Aggregation in *Azospirillum brasilense*: effects of chemical and physical factors and involvement of extracellular components

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A medium for consistent induction of aggregation of *Azospirillum brasilense* cells was developed and used to study the effects of chemical and physical factors as well as extracellular components involved in this phenomenon. Growth of *A. brasilense* strain Cd in a high C:N medium using fructose and ammonium chloride as C and N sources, respectively, resulted in flocculation visible to the naked eye after 24 h. No cell aggregates were formed after 72 h growth in low C:N medium. Aggregating cells, but not cells grown under low C:N, accumulated high amounts of poly- β -hydroxybutyrate and the cell envelope contained a well-defined electron-dense layer outside the outer membrane. Suspending the aggregates in 0.2 or 0.5 M urea was the only treatment effective for disrupting aggregates. The concentration of exopolysaccharide produced by four different strains of *A. brasilense*, differing in their capacity to aggregate, strongly correlated with the extent of aggregation. Electrophoretic protein profiles from different fractions of aggregating and non-aggregating cells were compared. Differences were observed in the pattern of low-molecular-mass proteins and in the polar flagellin that has previously been proposed to be involved in adhesion processes. However, a mutant lacking both lateral and polar flagella showed the strongest aggregation. The involvement of polysaccharides and/or proteins in aggregation of *A. brasilense* is discussed.

Keywords: *Azospirillum brasilense*, aggregation, flocculation, exopolysaccharides

INTRODUCTION

The free-living N₂-fixing rhizobacteria of the genus *Azospirillum* live in close association with plant roots, where they exert beneficial effects on plant growth and yield of many crops of agronomic importance (Okon & Labandera-Gonzalez, 1994).

Cell aggregation is a widespread phenomenon in the microbial world. It can be defined as the gathering of cells to form fairly stable, contiguous, multicellular associations, occurring under certain physiological conditions (Calleja, 1984). Free-living N₂-fixing bacteria such as *Azospirillum*, *Klebsiella* and *Azotobacter* are known for their capacity to aggregate and flocculate; this property may positively affect their dispersal and

survival in soil (Nur *et al.*, 1981; Sadasivan & Neyra, 1985; Madi *et al.*, 1988; Madi & Henis, 1989). Along with the fact that flocs can be produced on a large scale and separated easily from the growth medium, the phenomenon of bacterial aggregation is of great interest in the production of bacterial inoculants. Neyra *et al.* (1995) proposed the generation of inoculants containing flocs of *Azospirillum* and *Rhizobium* for common bean plants.

The bacterial surface plays an important role in the establishment of bacterial–plant associations as well as in bacterial aggregation (De Troch & Vanderleyden, 1996). Data suggesting the involvement of extracellular polysaccharides in *Azospirillum* aggregation have been published. The production of surface polysaccharides by *Azospirillum* has been demonstrated by growing colonies on media containing the fluorescent dye calcofluor, which binds predominantly to 1,4- β and 1,3- β

Abbreviations: EPS, exopolysaccharide; PHB, poly- β -hydroxybutyrate; SEM, scanning electron microscopy; TEM, transmission electron microscopy

linked glucans (Wood & Fulcher, 1978). Michiels *et al.* (1990, 1991) showed that non-fluorescent mutants have lost the ability to form flocs and to anchor to wheat roots, indicating that the calcofluor-binding polysaccharide may be necessary for flocculation and anchoring to roots. Sadasivan & Neyra (1985) related floc formation in *A. brasilense* Sp7 and *A. lipoferum* to β -linked exopolysaccharides. It has recently been found that a spontaneous mutant of Sp7, which forms colonies that stain weakly with Congo Red, does not flocculate and lacks cell-surface materials found in the parental strain (Katupitiya *et al.*, 1995).

Electron microscopy studies on aggregation of *Azospirillum* revealed the presence of an extracellular layer (Madi *et al.*, 1988). Treatment of cells with EDTA resulted in the dispersal of aggregates, and addition of the dialysed EDTA extract to the treated bacterial cells restored their aggregation capacity. This evidence indicates the possibility of the involvement of proteins in this phenomenon (Madi & Henis, 1989). Direct evidence for the involvement of extracellular proteins in the adhesion process of *Azospirillum* to abiotic surfaces has been provided (Dufrêne *et al.*, 1996a, b; Dufrêne & Rouxhet, 1996). Extracellular proteins were suggested or demonstrated to directly participate in aggregation of various bacterial species from diverse environments (Eggset *et al.*, 1983; Calleja, 1984; Reniero *et al.*, 1992; Wang *et al.*, 1994; Marsh & Bradshaw, 1995; Ma *et al.*, 1996; Wai *et al.*, 1996).

Azospirillum cells are able to aggregate, leading to the formation of visible flocs under diverse stress conditions (Bashan & Holguin, 1997). Following transfer from a rich medium to a N-limited medium (low and high C:N, respectively), a switch from a dispersive growth pattern to a mode of increased aggregation, clumping and flocculation occurs. A protocol including repeatable and measurable values of aggregate formation and aggregation intensity, respectively, was developed. This system was used to study the nature of the molecular interactions that occur between aggregating cells. In this work, we studied the effects of various chemical and physical factors on the stability of aggregates, and focused on the differential aggregation capacity of various strains of *A. brasilense* to systematically compare biochemical fractions in the search for the 'aggregation principle'.

METHODS

Bacterial strains and growth conditions. *Azospirillum brasilense* wild-type strains Cd (Eskew *et al.*, 1977) and Sp7 (Tarrand *et al.*, 1978), and mutant strains FAJ0204 (a Tn5 mutant of Sp7 defective in the production of both lateral and polar flagella; kindly supplied by J. Vanderleyden, Catholic University of Leuven, Belgium) and Sp72002 (a pleiotrophic Tn5 mutant of Sp7 affected in its aggregation capacity; kindly supplied by C. Elmerich, Institut Pasteur, Paris, France) were maintained on nutrient agar (Difco) slants. Kanamycin was added at $50 \mu\text{g ml}^{-1}$ for the Tn5 mutant strains. For aggregation assays and extracellular material extraction, bacteria were grown in 250 ml Erlenmeyer flasks, in 100 ml liquid

medium. Aggregation-inducing medium (high C:N) contained (g l^{-1}) D-fructose (6.67), MgSO_4 (0.2), NaCl (0.1), CaCl_2 (0.02), K_2HPO_4 (6.0), KH_2PO_4 (4.0), yeast extract (Difco) (0.1), NH_4Cl (0.214) and microelements as described by Okon *et al.* (1977). Low C:N medium contained the same components, but with NH_4Cl at 0.963 g l^{-1} . Liquid media were adjusted to pH 6.8. Flasks were inoculated with exponential-phase cultures to an initial OD_{540} of approximately 0.05 (about 10^7 c.f.u. ml^{-1}) and incubated on a rotary shaker (150 r.p.m.) at 30°C .

Scanning electron microscopy (SEM). *A. brasilense* Cd cells were washed by centrifugation (4000 g, 10 min, twice), resuspended in 10 mM phosphate buffer (pH 7.2), fixed in 2% (v/v) glutaraldehyde for 3 h, dehydrated through a graded ethanol series and critical-point dried. Dried bacteria were coated with gold by a diode sputtering system (Polaron E5000) and observed with a JEOL JSM-35C scanning electron microscope.

Transmission electron microscopy (TEM). Bacteria were washed by centrifugation (4000 g, 10 min, twice), resuspended in 10 mM phosphate buffer (pH 7.2), fixed in 3% (v/v) glutaraldehyde for 3 h, postfixed in 1% (w/v) OsO_4 for 2 h and rinsed three times in 50 mM sodium cacodylate buffer (pH 7.2). Rinsed bacteria were then dehydrated through a graded ethanol series and embedded in a low-viscosity epoxy resin. Samples were dried at 60°C for 24 h. Thin sections were cut with a diamond knife on an 8800 ultramicrotome (LKB), stained with uranyl acetate and lead citrate, and examined on a JEOL 100-CX transmission electron microscope.

Quantitative measurement of aggregation. The extent of aggregation was measured during growth or following different treatments according to Madi & Henis (1989), with some modifications. Aliquots of liquid culture containing aggregates were transferred to a conical tube and allowed to stand. After 20 min, aggregates had settled to the bottom of the tube and the suspension was mostly composed of free cells. Turbidity was measured from the suspension using a Genesis 5 spectrophotometer (Spectronic Instruments) at 540 nm (OD_s). The culture was then dispersed by treatment in a tissue homogenizer (Heidolph RZR 50) for 1 min and the total turbidity was measured (OD_t). The percentage aggregation was estimated as follows: % aggregation = $[(\text{OD}_t - \text{OD}_s) \times 100] / \text{OD}_t$.

Effects of chemicals and pH on the stability of aggregates. Bacteria were grown for 24 h in a high C:N medium, washed by centrifugation (4000 g, 10 min, twice) and resuspended in a saline solution (0.9%, w/v, NaCl). Aggregates were dispersed by homogenization and bacteria were transferred to 10 vols treatment solution. Chemical treatments were applied at different concentrations in 10 mM phosphate buffer (pH 7.0). For pH experiments, washed bacteria were resuspended in citrate/phosphate buffer (0.1 M citric acid, 0.2 M Na_2HPO_4 , pH 3–7) or in 0.1 M Tris/HCl buffer (pH 8 and 9). The extent of aggregation was measured as described above, after shaking for 30 min (150 r.p.m., 30°C) at the various selected pH levels or at different times of incubation for the chemical treatments.

Isolation of proteins from different fractions. Proteins were isolated from different fractions of bacteria grown for 24 h, to compare protein profiles between strains and between the two types of media (low and high C:N). Bacteria were pelleted by centrifugation (4000 g, 20 min). The two fractions tested were the supernatant (growth medium) and the pelleted bacteria. To remove proteins associated with the cell surface, the pellet was resuspended in 5 ml water and passed five times through a $25\text{G} \times 5/8$ inch needle or sonicated for 20 s, then recentri-

fuged (4000 g, 20 min). Outer-membrane proteins were extracted according to Schlöter *et al.* (1994). All fractions were filtered through a 0.22 µm membrane and dialysed against distilled water for 24 h at 4 °C, using a dialysis membrane with a molecular-mass cut-off of 10 kDa.

Gel electrophoresis. Proteins were separated by electrophoresis in SDS-polyacrylamide gels (12 %, w/v, acrylamide) and stained with Coomassie brilliant blue G250, using standard methods (Hancock & Carey, 1979). The molecular masses of the proteins were estimated using low-range prestained standards (Bio-Rad). Glycoproteins were detected using Schiff's reagent (Sigma).

Western blotting. Gels were electroblotted onto Immobilon-P membranes (Millipore). Immunodetection of the polar flagellin was achieved with AS-Fla1, an antiserum against the polar flagellum of *A. brasilense* (Croes *et al.*, 1993), a gift of J. Vanderleyden (Catholic University of Leuven, Belgium). Horseradish-peroxidase conjugated anti-rabbit IgG (Bio Makor) secondary antiserum was used with 4-chloro-1-naphthol as a substrate to develop the blots (Harlow & Lane, 1988).

Quantification of polysaccharides and proteins from growth medium. Exopolysaccharides (EPSs) were quantified by a method described by Del Gallo & Haegi (1990) with some modifications. Bacteria grown for 24 h were removed by centrifugation (4000 g, 20 min). The supernatant was then filtered through a 0.22 µm membrane. Ethanol-insoluble EPS was precipitated overnight at 4 °C with 3 vols cold ethanol. After centrifugation (30000 g, 20 min), the EPS was resuspended in and dialysed against distilled water for 3 d at 4 °C, using a dialysis membrane with a 2 kDa molecular mass cut-off. The amounts of sugar in the polysaccharide fractions were determined by the anthrone method, using glucose as a standard (Dische, 1962). The amounts of protein in dialysed growth medium were determined by the method of Bradford (1976), using BSA as the standard.

All experiments were carried out at least three times and were highly reproducible. Therefore, data from one of each experiment are presented.

RESULTS AND DISCUSSION

Microscopic studies on aggregation of *A. brasilense* Cd in high C:N medium

Relatively high C:N ratios may induce aggregation of *A. brasilense* cells in liquid media (Madi & Henis, 1989; Arunakumari *et al.*, 1992). However, the use of organic acids as carbon sources alters the pH of the medium during the course of growth (Madi & Henis, 1989), and aggregation is not always consistent. To study chemical, physical and extracellular factors involved in the aggregation process, a medium consistently yielding aggregates was developed. Cells of *A. brasilense* strain Cd growing on fructose (37 mM) as the sole C source with 4 mM NH₄Cl (high C:N) aggregated, yielding cell flocs visible after 24 h. In a fructose medium containing 18 mM NH₄Cl (low C:N), no aggregates of this strain were formed even after 72 h of growth.

A similar pattern of aggregation with high C:N as compared with low C:N was observed using arabinose, galactose and glycerol as C sources. Use of malate and succinate led to non-reproducible results, probably due to the observed drastic changes in pH occurring during

growth. It is known that under various stress conditions, bacteria are capable of floc formation, which may improve survival (Bashan & Holguin, 1997).

Aggregated and non-aggregated cultures differed greatly in their morphological features, as revealed by SEM and TEM (Fig. 1). Under aggregation-inducing conditions, cells grown for 24 h appeared to be connected by fibrillar bridges (Fig. 1a, c) that were not observed at the low C:N ratio (Fig. 1b, d). Natural components produced by micro-organisms as extracellular products can function as bridging polymers (Harris & Mitchell, 1973) and many floc-forming bacteria have been shown to produce extracellular fibrils that are efficient in polymer bridging; such fibrils are of a cellulosic nature in many species (Marshall, 1976). Del Gallo *et al.* (1989) suggested the involvement of a cellulosic polysaccharide in aggregation of *Azospirillum*; however, no cellulose fibrils were detected in *A. brasilense* strain Cd by Madi & Henis (1989).

Aggregating cells accumulated high amounts of poly-β-hydroxybutyrate (PHB) in granules (Fig. 1e, g) and the cell envelope contained a well-defined electron-dense layer above the outer membrane (Fig. 1g, i). In non-aggregating cells, no PHB was seen after 24 h and the cell envelope appeared to be diffuse and less well defined (Fig. 1f, h, j). These results are similar to those of Madi *et al.* (1988), which revealed the presence of an extracellular layer on the outer surface of aggregating cells grown in a malate synthetic medium. PHB accumulation and the formation of a thick layer of EPSs were also observed during flocculation and encystation of *A. brasilense* Sp7 by Sadasivan & Neyra (1985).

Quantitative measurement of aggregation of various *A. brasilense* strains

Strain Sp7 aggregated almost three times as much as strain Cd under high C:N conditions (Table 1). Sp72002, a pleiotropic Tn5 mutant, did not aggregate even after 72 h. The other mutant, FAJ0204, which lacks both lateral and polar flagella, is a superaggregating strain, reaching more than 70 % aggregation after 72 h. Strains FAJ0204 and Sp7 also aggregated in low C:N, but to a much lower extent than in high C:N medium (Table 1).

Aggregate formation started within a few hours of growth in high C:N medium, except for strain Sp72002. It was necessary to differentiate between micro- and macro-aggregates (flocs). The micro-aggregates consist of relatively small clumps of cells that were only visible under the microscope (Fig. 2a); they were formed in the early exponential phase, causing the observed rapid increase in percentage of aggregation. The macro-aggregates consisted of large aggregates and were visible to the naked eye (Fig. 2b). Flocs were observed during the early to mid-exponential phase of growth in strains Sp7 and FAJ0204, but only towards the end of the exponential phase in strain Cd. The appearance of flocs in this strain did not cause a further increase in the percentage of aggregation, which is a relative value: the number and size of aggregates increased along with the

Fig. 1 (a)–(h). For legend see facing page.

Table 1. Growth and aggregation parameters and concentration of EPS and proteins in growth medium for several strains of *A. brasilense* after 24 h growth under high or low C:N

Each value represents the mean and SE (in parentheses) of three replicates from one representative experiment. Each experiment was carried out three to five times and yielded similar results.

Strain ... C:N ratio ...	FAJ0204		Sp7		Cd		Sp72002	
	High	Low	High	Low	High	Low	High	Low
Dry weight*	0.105 (0.007)	0.164 (0.006)	0.105 (0.003)	0.160 (0.005)	0.100 (0.005)	0.162 (0.010)	0.098 (0.004)	0.166 (0.006)
Aggregation (%)	58.0 (8.2)	22.2 (4.2)	39.2 (4.6)	6.1 (2.7)	15.3 (3.0)	—†	—†	—†
EPS‡	7.52 (1.17)	11.99 (1.90)	4.89 (0.46)	4.59 (0.99)	2.76 (0.15)	4.09 (0.22)	2.74 (0.31)	2.12 (0.10)
Protein‡	1.09 (0.16)	4.46 (1.18)	1.89 (0.18)	3.70 (1.73)	1.10 (0.20)	4.17 (0.49)	2.02 (0.12)	2.70 (1.24)
Protein:EPS ratio	0.14 (0.05)	0.37 (0.09)	0.39 (0.03)	0.81 (0.21)	0.40 (0.07)	1.02 (0.12)	0.74 (0.10)	1.27 (0.49)

* Values are expressed as g dried bacteria (100 ml growth medium)⁻¹.

† No aggregation was obtained even after 72 h.

‡ Values are expressed as mg sugar or protein (g dried bacteria)⁻¹.

number of free cells in the culture, leading in the case of strain Cd to a plateau after 12 h growth. However, the percentage of aggregation continued to increase in strain Sp7 up to late exponential phase. In mutant FAJ0204, a weak increase in aggregation was observed even during the stationary phase.

As expected, under N-limiting conditions, the growth of all tested strains resulted in lower cell yields than growth in low C:N medium (Table 1). No differences in growth

capacity were observed among strains in each medium, as observed by the similar dry weights obtained (Table 1). Thus, differences in aggregation intensity cannot be related to differences in growth.

Differences in the extent of aggregation among the strains after 24 h growth are illustrated in Fig. 3. The different behaviour of these strains with respect to aggregation may be a useful tool for the investigation of the aggregation process by providing detectable dif-

Fig. 1. Scanning electron micrographs of Cd cells grown for 24 h in high- (a, c) and low- (b, d) C:N media. Transmission electron micrographs of Cd cells grown for 24 h in high- (e, g, i) and low- (f, h, j) C:N media. Arrows: 1, fibrillar 'bridges' between cells; 2, PHB granules; 3, extracellular electron-dense layer. Bars, 1 µm (a–h), 10 µm (i and j).

Fig. 2. Light microscopic observation of (a) micro-aggregates and (b) macro-aggregates (flocs) of high-C:N-grown Cd cells. Bars, 1 µm.

Fig. 3. Aggregation of the four strains after 24 h growth in low- and high-C:N media. Bacteria were transferred to Petri dishes before the photograph was taken.

ferences in surface components. In a hydrophobicity test using a two-phase water-hydrocarbon partitioning system (according to Arunakumari *et al.*, 1992), all strains were found to be surface-hydrophilic under both high and low C:N conditions.

Effects of physical and chemical factors on aggregation of *A. brasilense*

Mechanical homogenization caused a momentary disruption of aggregates, which re-formed within a few seconds after the termination of this treatment. A more

effective disruption was obtained by sonicating the cells for 20 s (Table 2), although a small decrease in cell viability was observed, as estimated by observation of bacteria under a microscope and by plating. Prolonged sonication (1, 2 and 5 min) caused a stronger disruption of aggregates, but concomitantly greatly reduced cell viability. Heat treatment of high-C:N-grown cells (80 °C, 20 min) strongly promoted aggregation (Table 2) and an autoclave treatment (121 °C, 20 min) of the cell suspension further enhanced the process (data not shown). It is important to note that both the sonication and the heat treatments first caused aggregate dispersion, but aggregates re-formed to a greater extent after incubation than the controls in the heat-treated

Table 2. Effects of sonication and heat treatment on aggregate stability of *A. brasilense*

Cells were grown for 24 h in high-C:N medium, washed by centrifugation (4000 g, 10 min, twice) and resuspended in saline solution. Aggregate-containing suspensions were then sonicated for 20 s or heated at 80 °C for 20 min. Controls were untreated suspensions. Percentage aggregation was measured according to the described methodology, after shaking for 30 min (150 r.p.m., 30 °C). Each value represents the mean of three replicates from one representative experiment. This experiment was carried out three times and yielded similar results. Different letters within columns indicate significant differences at $P = 0.05$ according to one-way analysis of variance.

Treatment	Percentage aggregation		
	FAJ0204	Sp7	Cd
Control	29.7 ^a	19.0 ^b	16.6 ^b
Sonication	15.7 ^b	8.9 ^c	4.6 ^c
Heat treatment	36.2 ^a	37.5 ^a	33.8 ^a

cells (Table 2). Physical methods of aggregate dispersion have been found to be reversible in a number of systems, even when causing cell death, as heat does (Calleja, 1984).

The effects of some chemicals, including the protein-denaturing agents β -mercaptoethanol, urea and SDS, and the chelators EDTA and EGTA, on aggregation of *A. brasilense* Cd were tested. Aggregate dispersion caused by some of these chemical treatments may point to the involvement of proteins in cell-to-cell adhesion. Moreover, it can indicate the nature of the forces implicated in cell cohesion (Calleja, 1984). Treatments with 0.5–2.5 % (v/v) β -mercaptoethanol, 1.0–2.5 M urea, 0.05–0.25 % (w/v) SDS and 0.5–5.0 mM EDTA were unable to prevent reaggregation of cells after disruption by mechanical homogenization. Higher concentrations of these compounds drastically reduced cell viability and caused partial lysis of bacteria with a few aggregates still visible, except for 0.5 % (w/v) SDS, in which cell lysis was almost complete. As for untreated controls, treated cells reaggregated immediately after the termination of the disruption; the extent of relative aggregation of treated cells was marginally smaller (varying between approximately 85 and 90 %) in comparison to controls (100 %). Re-formed aggregates were very stable and remained intact for several days. After rehomogenization of cells treated for 1 h, aggregates also re-formed. EGTA, a more specific Ca^{2+} chelator, also failed to prevent reaggregation.

Low concentrations of urea (0.2 and 0.5 M) led to efficient disruption of aggregates without apparent loss of viability. Similar to the other treatments, reaggregation occurred immediately after homogenization of the tested suspension. However, in 0.2 M urea, the aggregates were completely disrupted after 4 h of incubation, during which homogenization was per-

Table 3. Effects of different concentrations of urea (in 0.1 M phosphate buffer) on aggregate stability of *A. brasilense* strain Cd.

Percentage aggregation was measured according to the described methodology, after shaking for 4 h (150 r.p.m., 30 °C), during which homogenization was either performed every hour or not. Each value represents the mean of three replicates from one representative experiment. This experiment was carried out three times and yielded similar results. Different letters within columns indicate significant differences at $P = 0.05$ according to one-way analysis of variance.

Urea concn (M)	Percentage aggregation	
	With homogenization	Without homogenization
0 (control)	11.6 ^a	15.0 ^a
0.2	0.0 ^b	6.8 ^c
0.5	6.6 ^a	10.1 ^b
1.0	11.0 ^a	11.7 ^{ab}
2.5	11.3 ^a	12.4 ^{ab}

formed every hour (Table 3), without affecting cell viability. Aggregation was reduced in 0.5 M urea within the same time span (Table 3), and complete disruption of aggregates was observed after 6 h (data not shown). A longer incubation period was needed to achieve aggregate disruption without hourly homogenization (Table 3). High concentrations of urea, like high concentrations of salt, can act as a non-specific aggregating agent. This may explain why aggregate disruption was only achieved at relatively low concentrations of urea.

The results obtained with the various chemicals used suggest that neither disulfide bonds nor divalent cations and hydrophobic interactions play a major role in the aggregation of *A. brasilense* Cd. Disruption of aggregates by relatively low concentrations of urea could be achieved by changes in the conformation of molecular groups directly involved in aggregation. This may indicate that hydrogen bonds are implicated in cell-to-cell adhesion. However, it is possible that this treatment disperses aggregates by releasing the functional 'aggregation molecule(s)' from the cell surface. Bacterial suspensions treated with 0.2 or 0.5 M urea, and then washed by centrifugation and dialysis to remove the urea, were unable to reaggregate. This indicates that molecules involved in cell-to-cell interactions leading to aggregation were irreversibly altered, or rinsed from the cell surface.

Effects of pH on the stability of aggregates

The effects of pH on aggregation were studied to test the involvement of charged groups in this process. Strains Cd and FAJ0204 showed increased reaggregation at the lower pH levels, with the highest values obtained at

Fig. 4. (a) SDS-PAGE of different fractions of *A. brasilense* Cd cells after 24 h growth in high- (lanes 1, 3, 5) or low- (lanes 2, 4, 6) C:N medium. Lanes: M, prestained markers; 1, 2, outer-membrane proteins; 3, 4, proteins extracted after passage through a hypodermic needle; 5, 6, proteins extracted from growth media. (b) SDS-PAGE from sonicated fractions of *A. brasilense* cells after 24 h growth in high- (lanes 1–4) or low- (lanes 5–8) C:N medium. Lanes: M, prestained markers; 1, 5, FAJ0204; 2, 6, Sp7; 3, 7, Cd; 4, 8, Sp72002. (c) Reaction of lanes 3 to 6 from (a) with Schiff's reagent. (d) Western blotting of extracts from *A. brasilense* cells grown for 24 h after passage through a hypodermic needle using AS-Fla1, an antiserum against the polar flagellum. Lanes: M, markers; 1, 2, Sp7; 3, 4, Sp72002; 5, 6, FAJ0204. High-C:N media (lanes 1, 3, 5), low-C:N media (lanes 2, 4, 6). Asterisks and bracket indicate band positions mentioned in the text.

pH 3 (data not shown). A weak tendency of increased reaggregation at alkaline pH was also observed in these strains. The extent of reaggregation of strain Sp7, in contrast to strains Cd and FAJ0204, was not affected by pH.

The increase in the extent of reaggregation at acidic pH levels in strains Cd and FAJ0204 was inversely correlated with aggregate size. The more basic or acidic the pH, the more cell viability was affected. Aggregates at all pH levels were stable and remained intact for several days. Moreover, aggregates re-formed after various rehomogenization rounds, even when cell viability was lost. These findings may indicate the involvement of charged groups in this phenomenon, at least for strains Cd and FAJ0204, which showed a differential response to pH. Bacterial cells have predominantly negative charges at physiological pH (Bitton & Marshall, 1980; Foster & Bowen, 1982). At acidic pH, negative ionized groups can be neutralized by protonation, thus diminishing the strength of the repulsive forces between bacteria and leading to increased aggregation.

Profile of extracellular proteins of different strains in high- and low-C:N media

Proteins were extracted from several fractions of *A. brasilense* cells grown in low- or high-C:N media and their electrophoretic profiles were determined by SDS-PAGE. No differences were found in the profiles of outer-membrane proteins (Fig. 4a) from aggregating and non-aggregating Cd cultures. Both extracts showed high concentrations of the major 42 kDa protein, which has been shown to constitute about 60% of the total outer-membrane proteins (Bachhawat & Ghosh, 1987). A clear difference in the intensity of an approximately 100 kDa double band was detected: the lower band of the spent-medium extract of aggregated cells was strong, whereas that of non-aggregating cells was barely visible. Recovery of the double band was more efficient by passing cells through a hypodermic needle than by direct extraction from the medium, and its intensity was stronger in hypodermic-needle extracts from aggregated cultures than from non-aggregating ones (Fig. 4a). These bands react positively with Schiff's reagent (Fig. 4c) and

may therefore be glycoproteins. The same was found for strains Sp7 and Sp72002, although the double band appeared to be weaker. This band also reacted positively with AS-Fla1, an antiserum against the polar flagellum (Fig. 4d).

Madi & Henis (1989) suggested that a glycoprotein (97 kDa) located on the outer surface of the cells is involved in the aggregation of *Azospirillum* cells. Moens *et al.* (1995) have recently shown that the flagellin of the polar flagellum of *Azospirillum* consists of a glycoprotein with a similar molecular mass. Moreover, it has been repeatedly observed that two cross-reactive closely spaced bands appear, indicating possible differences in the extent of post-translational modifications (Moens *et al.*, 1995). According to these data, and since no other bands from extracellular-protein extracts reacted positively with Schiff's reagent, it can be suggested that the putative glycoproteins detected in the two studies cited above and in the present work are the same. It has been shown that the first step in the adhesion of *A. brasilense* to wheat roots is mediated by an adhesin, closely associated with the polar flagellum (Croes *et al.*, 1993). Cells lacking the polar flagellum fail to adsorb to wheat roots, whereas purified polar flagella specifically adsorb onto the root surface.

However, the non-motile mutant strain FAJ0204 aggregates to a greater extent than the wild-type. This mutant does not produce a polar flagellum. Moreover, no band in the protein profiles of this strain reacted positively to Schiff's reagent and none was detected in the Western blot against AS-Fla1 (Fig. 4d), indicating that no peptide reminiscent of such a glycoprotein is produced by this strain.

Proteinaceous adhesins participate in many adherence and attachment processes in which bacteria are involved. In some of these processes, adhesive properties have been attributed to bacterial flagella. This, however, has been postulated rather than proven, with the flagella being only of secondary importance (Moens & Vanderleyden, 1996). Results from this study cast doubt on the possible involvement of the flagellin of the polar flagellum as a main determinant of aggregation in *A. brasilense*. This is in spite of the observed differences in the electrophoretic pattern of the detected glycoprotein between aggregating and non-aggregating cultures in wild-type strains.

Strain FAJ0204 is one of several Tn5 mutants obtained from wild-type Sp7 which are affected in the production of both lateral and polar flagella. Analysis of the DNA flanking the transposon insertion site revealed a high level of similarity to the *fliG* gene of *Caulobacter crescentus* (S. Moens, personal communication), which encodes a protein involved in the regulation of both the rotation and the biogenesis of the bacterial flagellum (Ramakrishnan *et al.*, 1994). This, along with findings from the present work, indicates that even if the role of the polar flagellum in the aggregation process is not clear, both aggregation and flagellation processes could share one or more regulator genes.

Since aggregates were found to be very stable even when using drastic disruption methods, components directly involved in cell-to-cell adhesion may not be readily extractable. A more thorough treatment was applied by sonicating the cells for 20 s (Table 2). The supernatant was then analysed. As can be observed in Fig. 4(b), very different protein profiles were obtained between low- and high-C:N cultures of the four strains. Great differences were apparent in the range of low-molecular-mass proteins (less than 30 kDa). The protein profile of FAJ0204 grown under low C:N is similar to the profile of the other strains, including itself, obtained from high-C:N cultures. As was previously established, this strain aggregates strongly, even under low C:N (Table 1).

It is possible that some of the differential bands from sonicated suspensions are intracellular proteins that were released during sonication. Thus, the differences observed may result from differences in the metabolism between low- and high-C:N conditions. However, it is still possible that one or more bands represent extracellular protein(s) involved in cell-to-cell adhesion.

Several experiments were conducted to determine whether proteolytic enzymes are able to disrupt aggregates. Trypsin and proteinase K, at various concentrations, were not able to induce aggregate dispersion even when several rounds of homogenization were applied. However, more work is needed to elucidate the question of the possible involvement of extracellular proteins in aggregation of *Azospirillum*.

Possible involvement of EPSs in aggregation

Previous studies have shown that EPSs may be involved in aggregation (Sadasivan & Neyra, 1985; Del Gallo *et al.*, 1989; Michiels *et al.*, 1990; Arunakumari *et al.*, 1992; Katupitiya *et al.*, 1995). To further investigate the possible contribution of secreted polysaccharides in aggregation, the concentration of EPS from different strains was determined. Differences in the concentration of EPS were detected between high- and low-C:N conditions and between strains (Table 1). The extent of aggregation among strains correlated with amounts of EPS in both low and high C:N media.

If the effect of EPS on aggregation were determined only by its concentration, it would be expected that a higher EPS concentration would be found in high C:N cultures than in low C:N ones. However, this is not the case in cultures of strains FAJ0204 and Cd (Table 1). This indicates that not only the amount of EPS produced, but also its structure and composition, may play a role in aggregation. Moreover, it was observed that in high-C:N EPS extracts, the ethanol-insoluble fractions precipitated and strongly adhered to the bottom of the bottle, whereas in the low C:N extracts, ethanol-insoluble fractions tended to flutter and could be isolated only by centrifugation.

No correlation was found between aggregation and protein concentration in the growth medium. Under both low and high C:N, the ratio of protein to ethanol-

insoluble EPS decreased with aggregation. These findings support the hypothesis that polysaccharides play a major role in aggregation of *Azospirillum* cells.

Conclusions

This work presents a consistent method enabling the study of aggregation of *A. brasilense* in high C:N media. Fibrillar bridges were found to link cells, as observed by SEM. We are interested in investigating the nature of these adherent fibrils.

Studies on the effect of several chemical and physical factors on aggregation capacity demonstrate the high cohesiveness of aggregates formed under high C:N. Only resuspension of the culture in low concentrations of urea allowed complete disruption of aggregates, whereas reversible disaggregation occurred in heated suspensions. These findings indicate the possible involvement of hydrogen bonds, as has been observed in other bacterial and yeast systems (Calleja, 1984). Aggregation of *A. brasilense* cells is an active process, since its induction occurs under certain physiological conditions. However, when aggregates are disrupted, even by treatments that affect bacterial viability, such as exposure to heat, drastic pH, sonication and various chemicals, aggregates are re-formed, sometimes to higher extents than in untreated controls, suggesting that the molecules involved are very stable.

The differences in EPS concentration observed by using different strains and varying C:N ratios support the hypothesis that EPSs play a role in the aggregation process. With respect to the role of extracellular proteins, no sharp differences explaining the aggregating behaviour of the various strains could be detected. Clear variation was observed in extracts of high- and low-C:N grown cells after sonication, especially in their profile of relatively low-molecular-mass proteins. However, the differences in the aggregation capacity of cultures of the various strains grown at high C:N were not reflected in their protein profiles. We cannot discard the possibility that the putative protein may be constitutively expressed, and in this case, aggregating behaviour could be determined by structural modifications rather than by synthesis *de novo*. The glycoprotein detected has a molecular mass similar to the one previously described as participating in the adherence of cells to roots (Croes *et al.*, 1993) but is probably not involved in the aggregation process as it could not be found in FAJ0204, the 'superaggregating' strain.

Based on these findings, it is proposed that EPSs of *A. brasilense* participate in cell-to-cell adhesion during aggregation by acting as non-specific flocculants, or through interaction with proteins or other molecules attached to the bacterial envelope. Cell-surface lectins of *A. brasilense* and *A. lipoferum* have been suggested to be involved in the adhesion of these bacteria to wheat roots (Nikitina *et al.*, 1996). In this study, differences in the extent of aggregation among the tested strains could be

related to the concentration of EPS present and/or to its composition, and not to a protein constituent that may be present in all strains to the same degree. The nature of the EPS and the differentially expressed low-molecular-mass proteins obtained after sonication are now under study. A bioassay for the detection of added surface extracts from aggregating cells involved in aggregation is also being developed. This assay may help isolate and define factors directly involved in the aggregation of *Azospirillum* cells.

ACKNOWLEDGEMENTS

We thank J. Vanderleyden and S. Moens from the F. A. Janssens Laboratorium voor Genetica of the Katholieke Universiteit Leuven (Belgium) for the antiserum Fla1 and the Tn5 mutant FAJ0204. We also thank C. Elmerich from the Unité de Physiologie Cellulaire, Département des Biotechnologies of the Institut Pasteur (France) for the Tn5 mutant Sp72002.

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Received 3 November 1997; revised 16 March 1998; accepted 18 March 1998.