Involvement of outer-membrane proteins in the aggregation of *Azospirillum brasilense*

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A bioassay was developed to investigate biological factors involved in the aggregation of *Azospirillum brasilense* strain Cd. Cells were grown for 24 h under aggregation-inducing and non-aggregation-inducing conditions (high and low C:N, respectively) and sonicated for 20 s. The cells were washed by centrifugation and resuspended in potassium phosphate buffer containing the two types of sonication extract. A greater extent of aggregation and higher flocculation were observed after 2–3 h incubation in the presence of sonicates from cells grown at high C:N (H-cells) compared to cells grown at low C:N. Flocculation did not occur after incubation of these cells in phosphate buffer. Boiled or proteinase K-treated sonicates originating from H-cells had lower aggregation-inducing capacity. After fractionation of the crude sonicate, both the outer-membrane protein (OMP) and the total membrane (mostly OMP) fractions possessed relatively high aggregation specific activities. The aggregation-inducing capacity of the OMP fraction strongly correlated with its protein concentration in the bioassay. Treatment of this fraction with proteinase K also decreased its aggregation-inducing activity. These findings suggest that OMPs are involved in the aggregation process of cells of *A. brasilense*.

Keywords: *Azospirillum brasilense*, aggregation, flocculation, outer-membrane proteins

INTRODUCTION

The free-living N₂-fixing rhizobacterium *Azospirillum brasilense* lives in close association with plant roots and may exert beneficial effects on plant growth and yield of many crops of agronomic importance (Okon & Labandera-Gonzalez, 1994). This and other plant-growth-promoting rhizobacteria (PGPR) are known for their capacity to aggregate and flocculate, and 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). Thus, the phenomenon of bacterial aggregation has considerable importance in the production of inoculants for agriculture.

The bacterial surface plays an important role in the establishment of bacteria–plant associations as well as in bacterial aggregation (De Troch & Vanderleyden, 1996). Data suggesting the involvement of extracellular

polysaccharides and proteins in aggregation of *Azospirillum* have been published (Sadasivan & Neyra, 1985; Madi & Henis, 1989; Michiels *et al.*, 1990, 1991; Katupitiya *et al.*, 1995; Burdman *et al.*, 1998). However, the mechanism of aggregation and the molecules involved in cell-to-cell adhesion are still unknown. Recently, a regulatory gene of *A. brasilense* was identified and proposed to be involved in the regulation of aggregation, capsule production and colonization of root surface (Pereg-Gerk *et al.*, 1998). Nikitina *et al.* (1996) and Castellanos *et al.* (1998) suggested the presence of cell-surface lectins in *Azospirillum* that could be involved in cell aggregation and bacterial adhesion to roots.

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 have been suggested to participate directly in the aggregation of various bacterial species from diverse environments (Eggset *et al.*, 1983; Calleja, 1984; Marsh & Bradshaw, 1995; Wai *et al.*, 1996).

Abbreviations: EPS, exopolysaccharide; H-cells, high C:N-grown cells; L-cells, low C:N-grown cells; (M)OMP, (major) outer-membrane protein.

Azospirillum cells aggregate and flocculate under diverse stress conditions and in the presence of various carbon sources, this phenomenon being generally accompanied by an accumulation of polyhydroxybutyrate (PHB) granules (Sadasivan & Neyra, 1985; Burdman *et al.*, 1998). In previous work (Burdman *et al.*, 1998) we developed a medium for consistent induction of aggregation of *A. brasilense* cells and studied the effects of chemical and physical factors on this phenomenon. Growth of *A. brasilense* strain Cd in a medium with a high C:N ratio (using fructose and ammonium chloride as C and N sources, respectively) resulted in flocculation visible to the naked eye after 24 h, whereas no cell aggregates were formed after 72 h of growth in low C:N medium. The same study describes an effective method for temporarily disrupting flocs by sonication of the cells for a relatively short period (20 s). This treatment was shown to be very effective in releasing proteins (most probably those attached to the cell surface) without a drastic reduction in cell viability (Burdman *et al.*, 1998). Based on these findings, a suitable bioassay was developed to investigate the nature of biological factors involved in cell-to-cell adhesion leading to aggregation. This bioassay procedure is described in this paper together with the first results using this system.

METHODS

A. brasilense strains and growth conditions. Wild-type strains Cd (Eskew *et al.*, 1977) and Sp7 (Tarrand *et al.*, 1978), and 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 (Pereg-Gerk *et al.*, 1998), 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 g ml⁻¹ for the Tn5 mutant strains. Bacteria were grown under aggregation- or non-aggregation-inducing conditions (high and low C:N, respectively). The high C:N medium contained (g l⁻¹) D-fructose (6.67), MgSO₄ (0.2), NaCl (0.1), CaCl₂ (0.02), K₂HPO₄ (6.0), KH₂PO₄ (4.0), yeast extract (Difco) (0.1), NH₄Cl (0.214) and microelements as described by Okon *et al.* (1977). The low C:N medium contained the same components, but with NH₄Cl at 0.963 g l⁻¹. Liquid media were adjusted to pH 6.8. Flasks with 100 ml medium were inoculated with exponential-phase cultures at an initial OD₅₄₀ of approximately 0.05 (about 10⁷ c.f.u. ml⁻¹) and incubated on a rotary shaker (150 r.p.m.) at 30 °C for 24 h (until late exponential phase in both media).

Quantitative measurement of aggregation. The extent of aggregation was measured according to Madi & Henis (1989) with some modifications. Aliquots of suspensions containing aggregates were transferred to a conical tube and allowed to stand. After 20 min at 24 °C, aggregates had settled to the bottom of the tube and the suspension was mostly composed of free cells. The turbidity of the suspension was measured using a Genesis 5 spectrophotometer (Spectronic) 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 immediately (OD_t). The percentage aggregation was estimated as follows: percentage aggregation = (OD_t - OD_s) × 100/OD_t. While aggregation is reflected in the value measured as described above, flocculation, on the

other hand, is defined as the formation of visible aggregates (flocs) and was expressed in this study as a qualitative parameter.

The aggregation bioassay. Cells were grown in high or low C:N medium (H- and L-cells, respectively; 300 ml for H-cells and 200 ml for L-cells) as described above. Approximately 0.3 g total bacterial dry weight was used in each experiment. Cells were centrifuged (4000 g, 10 min, twice), resuspended in 10 mM potassium phosphate buffer (pH 6.8) and finally sonicated in an ultrasonic disintegrator (MSE) at 1.2 A for 20 s on ice. The centrifuged supernatant of sonicated cells (5000 g, 15 min) was filtered (0.45 µm) and designated the sonicate extract (or sonicate). The final extraction volume was made up to 60 ml with phosphate buffer. Pelleted sonicated cells were resuspended in 10 ml phosphate buffer. Bioassays were carried out in 50 ml conical tubes (Miniplast Ein-Shemer) as follows: 10 ml sonicate or its fractions (see below) were added to 0.5 ml bacterial suspension in phosphate buffer and made up to a total volume of 15 ml. Negative controls were supplemented with phosphate buffer alone. Tubes were incubated at 30 °C with shaking (150 r.p.m.). Minor variations are described in Results and Discussion.

Effects of boiling and treatment with proteases on the aggregation-inducing activity of the sonicate extract. Sonicates from H-cells were treated with proteinase K (EC 3.4.21.64, from *Tritirachium album*; Sigma) or trypsin (EC 3.4.21.4, from beef pancreas; BDH) at 1.0 mg ml⁻¹ for 3 h at 37 °C, or alternatively boiled for 20 min before the onset of the bioassay in which sonicated L-cells were used. Controls included phosphate buffer alone, sonicate incubated at 37 °C for 3 h without proteases or with boiled enzymes, and sonicate alone kept at 4 °C until the bioassay (fresh sonicate).

Fractionation of the sonicates. Cells were grown in high C:N medium for 24 h, centrifuged (4000 g, 10 min, twice), resuspended in 10 mM phosphate buffer and then sonicated for 5 min. After centrifugation (5000 g, 15 min) and filtration (0.45 µm) the supernatant was divided into two equal volumes: the 'crude sonicate' and a second fraction that was further purified by ultra-centrifugation (100 000 g, 1 h). The supernatant of this centrifugation stage contained the 'free protein fraction' (extracellular proteins weakly attached to the cell wall and cytoplasmic proteins, both released by sonication). The pellet was resuspended in 20 mM Tris/HCl (pH 7.2) and divided into two equal volumes. One volume contained the 'total membrane protein fraction'. The outer-membrane protein (OMP) fraction was obtained from the second volume according to Schlöter *et al.* (1994). The bioassay was carried out on sonicated L-cells by applying the different fractions obtained from the same amount of bacterial dry weight as previously described.

Protein determination. The bioassay was initially developed on the basis of material extracted from a known dry weight of cells. In bioassays conducted after treatments such as boiling, proteases and fractionation, the protein concentration in the bioassay was determined according to Bradford (1976).

Gel electrophoresis. Proteins were separated by SDS-PAGE (12%, w/v, acrylamide) and stained with Coomassie brilliant blue, using standard methods (Laemmli, 1970). The molecular masses of the proteins were estimated using low-range prestained standards (Bio-Rad). Relative quantification of proteins in SDS-PAGE gels was performed using NIH Image (1.61) software.

Statistics. Each experiment was carried out three times with three replicates per treatment in each one. Since results were

highly reproducible, data from representative experiments are shown. Data were subjected to one- or two-way analysis of variance (ANOVA) for determination of significant differences between treatments.

RESULTS AND DISCUSSION

Characterization of the bioassay system

Sonicates obtained from various strains of *A. brasilense* differing in their aggregation capacity were tested for their effects on aggregation of H-cells of strain Cd (aggregating H-cells). The other strains were also grown under high C:N. FAJ0204 and Sp72002 are Tn5-induced strains of Sp7. The first has a strong aggregative phenotype whereas the latter does not aggregate at all under the tested growth conditions. In previous work, wild-type strain Sp7 was shown to aggregate less than mutant FAJ0204, but more than wild-type Cd (Burdman *et al.*, 1998).

There is a clear effect of adding sonicates in inducing aggregation of H-cells (Table 1). The aggregation rates of the various strains as measured by Burdman *et al.* (1998) and the aggregation-inducing activity of their extracts were positively correlated ($r^2 = 0.956$). Sonicates from the highest aggregating strains (FAJ0204 and Sp7) significantly differed from controls and a

clearly more intensive flocculation was observed when H-cells were resuspended in sonicates originating from the three aggregating strains in comparison to controls and sonicates from strain Sp72002. Although some differences in percentage aggregation were not found to be statistically significant, the same picture was observed in two other separate experiments.

The aggregative capacity of the sonicates was further investigated using H- and L-cells. Strain Cd cells were grown under both conditions, sonicated for 20 s and resuspended in phosphate buffer alone and in buffer containing sonicates of H- or L-cells. In both cases, resuspension in sonicates from aggregating cells (H-cells) showed significantly higher extents of aggregation in comparison to those obtained from non-aggregating cultures (L-cells) and controls (Table 2). It was also observed that sonicates from L-cells were more aggregation-inducing than controls with phosphate buffer alone. As expected, aggregation of H-cells was higher and flocs appeared faster when compared to L-cells. H-cells flocculated within a few minutes after resuspension, whereas visible flocs of L-cells appeared only after 2–3 h. Flocculation (appearance of visible aggregates) occurred in all treatments, except for L-cells resuspended in phosphate buffer, for which low extents of aggregation were still obtained. This result is explained by the formation of small clumps of cells (microaggregates) with a polar configuration that are visible only under the microscope, in both high and low C:N cultures of strain Cd (Burdman *et al.*, 1998).

The same procedure was applied to the non-aggregating mutant strain Sp72002. No flocculation occurred even after 24 h incubation of this strain in sonicates obtained from H-cells of strain Cd (not shown). This mutant strain produces the least exopolysaccharide (EPS) of the four tested strains (Burdman *et al.*, 1998), lacks thickening of the extracellular coat, does not form a polysaccharide net between cells (as observed for wild-type Sp7 during flocculation) and does not bind Congo Red like wild-type strains (Pereg-Gerk *et al.*, 1998). Aggregation in our bioassay system may involve interaction between factor(s) from the sonicate and polysaccharides in the cell wall; the absence of some of the latter could explain the observed lack of aggregation.

Table 1. Aggregation of H-cells of *A. brasilense* strain Cd after 1 h incubation in 20 s sonicates of different strains of *A. brasilense* grown for 24 h under high C:N

Sonicate origin	Aggregation (%)*	Flocculation
Control†	11.0 ^c	Intermediate
Sp72002	11.6 ^{bc}	Intermediate
Cd	13.2 ^{abc}	Strong
Sp7	14.3 ^{ab}	Strong
FAJ0204	15.1 ^a	Strong

* Different letters (a–c) indicate significant differences between treatments at $P = 0.05$.

† In the control treatment, H-cells were resuspended in phosphate buffer.

Table 2. Aggregation of H- and L-cells of *A. brasilense* strain Cd after incubation in their own or reciprocal sonicates

Controls were resuspended in phosphate buffer. H-cells were incubated for 1 h, whereas L-cells were incubated for 2 h. Different letters (a–c) indicate significant differences between treatments at $P = 0.05$ (sonicate origins) in each type of cell.

Sonicate origin	L-cells		H-cells	
	Aggregation (%)	Flocculation	Aggregation (%)	Flocculation
Control	3.1 ^c	None	8.4 ^b	Intermediate
L-cells	7.3 ^b	Weak	9.9 ^b	Intermediate
H-cells	8.7 ^a	Intermediate	13.9 ^a	Strong

Table 3. Effects of pre-sonication on L-cells of *A. brasiliense* strain Cd used in the bioassay

The extent of aggregation after 4 h incubation of sonicated and non-sonicated cells in phosphate buffer (control) and 20 s sonicate of L- and H-cells is shown. Different letters (a–b) indicate significant differences between the different type of cells at $P = 0.05$ (sonicated or not) in each incubation treatment.

Sonicate origin	Sonicated L-cells		Non-sonicated L-cells	
	Aggregation (%)	Flocculation	Aggregation (%)	Flocculation
Control	13.0 ^a	—	2.9 ^b	—
L-cells	22.8 ^a	+	9.4 ^b	—
H-cells	26.4 ^a	+	11.2 ^b	—

Table 4. Effects of different treatments on the aggregation-inducing activity of 20 s sonicates from *A. brasiliense* strain Cd H-cells, as determined by the percentage of aggregation of L-cells after 2 h incubation

Controls were L-cells incubated in phosphate buffer. Each value represents the mean of three replicates from one of three similar experiments. Different letters (a–c) indicate significant differences at $P = 0.05$.

Treatment	Aggregation (%)	Protein content (mg ml ⁻¹)*	Flocculation
Fresh sonicate†	8.6 ^a	0.1	+
37 °C, 3 h	7.6 ^a	0.09	+
Proteinase K, 3 h	3.9 ^b	0.02	—
100 °C, 20 min	2.8 ^{bc}	0.03	—
Control	2.0 ^c	—	—

* Values represent approximate protein concentration after subtracting the added proteinase K.

† Sonicate kept at 4 °C prior to bioassay.

Effects of sonication on the target cells used in the bioassay

In the first experiments, cells used in bioassays were exposed to their own sonicate. The extent of aggregation between sonicated and non-sonicated L-cells to which sonicates were added was compared to estimate the role of sonication in aggregation. Sonicated cells showed a significantly higher extent of aggregation than the non-sonicated ones (Table 3) in all suspensions. Flocs of sonicated L-cells were again visible after 2–3 h incubation with sonicates. In sharp contrast, non-sonicated L-cells showed only weak flocculation after 20 h incubation (which may have resulted from stress induced by a long incubation period). Sonicated L-cells and sonicates from aggregating H-cells were therefore used for further studies.

The fact that sonicated L-cells flocculated faster and more extensively than non-sonicated cells may be due to the removal of an extracellular cell-bound layer or component that impedes adhesion in non-sonicated L-cells. When centrifuged, the bacterial pellet obtained from H-cells is rough and cohesive, the cells clustering together with the pellet strongly attached to the bottom

of the tube. Pellets obtained from L-cells are smooth, attach weakly to centrifuge tubes and are easily resuspended. Following sonication, the attachment of L-cells to the bottom of the centrifugation tubes is enhanced, resembling the behaviour of H-cells.

Influence of physiological status on aggregation

The role of the metabolic status of the cells in the aggregation bioassay was investigated by adding 0.1% NaN₃ to the sonicated bacteria. Bacteria were immediately affected by this treatment as observed by microscope (loss of swimming behaviour and disruption of microaggregates) and plating (decrease in viability). NaN₃-treated L-cells showed a significant decrease in the extent of aggregation in comparison to untreated L-cells. Flocculation did not occur in any NaN₃ treatment even after 20 h incubation.

Addition of NaN₃ to aggregating Cd cultures did not disrupt flocs but did disrupt microaggregates. Microaggregates were also disrupted by NaN₃ using L-cells, explaining how the extent of aggregation in the NaN₃-treated controls (resuspended in phosphate buffer with NaN₃) could reach 0% (not shown).

It is important to state that the effects of NaN_3 are due to the azide and not the sodium ion, since resuspension of cells in saline solution (150 mM NaCl) does not cause a disruption of microaggregates and does not impede flocculation (Burdman *et al.*, 1998).

Effects of boiling and proteases on the aggregation-inducing activity of sonicates

Both boiling and protease treatment using proteinase K significantly reduced the aggregation-inducing activity of the sonicate (Table 4). Moreover, L-cells did not flocculate in these treatments, in contrast to the flocculation observed with sonicates kept at 4 or 37 °C.

Treatment of the sonicate with boiled proteinase K (not shown) reduced aggregation in comparison to the untreated sonicate, but to a significantly lesser extent than unboiled proteinase K. Such a reduction in aggregation in comparison to the untreated sonicate could be due to residual enzymic activity, as proteinase K is known to be highly heat-stable. Treatment of the crude sonicate with trypsin did not affect its aggregation-inducing capacity significantly. Protein content of sonicates was reduced by trypsin, but not as extensively as with proteinase K (not shown).

These results suggest that adhesive proteins present in the sonicate are at least partially responsible for the aggregation-inducing activity. Addition of bovine serum albumin to sonicated L-cells at a protein concentration similar to that of sonicates used in the bioassay did not increase aggregation above the control level (phosphate buffer) and flocculation was not observed (not shown). This indicates that increase in aggregation is not due to an increase in general protein content in the suspension.

Involvement of OMPs in aggregation

OMPs have been proposed to play a role as adhesins in invasion and adhesion processes in various Gram-negative bacteria. De Mot & Vanderleyden (1991) purified the major OMP (MOMP) of *Pseudomonas fluorescens* and showed that it can strongly and selectively adsorb to plant roots. Similar results were obtained by Achouak *et al.* (1995) with a MOMP of *Rahnella aquatilis*. In both cases, these MOMPs were proposed to play a role in attachment and colonization of plant roots by the producing bacteria. Adhesive OMPs (generally porins) occur in *Vibrio cholerae* (Sasmal *et al.*, 1992), *Aeromonas hydrophila* (Quinn & Flower, 1995; Lee *et al.*, 1997) and in an entero-aggregative strain of *Escherichia coli* (Debroy *et al.*, 1995). Porins were shown to be involved in the invasion of epithelial cells by *Salmonella typhimurium* (Dorman *et al.*, 1989) and *Shigella flexneri* (Bernardini *et al.*, 1993).

To obtain a greater amount of the OMPs of *Azospirillum*, H-cells were sonicated for 5 min while L-cells used in the bioassay were sonicated for 20 s. Controls included a 20 s sonicate from H-cells as in previous experiments. No qualitative differences be-

Fig. 1. SDS-PAGE of different fractions obtained from H-cells of *A. brasilense* strain Cd tested for their aggregation-inducing capacity. Lanes: M, prestained markers; 1, 5 min crude sonicate; 2, free proteins; 3, membrane proteins; 4, OMPs. The arrows indicate the position of bands discussed in the text.

tween the protein profile of the 20 s and the 5 min sonicates were observed in SDS-PAGE (not shown), but twice as much protein was extracted with the 5 min sonicate (Table 2).

The total membrane protein fraction (Fig. 1, lane 3) was mostly composed of OMPs (approx. 82%, based on image analysis). The aggregation-inducing capacity of the total membrane fraction was similar to that of the 20 s sonicate and was slightly higher than that of the free protein fraction. It should be pointed out that this result was obtained with a much lower protein concentration than with the crude extracts and with the free protein fraction. This is reflected in the high relative-enrichment value for the total membrane protein fraction (Table 5) in comparison to crude and free protein fractions.

Fractionation of the crude sonicates resulted in enrichment of OMPs as can be concluded by comparing the SDS-PAGE profile from this study (Fig. 1, lane 4) with the one obtained by Bachhawat & Ghosh (1987), who previously isolated and characterized the OMPs from *A. brasilense*. The 23, 28, 32 and 42 kDa bands are shared by both profiles. Image analysis revealed the level of contamination of the OMP fraction by proteins other than OMPs to be approximately 5%. The 42 kDa major protein, found to be about 35% of total protein in the total membrane fraction (based on image analysis), constituted approximately 60% of the total protein in the OMP fraction.

The OMP fraction showed a significantly lower relative aggregation when compared to the 5 min sonicate fraction. However, the aggregation-inducing capacity of this fraction was significantly higher than that of the phosphate buffer control. Flocculation was also

Table 5. Aggregation of L-cells of *A. brasilense* strain Cd after 2 h bioassay with different protein fractions from H-cells

Each fraction was obtained from the same amount of bacteria sonicated for 5 min (except for the 'crude, 20 s sonication' treatment). Controls were L-cells incubated in phosphate buffer. Each value represents the mean of three replicates from one of three similar experiments. Different letters (a-c) indicate significant differences at $P = 0.05$. 'Relative enrichment' values express the enrichment of the aggregation-inducing capacity of each fraction and are calculated as percentage aggregation/protein content.

Protein fraction	Aggregation (%)	Protein content (mg ml ⁻¹)	Relative enrichment	Flocculation
Crude, 5 min sonication	11.6 ^a	0.22	53	+
Crude, 20 s sonication	9.3 ^{ab}	0.1	93	+
Free proteins	6.7 ^b	0.2	34	+
Membrane proteins	8.6 ^{ab}	0.03	287	+
OMPs	6.3 ^b	0.01	630	+
Control	1.1 ^c	—	—	—

Fig. 2. Extent of aggregation of L-cells of *A. brasilense* strain Cd incubated at different concentrations of OMPs (●) or in the 20 s crude sonicate at 0.1 mg ml⁻¹ (solid bar). Different letters (A-C) indicate significant differences ($P = 0.05$) according to one-way analysis of variance.

observed in this treatment. Decrease in activity of the OMP fraction may be caused by loss or partial denaturation of proteins extracted from the membranes by use of a detergent. However, the relative-enrichment value of this fraction was the highest (Table 5).

As indicated before, the relatively highly active membrane protein fraction was mostly composed of OMPs. Furthermore, from the nature of the aggregation process itself, it can be inferred that if membrane-bound proteinaceous compounds that show adhesive properties are involved in the naturally occurring aggregation of *A. brasilense*, they should be OMPs, if not extracellular.

Concentration-dependent bioassays using the OMP fraction were carried out, showing that aggregation as well as flocculation increased with OMP concentration (Fig. 2). The extent of aggregation and flocculation

reached using 0.02 mg purified OMP fraction ml⁻¹ necessitated 0.1 mg crude sonicate ml⁻¹, therefore demonstrating an enrichment of aggregative capacity in the OMP fraction. Although 0.1 mg purified OMP ml⁻¹ caused an aggregation level significantly higher than that of the crude sonicate, the plateau reached indicates that a culture of cells has a limited potential for aggregation. This maximal aggregation could be determined by binding saturation of other compounds (such as EPS) involved in cell-to-cell adhesion. Treatment of the OMP fraction with proteinase K prevented aggregation and flocculation as observed with a 20 s crude sonicate (not shown).

To address the specificity of OMPs in the aggregation process of *A. brasilense*, OMPs from strain Cd were compared to OMPs from *E. coli* strain ML85 (obtained from H. Williams, University of Maryland, USA) and from *P. fluorescens* strain 417 (obtained from P. A. H. M. Bakker, Utrecht University, The Netherlands). Both the *E. coli* and *P. fluorescens* OMP fractions showed activities similar to that of Cd (not shown). Proteins with adhesive properties are thought to occur in the outer membrane of these genera (De Mot & Vanderleyden, 1991; Debroy *et al.*, 1995), not only in aggregative bacteria like *Azospirillum*. Although ruling out an exclusive role for OMPs in aggregation, it is possible that a more specific reaction of one or more of these proteins with other surface components forms the trigger for the aggregation reaction.

Conclusions

A bioassay that enables the study of biological factors involved in aggregation of *Azospirillum brasilense* was developed. The first results from this bioassay system suggest the involvement of protein(s) from the outer membrane in the aggregation phenomenon. Previous reports have suggested adhesive properties of OMPs (generally porins) of Gram-negative bacteria. The fact

that the aggregation-inducing capacity of the crude sonicate was strongly affected by proteinase K but not by trypsin indicates that specific OMPs, such as the trypsin-insensitive 42 kDa protein (Bachhawat & Ghosh, 1987), may take part in that process. In addition, we have purified and are in the process of characterizing the 42 kDa major protein from the OMP fraction (Fig. 1, lane 4). Partial amino acid sequencing of this protein showed a region with high similarity to other porins (unpublished results), thus strengthening the hypothesis that this protein is located in the outer membrane.

Protein profiles of different *A. brasilense* strains differing in their aggregation capacity were obtained using different extraction methods (Burdman *et al.*, 1998). No differences in protein profiles that explain the differential aggregation behaviour between strains grown under the same conditions were found. The polar flagellum previously proposed to be involved in the first step of bacterial attachment to roots (Croes *et al.*, 1993) has been shown not to be involved in aggregation (Burdman *et al.*, 1998).

Previously published data support the hypothesis that EPS is involved in aggregation of *Azospirillum* (Sadasivan & Neyra, 1985; Del Gallo *et al.*, 1989; Michiels *et al.*, 1990; Arunakumari *et al.*, 1992; Katupitiya *et al.*, 1995; Pereg-Gerk *et al.*, 1998; Burdman *et al.*, 1998). We propose that under certain growth conditions, protein(s) from the outer membrane could interact with EPS, leading to aggregation and flocculation. Recently, Castellanos *et al.* (1998) suggested that several cell-wall proteins extracted from various *A. brasilense* strains exhibit lectin-like activities. The strains differing in aggregation capacity had the same OMPs profile and no strain showed differences caused by growth under a high or low C:N ratio (unpublished data). However, the strains did differ in EPS content with correlation to aggregation extent (Burdman *et al.*, 1998). Therefore, we suggest that the OMP(s) involved in aggregation is (are) constitutively present in *A. brasilense*. Differences in the extent of aggregation between the various strains might be related to the amount, composition and structure of EPS present. The interaction between the molecular factors leading to aggregation does not seem to be specific, since OMPs from *E. coli* and *P. fluorescens* induced aggregation as effectively as OMPs of *Azospirillum*.

We are currently investigating the differences in EPS composition and structure between different strains and under different growth conditions. An in-depth study on the OMPs of *A. brasilense* (especially the 42 kDa MOMP) is also being undertaken to elucidate the 'participants' involved in cell-to-cell adhesion of *A. brasilense* during aggregation.

ACKNOWLEDGEMENTS

We thank J. Vanderleyden and S. Moens from the F. A. Janssens Laboratorium voor Genetica of the Katholieke Universiteit Leuven (Belgium) for the Tn5 mutant FAJ0204. We also thank C. Elmerich from the Unité de Physiologie

Cellulaire, Département des Biotechnologies, Institut Pasteur (France) for the Tn5 mutant Sp72002. This research was supported by the Israel Science Foundation, founded by the Academy of Sciences and Humanities, and by the Hebrew University Intramural Fund Basic Research Awards.

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Received 11 November 1998; revised 3 February 1999; accepted 16 February 1999.