reduction method, and nitrate utilization was assayed using cells grown anaerobically in the presence of 25 mM KNO$_3$, as previously described (Bani *et al.*, 1980).

Adsorption of *A. brasilense* strains to plant surfaces. Bacteria were adsorbed to wheat roots, as described by Michiels *et al.* (1990), with the following modifications: cultures of *A. brasilense* SPF94, *A. brasilense* SPFE6 and *E. coli* HB101 (as negative control) were labelled by inoculating 4 ml LB medium supplemented with 8 μCi AA^{3}H (Tritiated Amino Acid Mixture; Amersham Pharmacia) with a single colony. This culture was grown to OD$_{600}$ = 1·2 (about 5 × 10^8 c.f.u. ml^{-1}). The cells were then washed twice in saline solution (0·85 % NaCl) and resuspended in 4 ml MM medium to a final concentration of 10^7 bacteria ml^{-1}. Ten-day-old sterile wheat-seedling roots (*Triticum aestivum* cv. Aurelio) were cut into 2 cm pieces. Five pieces were added to each tube of MM. The negative control consisted of tubes containing the same amount of heat-killed SPF94 (15 min at 80 °C). After 1 h at 33 °C in a rotary shaker at 65 r.p.m., the roots were washed three times in 10 ml saline solution, and the associated radioactivity was measured in a liquid scintillation counter. The percentage of the total added radioactivity remaining on the roots was calculated.

Potato cells isolated from callus were cultured on LS medium (Linsmaier & Skoog, 1965) to a density of 1 × 10^5 cells ml^{-1}, and filtered through a nylon filter (200 μm pore size) every ten days. The bacteria were grown on LB at 30 °C up to 5 × 10^8 c.f.u. ml^{-1}. The bacteria were then washed with saline solution and added to the potato-cell suspension at a final concentration of 3 × 10^5 ml^{-1}. The mixture was incubated for 1 h at 30 °C with gentle shaking, filtered through a nylon filter (10 μm pore size), washed with two volumes of saline solution and ground in a mortar. Disrupted cells were diluted in saline solution, plated on LB plates and potato-cell-bound bacteria were counted.

RESULTS

Isolation of mutant

A. brasilense SPF94 mutants were obtained after Tn5 mutagenesis using pGS9 as a suicide vector. About 5000 kanamycin-resistant mutants were assayed on MM agar plates containing CFW. Macroscopic examination under UV light revealed five colonies with altered fluorescence. One of these mutants, SPFE6, was much more fluorescent than the parental and the other mutant strains and was thus chosen for further characterization.

We used Southern blotting with a Tn5 probe to ensure that the mutant contained Tn5. When total SPFE6 DNA was digested with *Eco*RI (which does not cut the transposon sequence), a single 18 kb band was observed (not shown). Double digests with *Eco*RI and *Sal*I (which cuts Tn5 once) produced two bands of approximately 7 and 11 kb. Thus, SPFE6 only contains one copy of the transposon within a 13 kb *Eco*RI fragment.

Sequence editing

Sequence analysis showed that the 2012 bp nucleotide sequence surrounding Tn5 contained an ORF (from nt 697, ATG start codon, to nt 1819, TGA stop codon). The 374-amino-acid sequence was highly homologous to the MreB-like proteins of *Magnetospirillum magnetotacticum* (identity 90 %, similarity 93 %, accession no. ZP00055538), *Caulobacter crescentus* (identity 72 %, similarity 84 %, accession no. NC002696) and *Rickettsia prowazekii* (identity 71 %, similarity 82 %, accession no. NC000963). The sequence of the *A. brasilense mreB*-like gene was submitted to the NCBI database (accession no. AF438483). No recognizable promoter sequences were found upstream of the ATG start codon. The conserved domain BLAST analysis program revealed two domains typical of MreB proteins: the HSP70-like domain corresponding to the first 200 amino acids and the FtsA-like domain corresponding to amino acids 140 to 320.

The phenotype of SPFE6 was highly pleiotropic; thus, to exclude the possibility that this pleiotropy was due to secondary mutations that accidentally occurred during Tn5 mutagenesis, we constructed two new *mreB* mutants using the Kan cassette. All of the characteristics described below were identical in the new mutants and in SPFE6.

Cell-surface properties

We compared the cell-surface properties of SPFE6 with those of the parental strain. The aggregation assay demonstrated that the mutant strain was four to five times more likely to form microaggregates than the parental strain. Accordingly, SPFE6 cells in stationary phase dispersed more slowly than the parental cells (data not shown). The hydrophobicity test and the net-electrical-charge assay did not show any significant differences between the mutant and parental strains, both of which were negatively charged (data not shown). The cohesiveness assay suggested that there may be differences in surface electrical charge at different pHs. The mutant strain agglutinated after long incubation at low pHs; in particular, after 7 h at pH 4·0 the OD_{600} of the mutant suspension was $0·25 \pm 0·1$, while that of the parental strain was $1·4 \pm 0·2$.

Staining also revealed some differences between the parental and mutant strains. Both strains bound comparable amounts of Congo Red, but the parental strain bound much more Alcian Blue than the mutant (data not shown), indicating that the mutant produces a larger amount of acidic PS.

Capsule composition

Quantitative analysis showed some clear differences in the composition of the capsule in the mutant and parental strains. There was up to tenfold more capsule protein in the mutant than in the parental strain, whereas both strains contained similar amounts of PS (Table 1). HPLC analysis (Table 1) showed that the mutant contained twofold more

Table 1. Glycocalyx composition of *A. brasilense* strains SPF94 and SPFE6

Characteristic	Strain	
	SPF94	SPFE6
Total PS	5·30*	2·80*
Glucose	1·19*	0·75*
Galactose	0·74*	0·95*
Rhamnose	0·76*	0·81*
Fucose	0·88*	1·91*
Total protein	38·00*	141·00*
Proteins per cell	$7·6 \times 10^{-8}$	$57·6 \times 10^{-8}$
PS per cell	$1·1 \times 10^{-8}$	$1·1 \times 10^{-8}$
c.f.u. ml^{-1}	$5·0 \times 10^{8}$	$2·5 \times 10^{8}$
Cell dry weight	0·52†	0·33†

*Data expressed in mg (ml culture)$^{-1}$. Each value is the mean of three replicates. Fucose and total protein were the only characteristics that differed significantly (*t* test) between the two strains.
†Data expressed in mg ml^{-1}; difference not significant.

fucose than the parental strain, whereas both strains contained similar amounts of the other monosaccharides.

SDS-PAGE and blotting of the capsular proteins (Fig. 1) showed that the parental strain contained a reduced number of proteins compared with the mutant. The parental strain contained five distinct proteins of about 250, 100, 40, 32 and 30 kDa; the 40 kDa one being the most abundant. The mutant strain displayed a more heterogeneous pattern, without any distinctive proteins: the 250, 100 and 32 kDa proteins were less abundant, the 40 kDa protein was almost undetectable, and additional bands of between 60 and 100 kDa and between 35 and 45 kDa were present.

Glycoprotein patterns obtained with Alcian Blue or lectin staining confirmed the difference between mutant and parental strains. As mentioned above, the mutant strain was characterized by a lower capsular PS/protein ratio (Table 1). Thus, more mutant capsular protein was loaded to enable us to compare the proteins obtained from an equivalent number of cells. Alcian-Blue staining revealed three main glycoproteins in the parental strain (Fig. 1) at 250, 100 and 40 kDa. The mutant contained less of the 250 and 100 kDa proteins and did not contain the 40 kDa protein, whereas additional lower-molecular-mass glycoproteins were present. Similar results were obtained with the *Sambucus nigra* agglutinin (SNA), which is specific for sialic acid-containing proteins (Fig. 1). However, this stain did not detect the two largest proteins (250 and 100 kDa), indicating that they do not contain sialic acid residues.

The N-terminal sequence of the 40 kDa protein, which is specific to SPF94, was TTSSGINGQT. This sequence was compared with the SWISS-PROT protein databases by use of a BLAST search and found, in the absence of the signal peptide, to be 70 % identical to the N-terminal sequence
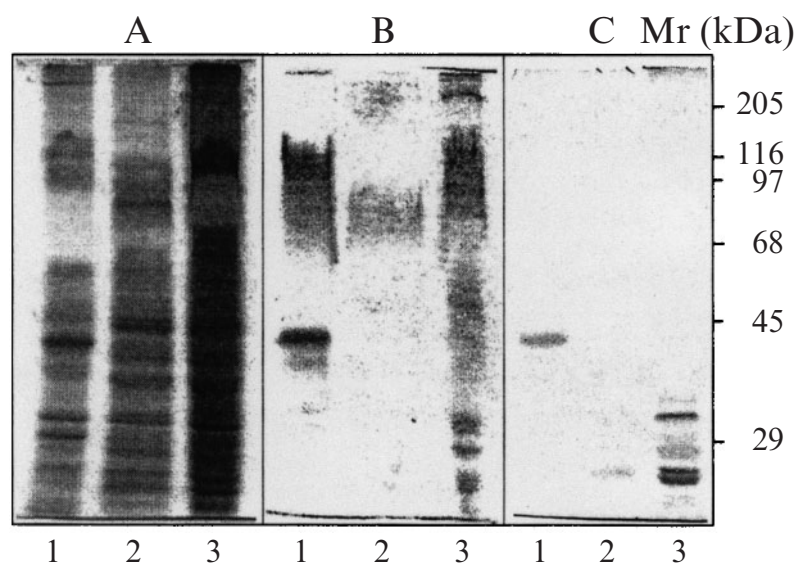
Fig. 1. SDS-PAGE analysis of capsular proteins obtained from SPF94 (lane 1) and SPFE6 (lanes 2 and 3). Panel A, Coomassie Brilliant Blue staining; panel B, Alcian Blue staining; panel C, *Sambucus nigra* agglutinin staining with the lectin detection kit (Boehringer) after gel transfer to Immobilon-P membrane. The same amount of protein was loaded in lanes 1 and 2, whereas seven times more protein was loaded in lane 3 to enable a comparison of the proteins obtained from an equivalent number of cells.

(FTSSGTNGKV; Labigne-Reussel *et al.*, 1985) of the afimbrial adhesin AFA-1 of uropathogenic *E. coli*.

Adsorption of *A. brasilense* strains to plant surfaces

The lack of an adhesin-like protein suggested that the mutant could be impaired in the capability to attach to plant surfaces. Thus, we compared the ability of the parental and mutant strains to adsorb to wheat roots and to a suspension of cultured potato cells. The adsorption capabilities of the two strains did not differ significantly (Table 2). Between six and nine times fewer *E. coli* cells adsorbed. This is comparable to the data obtained with the heat-killed *A. brasilense* cells. This suggests that a specific and active process is involved, and that this process does not involve the 40 kDa surface adhesin-like protein lacking in the mutant.

Table 2. Adsorption of *A. brasilense* and *E. coli* to plant roots and cells

Values show the percentage of the total added bacteria. Each value is the mean±SD of six measurements from two independent experiments with three replicates. ND, Not determined

Strain	Bacteria bound to:	
	Wheat roots	**Potato cells**
A. brasilense SPF94	11·1±2·5	2·3±0·5
A. brasilense SPFE6	15·2±3·1	1·8±0·3
E. coli HB101	1·6±0·5	0·3±0·1
A. brasilense SPF94, heat killed*	2·1±0·3	ND

*After labelling, bacteria were treated at 80 °C for 15 min before the binding assay.

Microscopy

Microscopy also revealed the phenotypic differences between the parental and mutant strains: SPFE6 cells were round, instead of spiral shaped (Fig 2A, B). Furthermore, the mutant cells appeared to be less motile. The mutant still formed cysts (larger cells), although these appeared at an earlier stage than in the parental strain. Epifluorescence microscopy confirmed that fewer parental strain cells bound to CFW (Fig. 2C, D). This is consistent with the finding that the mutant colonies were more fluorescent on CFW plates (data not shown).

Electron microscopy (Figs 3 and 4) further confirmed the remarkable differences in the cell shapes of the mutant and parental strains. SEM (Fig. 4) clearly demonstrated that the mutant cells were round, divided quite well and, despite being less motile, possessed an apparently normal polar flagellum. SEM also showed that the surface of the mutant cells was more compact and more dense than that of the parental strain. Furthermore, the external capsule of the mutant was thicker than that of the parental strain (Fig. 3). Some mutant cells contained considerably more poly-β-hydroxybutyrate granules (Fig. 3C) than the parental strain and encapsulated forms, which contained multiple central bodies (Fig. 3A).

Other characterizations

To determine whether the phenotype of SPFE6 is associated with thermal resistance, as is the case for *Azospirillum* cysts, we compared the resistance of SPFE6 cells with that of SPF94. Our results (data not shown) indicated that survival after heat treatment was identical in the parental and mutant strains.

The round shape of the mutant could also be due to the impaired synthesis of cell-wall components, giving rise to a kind of spheroplast. To test whether the cell wall of the
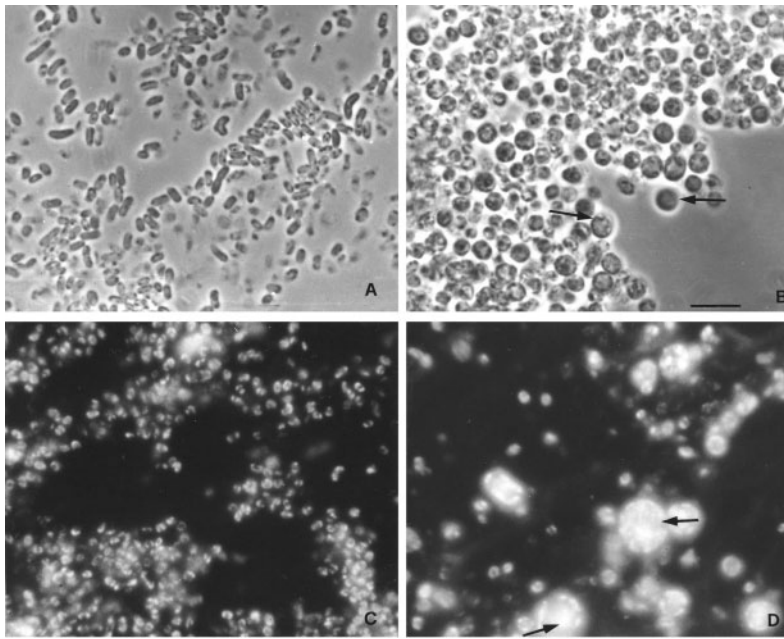
Fig. 2. Normal light (A, B) and epifluorescence (C, D) microscopic observation of *A. brasilense* SPF94 (A, C) and *A. brasilense* SPFE6 (B, D) cells stained with CFW. Arrows indicate cystic forms. Cells in A and B were grown in liquid media; cells in C and D on agar.

mutant was altered, we tested the sensitivity of the peptidoglycan layer to lysozyme. Our results (Fig. 5) clearly demonstrated that mutant cells were more resistant to lysozyme and to osmotic shock than the parental strain. Therefore, the rounded shape is not due to an impairment in the cell wall. In fact, the higher level of resistance could be related to the thicker envelope structure, as revealed by electron microscopy. This thick envelope may somehow prevent lysozyme from reaching the mutant cell wall.

We also showed that the mutation did not affect nitrogen metabolism (data not shown), as the parental and mutant strains were both equally able to fix atmospheric nitrogen and to grow on nitrate as sole nitrogen source.

DISCUSSION

We isolated and characterized an *A. brasilense* mutant with altered morphology and an altered cell surface. The
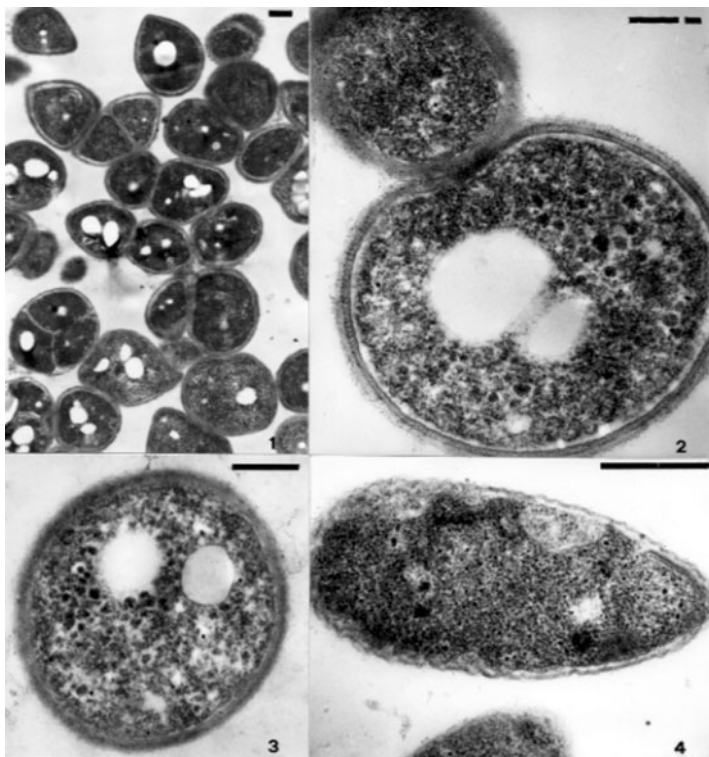

Fig. 3. Transmission electron micrographs of thin sections of *A. brasilense* strain SPFE6 (1, 2 and 3) and SPF94 (4). Bars, 0·5 μm.
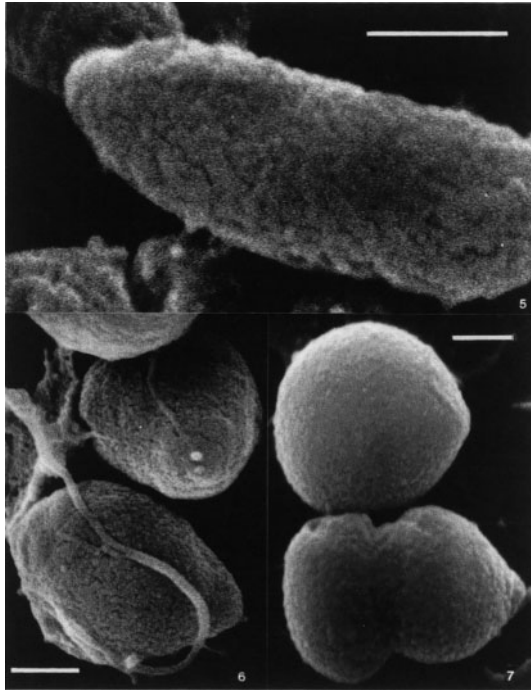
Fig. 4. Scanning electron micrographs of *A. brasilense* strains SPF94 (5) and SPFE6 (6 and 7). Bars, 0·5 μm.

Fig. 5. Effect of lysozyme treatment on survival of *A. brasilense* SPF94 and SPFE6. *x* Axis, time of exposure to 1 mg lysozyme ml^{-1}; *y* axis, log number of colony forming units recovered. SMM, diluted in SMM (isotonic) and plated out on PY agar plates; water, diluted in water (hypotonic). Viable cells were counted after incubation for 2 days at 33 °C.

insertion of Tn*5* into the *mreB* homologue is responsible for the pleiotropic phenotype of this mutant.

Although the shape of the mutant cells was similar to that of *E. coli mreB* mutants (Labigne-Reussel *et al.*, 1985; Matsuzawa *et al.*, 1972), the *A. brasilense* phenotype seemed to be more complex. The phenotype of SPFE6 cells was in many ways reminiscent of the differentiated 'cystic' forms that occur in cultures of *A. brasilense*. After particular environmental changes, *Azospirillum* species undergo a series of physiological modifications, including changes in the capsular composition (Sadasivan & Neyra, 1987). This in turn affects the ability to bind to CFW (De Troch *et al.*, 1992) and leads to changes in poly-*β*-hydroxybutyrate accumulation (Tal & Okon, 1985), cell shape and motility, while other metabolic functions become less active, tending to dormancy (Bastarrachea *et al.*, 1988; Becking, 1985; Sadasivan & Neyra, 1987). All of these changes eventually result in the formation of cyst-like forms that are larger, round and more resistant to high temperature and desiccation (Sadasivan & Neyra, 1985). Interestingly, the cells of many strains of *Azospirillum* form cyst-like structures when they colonize roots or other plant tissues (Bashan *et al.*, 1991; Berg *et al.*, 1979, 1980; Madi *et al.*, 1989; Michiels *et al.*, 1989; M. Grilli Caiola and others, unpublished results.). However, this process has been described in a rather fragmented manner, with phenotypes indiscriminately described as 'cysts', 'cyst-like forms', 'coccoid forms' (C-forms), etc., some related to stress
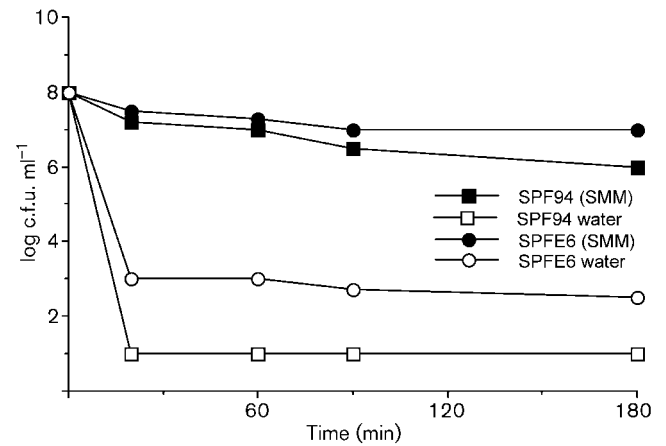
resistance, others associated with the colonization of plant tissues.

Genetic studies have improved our knowledge of the encystment of *Azospirillum* cells. Some mutants with altered encystment have been isolated and characterized (De Troch *et al.*, 1992; Michiels *et al.*, 1989, 1990). However, the relationships between these mutations and encystment have not been assessed, with the possible exception of the *enc* mutants (Bastarrachea *et al.*, 1988). Examination of the Sp7-S mutant described by Katupitiya *et al.* (1995) and Castellanos *et al.* (1997) revealed a relationship between the surface properties of the bacterium, its shape, and its ability to colonize plants. This mutant stained weakly with Congo Red, did not flocculate in the presence of fructose or nitrate, lacked the thick exopolysaccharide layer, had the same general nutritional properties and growth rate as the wild-type and had the same vibroid morphology and motility as the wild-type. Like Sp7-S, SPEF6 displayed a highly pleiotropic phenotype with respect to capsular protein production, Calcofluor staining abilities, aggregation in liquid cultures, resistance to lysozyme and resistance to osmotic shock.

Many of the cell-surface properties of the mutant strain SPEF6 differed from those of the parental strain: the mutant aggregated more readily than the parental strain, and its surface components bound much more CFW and less Alcian Blue. The study of *A. brasilense* 7030 mutants (Michiels *et al.*, 1989, 1990, 1991) also indicates a relationship between surface properties and aggregation. These mutants were Calcofluor dark and had lost the ability to form flocs in liquid cultures, and their exopolysaccharides were more acidic than those of the parental strain. These features were

similar, though different, to those of SPFE6. Mutants of *A. brasilense* strain Sp7, recently described by Burdman *et al.* (2000), showed instead a positive correlation between exopolysaccharide composition and aggregation. The monosaccharide composition of the SPF94 capsules was virtually identical to that of SPFE6 (Table 1), with the exception of fucose content, although the significance of this to the mutant phenotype is unclear. The protein composition of the capsular extracts, on the other hand, was quantitatively and qualitatively different in the two strains.

The larger amount of protein in the mutant extract may be related to an altered membrane permeability, resulting in the export of cytoplasmic proteins. As a further pleiotropic effect, the mutant lacked a specific glycosylated 40 kDa protein that is normally present in other related species. A 40 kDa protein has in fact been found in the capsular protein preparation of *Azospirillum lipoferum* strains grown under different conditions (E. G. Biondi and others, unpublished results), and also in *A. brasilense* Sp7 (Schloter *et al.*, 1994). Whether this protein was absent in the SPF94 mutant because it is not synthesized or because it is rapidly digested by proteases, we cannot decide on the basis of present data. The N-terminal sequence of the 40 kDa protein showed homology to the *E. coli* afimbrial adhesin AFA-1. Adhesins are extracellular proteins responsible for the attachment of bacteria to different surfaces, including animal (Hultgren *et al.*, 1996) and plant (Romantschuk, 1992) tissues. However, we cannot reach any conclusions on the role of this protein, since two adsorption assays both demonstrated that the parental and mutant strains did not differ greatly in this characteristic.

MreB is a structural protein, governing the shape of *B. subtilis* cells. The phenotype of the *Azospirillum mreB*-like mutant suggests that the inactivation of this gene also affects other functions: MreB may be involved in the definition of surface properties, and more generally in the control of cellular differentiation. The mutant constitutively showed some of the features of differentiated *Azospirillum* cells: it was round, produced large amounts of exocellular material, formed large aggregates and had multiple encapsulated cells containing abundant poly-*β*-hydroxybutyrate granules even during exponential growth. The mutant cells (metabolically active, actively dividing and able to fix nitrogen) resembled some of the forms observed in association with plants, instead of the resistant cyst-like forms. Thus, *mreB* may always be active during the vegetative growth of *Azospirillum* cells, and may be repressed when environmental conditions signal that it is time to start to differentiate. Though the absence of ORFs immediately downstream of *mreB* suggests that it is a single gene, we cannot exclude that the insertion affected the expression of other genes associated in the same putative operon of *mreB*, resulting in a sort of polar effect. Whatever its mode of action, the mutation in the *mreB*-like gene is responsible for a number of important phenotypic effects in *Azospirillum*, particularly in functions related to plant interaction, and should be further characterized.

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