Isolation and Characterization of the Outer Membrane Proteins of *Azospirillum brasilense*

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The outer membrane of *Azospirillum brasilense* was isolated from the total membrane fraction by sucrose density gradient centrifugation and by Sarkosyl extraction; both preparations showed an identical outer membrane protein profile in slab gels after electrophoresis under denaturing conditions. The profile showed a major 42 kDa protein constituting about 60% of the total outer membrane proteins. This major protein and a minor 40 kDa protein were tightly but non-covalently associated with peptidoglycan. In addition, a 23 kDa heat-modifiable protein, resistant to trypsin digestion, was detected in the outer membrane. Growth of *A. brasilense* under iron deficiency induced four additional high molecular mass proteins (87, 83, 78 and 72 kDa) in the outer membrane. A comparison of the outer membrane protein profiles of the three different *Azospirillum* species suggests that such profiles might be useful in taxonomic classification.

INTRODUCTION

Azospirillum species are Gram-negative soil bacteria that fix nitrogen under microaerophilic conditions both in isolated cultures and in their unique kind of association with roots of members of the grass family, including many important cereal crops (Von Bulow & Dobereiner, 1975; Umali-Garcia *et al.*, 1980; Van Berkum & Bohlool, 1980). Most of the bacteria belonging to the genus *Azospirillum* have been classified within the two species *A. brasilense* and *A. lipoferum*, depending on their nutritional characteristics and DNA homology (Tarrand *et al.*, 1978). Recently, a new species, *A. amazonense* (Magalhaes *et al.*, 1983), has been identified within this genus by modifying the selection procedure (Falk *et al.*, 1985), an approach which may reveal further new species of *Azospirillum* (Franche & Elmerich, 1981).

In addition to root colonization activity, chemotaxis of *Azospirillum* towards different nutrients has also been demonstrated (Barak *et al.*, 1983; Heinrich & Hess, 1985; Reinhold *et al.*, 1985). It is likely that the outer membrane has an important role in chemotaxis and in 'associative symbiosis', possibly via specific recognition properties inherent in some of the outer membrane proteins. Several workers have suggested the existence of species-specific differences in the cell surface of *Azospirillum* species based on their preferential association with specific plant roots (Baldani & Dobereiner, 1980; De-Polli *et al.*, 1980), but this has not been definitely established (Elmerich, 1984). Although the cell envelope proteins of many Gram-negative bacteria, particularly members of the *Enterobacteriaceae* (Lugtenberg & Van Alphen, 1983) are well characterized, no information is available on the outer membrane proteins of *Azospirillum* species. Differences in outer membrane protein profiles reflect changes in growth conditions (Sterkenburg *et al.*, 1984; Brown & Williams, 1985). For example, when iron becomes growth-limiting, induced synthesis of a number of proteins has been observed (Williams *et al.*, 1984; Hoe *et al.*, 1985; Neilands, 1982). Some of these act as receptors for high-affinity iron-siderophore uptake systems (Neilands, 1982). Studies with some rhizobacteria (Schroth &

Abbreviations: LBS, Luria broth-succinate; SMM, succinate minimal medium-NH₄Cl; LB Sucr, Luria broth-sucrose; EDDA, ethylenediamine-di(*o*-hydroxyphenylacetic acid).

Hancock, 1982) and the identification of siderophores in soil samples (Powell *et al.*, 1983) provide strong indications that soil microbes face an iron-deficient environment.

We report here the isolation of the outer membrane of *A. brasilense* and the identification of its associated proteins including those induced under iron deficiency. We have also compared outer membrane protein profiles of different members of the genus *Azospirillum* in an attempt to identify species-specific differences in their cell surfaces.

METHODS

Bacteria. The major part of this study was done with *A. brasilense* RG, a strain isolated as a spontaneous streptomycin-resistant colony (Maulik & Ghosh, 1986) from a culture of *A. brasilense* sp. 81 obtained from N. R. Krieg (Virginia Polytechnic Institute, USA). *A. brasilense* sp. 7, *A. lipoferum* 59b and *A. amazonense* Y1 were also obtained from N. R. Krieg. *A. brasilense* K67 was obtained from C. Elmerich (Pasteur Institute, Paris).

Media and growth conditions. *A. brasilense* and *A. lipoferum* were grown in Luria broth (Miller, 1972) supplemented with 0.1% sodium succinate and adjusted to pH 7.0, (LBS), or in the succinate minimal medium of Okon *et al.* (1976) supplemented with 0.1% NH_4Cl (SMM). *A. amazonense* Y1 was grown in Luria broth supplemented with sucrose (5 g l^{-1}) and adjusted to pH 6.0 (LB Sucr). The bacteria were grown at 32°C in a shaker-incubator (100 r.p.m.).

Preparation of crude cell envelope. Unless otherwise stated, all operations described below were done at $0-4^\circ\text{C}$. Exponential phase cells ($\text{OD}_{590} = 0.6-1.0$) grown in LBS or LB Sucr medium were harvested (6000 g, 5 min), washed with Tris/HCl buffer (10 mM, pH 8.0) (subsequently referred to as 'Tris buffer'), and resuspended in the same buffer containing 15% (w/v) sucrose. The cells were disrupted in an ultrasonic disintegrator and the cell lysate was digested with DNAase and RNAase [$20 \mu\text{g}$ of each (ml lysate) $^{-1}$] for 20 min. The lysate, after removing intact cells and cell debris by low-speed centrifugation, was diluted with Tris buffer (2 vols) and centrifuged for 60 min at 100000 g in a Beckman ultracentrifuge. The pellet, constituting the 'crude envelope fraction', was resuspended in Tris buffer and further purified by washing and centrifuging at 100000 g using the same buffer.

Isolation of the outer membrane. (a) *By density gradient centrifugation.* The procedure was a modification of that of Hancock & Carey (1979). The 'crude envelope fraction' was centrifuged, after layering onto a 45–70% linear sucrose gradient in Tris buffer for 5.5 h in a Beckman 50.2 fixed angle rotor at 44000 r.p.m. (180000 g). The three distinct bands formed were collected as fractions, each diluted 10-fold with Tris buffer, and centrifuged at 100000 g for 1 h; the sedimented pellets were resuspended in Tris buffer and stored at -20°C .

(b) *By extraction with Sarkosyl NL97.* Outer membrane proteins were isolated from the washed crude envelopes by treatment with 0.5% Sarkosyl NL97 (Ciba-Geigy) for 30 min at 28°C (Filip *et al.*, 1973) followed by centrifugation at 100000 g for 60 min. The pellet containing the outer membrane was washed once with Tris buffer, collected by centrifugation at 100000 g, resuspended in Tris buffer and stored at -20°C .

Isolation of the peptidoglycan-protein complex. The presence of peptidoglycan-associated proteins was demonstrated by solubilizing the crude envelope in SDS-PAGE sample buffer (Lugtenberg *et al.*, 1975) at 60°C for 30 min and sedimenting the peptidoglycan along with the associated proteins by centrifugation at 180000 g for 90 min. The sedimented material was resuspended, washed with distilled water and collected by centrifugation at 180000 g for 90 min.

SDS-PAGE. Electrophoresis of membrane proteins was done in SDS-polyacrylamide gels as described by Lugtenberg *et al.* (1975). Protein bands were stained with Coomassie Blue R-250, and destained with 5% (v/v) methanol–7.5% (v/v) acetic acid solution. Densitometric scans were done with a LKB Laser densitometer (2202 Ultroskan). SDS (Sigma) was recrystallized from 80% (v/v) hot ethanol before use.

Enzyme assays. NADH oxidase and succinate dehydrogenase associated with the membrane preparations were assayed essentially as described by Osborn *et al.* (1972). One unit of NADH oxidase is defined as the amount of enzyme that catalyses the oxidation of $1 \mu\text{mol NADH min}^{-1}$. One unit of succinate dehydrogenase is defined as the amount that brings about a change of $1.0 \text{ OD unit min}^{-1}$ at 550 nm using phenazine methosulphate ($\epsilon = 1.93 \times 10^4$) in the presence of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide.

Protein was estimated by the dye binding method of Bradford (1976).

RESULTS

Isolation of outer membrane by sucrose density gradient centrifugation

Sucrose density gradient centrifugation separated the crude envelope into three bands which could be collected as three distinct membrane fractions with densities (g ml^{-1}) of 1.15, 1.19 and 1.24. The bands contained 0.56, 0.10 and 0.018 units of NADH oxidase, 2.29, 0.97 and 0.075 units of succinate dehydrogenase, respectively. These results indicate that the cytoplasmic

Fig. 1

Fig. 2

Fig. 1. SDS-PAGE (12%, w/v, acrylamide) of the membrane proteins of *A. brasilense* RG. A, Crude envelope; B, outer membrane proteins isolated by sucrose density gradient centrifugation; C, inner membrane proteins isolated by sucrose density gradient centrifugation; D, outer membrane proteins isolated by Sarkosyl extraction of crude envelope; E, peptidoglycan-associated proteins isolated as described in Methods. The pointers indicate molecular mass values: pointers on the left show the positions of standard molecular mass marker proteins; pointers in the middle indicate molecular masses of outer membrane proteins.

Fig. 2. SDS-PAGE (8%, w/v, acrylamide) showing the oligomeric and monomeric forms of the peptidoglycan-associated proteins. Proteins were released from the peptidoglycan by incubating the peptidoglycan-protein complex in 2% (w/v) SDS-PAGE sample buffer at 100 °C for 5 min (A) or at 60 °C for 30 min in sample buffer containing 0.4 M-NaCl (B).

membrane was concentrated in the top, lighter fraction. The bottom, heavier fraction banded at a density that corresponded to outer membrane associated with peptidoglycan (Burnell *et al.*, 1980). This was expected as our method of isolation did not involve digestion of the peptidoglycan. The outer membrane fraction in the bottom layer showed very little contamination with cytoplasmic membrane-associated enzymes but the intermediate fraction appeared to contain unseparated cytoplasmic and outer membrane.

Characterization of outer membrane proteins of *A. brasilense* RG by SDS-PAGE

SDS-PAGE gels of the total membrane, the cytoplasmic membrane and the outer membrane isolated by density gradient centrifugation and the Sarkosyl-extracted outer membrane of *A. brasilense* RG are shown in Fig. 1. There was very little cross-contamination between the outer membrane and the cytoplasmic membrane proteins prepared by density gradient centrifugation as revealed by SDS-PAGE analysis (Fig. 1; lanes B and C, respectively) and by enzyme analysis. The outer membrane prepared by the Sarkosyl extraction procedure gave a protein profile (Fig. 1; lane D) identical to that of the outer membrane isolated by density gradient centrifugation (Fig. 1; lane B). All subsequent experiments were therefore done with outer membrane proteins isolated by the more convenient Sarkosyl extraction procedure.

A major outer membrane protein of molecular mass 42 kDa constituted over 60% of the protein in the outer membrane of *A. brasilense* RG as determined by densitometer tracings of the gel. Other minor proteins were also observed in the gel and they were identified by their molecular mass values as shown in Fig. 1.

Fig. 3. (a) SDS-PAGE (12%, w/v, acrylamide) of the outer membrane proteins solubilized at different temperatures in 2% (w/v) SDS-PAGE sample buffer, showing the presence of a heat-modifiable protein: A, 30 min at 60 °C; B, 30 min at 70 °C; C, 5 min at 100 °C. Outer membrane proteins were prepared by the Sarkosyl extraction method from cells grown in SMM. hmp, hmp*, see Results. (b) Trypsin sensitivity of the outer membrane proteins of *A. brasilense* RG. Outer membrane proteins prepared as for Fig. 3(a) were incubated in Tris buffer with trypsin [$100 \mu\text{g (mg membrane protein)}^{-1}$] for 30 min at 37 °C. The digested membranes were collected and washed by centrifugation (100000 *g*, 60 min) and resuspended in Tris buffer. SDS-PAGE (12%, w/v acrylamide) of these samples was done before trypsin digestion (lane A) or after trypsin digestion (lane B); lane C, molecular mass markers.

Peptidoglycan-associated proteins

The peptidoglycan-associated proteins could be released from the peptidoglycan on incubation of the peptidoglycan-protein complex in 2% SDS-PAGE sample buffer at temperatures of 70 °C and above (Fig. 1, lane E; Fig. 2, lane A) or at 60 °C in the same buffer containing 0.4 M-NaCl (Fig. 2, lane B). This suggests that the major 42 kDa protein and a minor 40 kDa protein are tightly but non-covalently linked to the peptidoglycan. The peptidoglycan-associated proteins released from the peptidoglycan in the presence of 0.4 M-NaCl at 60 °C in 2% SDS-PAGE sample buffer remained in an aggregated or oligomeric form and appeared as high molecular mass bands (Fig. 2, lane B). The predominant oligomer observed on SDS-PAGE corresponded to a molecular mass of 70 kDa.

Heat-modifiable proteins

The solubilization experiments in 2% SDS-PAGE sample buffer at increasing temperatures revealed the presence of one outer membrane protein that was heat-modifiable (Fig. 3a). The heat-modifiable protein was solubilized even at 37 °C and had an apparent molecular mass of 23 kDa (hmp) below 80 °C, but above 90 °C the same protein had an apparent molecular mass of 28 kDa (hmp*). These experiments were done with cells grown in SMM which favoured production of the heat-modifiable protein (2–3-fold compared with cells grown in LBS as determined by Laser densitomer scans).

Fig. 4. SDS-PAGE (12%, w/v, acrylamide) of the crude envelopes (A, C, E, G and I) and outer membrane proteins (B, D, F, H and J) respectively, of different *Azospirillum* species: A, B, *A. brasilense* RG; C, D, *A. brasilense* sp. 7; E, F, *A. brasilense* K67; G, H, *A. lipoferum* 59b; I, J, *A. amazonense* Y1.

Trypsin-sensitivity of the outer membrane proteins

Sensitivity to proteolytic digestion of the outer membrane proteins, which is an indication of the surface exposure, was tested by treating the isolated outer membrane with trypsin. The trypsin digestion of the outer membrane preparation (Fig. 3b) showed that the 42, 40 and 32 kDa proteins and the heat-modifiable protein (28 kDa) were among the proteins insensitive to trypsin.

Profiles of outer membrane proteins of *Azospirillum* species

A comparison of the total membrane and Sarkosyl-extracted outer membrane proteins of *A. brasilense*, *A. lipoferum* and *A. amazonense* was made by SDS-PAGE analysis (Fig. 4). *A. brasilense* sp. 7 had an almost identical profile to that of *A. brasilense* RG. The outer membrane protein profile of strain K67, a strain classified as *A. brasilense* (Franché & Elmerich, 1981), was totally different from the others in that relatively larger proportions of the total membrane proteins were not solubilized by Sarkosyl treatment. SDS-PAGE analysis revealed that *A. lipoferum* 59b contained a single major outer membrane protein (35 kDa) that constituted approximately 70% of the total protein in the outer membrane. *A. amazonense* Y1 was characterized by the presence of numerous proteins (i.e. Sarkosyl-insoluble proteins) in the outer membrane, the most prominent of which was the 38 kDa protein.

Effect of iron-limitation on the outer membrane protein profile of *A. brasilense* RG

Growth of cells in iron-limiting conditions resulted in the production of four new proteins in the outer membrane. The molecular masses of these proteins as determined by SDS-PAGE were 87, 83, 78 and 72 kDa (Fig. 5). These iron-regulated proteins were solubilized at 60 °C by 2% SDS-PAGE sample buffer and therefore they were unlikely to be associated with the peptidoglycan. Furthermore, trypsin digestion of the outer membrane isolated from iron-starved cells resulted in the cleavage of the iron-regulated outer membrane proteins to yield lower molecular mass fragments (data not shown). This suggests that the iron-regulated proteins were exposed and that they probably function as receptors for iron compounds.

Fig. 5. SDS-PAGE (8%, w/v, acrylamide) of the outer membrane proteins of *A. brasilense* RG showing the induction of the iron-regulated proteins. A, outer membrane proteins in cells starved of iron by the addition of the iron chelator EDDA ($50 \mu\text{g ml}^{-1}$) to the growth medium (LBS) at an OD_{590} of 0.2, and harvested after 3 h at an OD_{590} of 0.6. B, Molecular mass markers.

DISCUSSION

As in many Gram-negative bacteria the outer membrane of *A. brasilense* RG contains only a few proteins, and a single 42 kDa protein constitutes over 60% of the total protein in the outer membrane. This protein, along with another minor 40 kDa protein, is associated with the peptidoglycan, and they probably function as non-specific diffusion channels or porins as has been shown with the peptidoglycan-associated proteins of *Escherichia coli*, *Pseudomonas aeruginosa* and *Salmonella typhimurium* (Lugtenberg & Van Alphen, 1983). Functional porins are generally formed from the oligomers of these proteins. The peptidoglycan-associated proteins of *A. brasilense* clearly have an oligomeric form although the nature of this oligomer has not been ascertained. The oligomer is stable even in 2% SDS at 60 °C and has a molecular mass of 70 kDa as determined by SDS-PAGE. However, the molecular masses of these complexes are not correctly reflected by SDS-PAGE as most of these proteins have an increased β -structure and the oligomers bind far less SDS than the monomer (Nakae *et al.*, 1979), which leads to their anomalous electrophoretic mobilities.

An unusual feature of the outer membrane proteins of *A. brasilense* RG is the trypsin-insensitivity of the heat-modifiable protein, as hitherto a characteristic feature of the heat-modifiable OmpA protein of *E. coli* and the highly conserved heat-modifiable OmpA-like proteins of other Gram-negative bacteria has been their sensitivity to cleavage by trypsin (Beher *et al.*, 1980).

Comparison of the SDS-PAGE outer membrane protein profiles of different *Azospirillum* species indicates that the profile is characteristic of each species and could be useful as an aid to taxonomy. For example, strain K67 was previously tentatively classified as *A. brasilense* (Franche & Elmerich, 1981) but its outer membrane protein profile now suggests that it may belong to a new species completely different from *A. brasilense*, *A. lipoferum* or *A. amazonense*. Although strain-specific chemotaxis has been recently demonstrated in *Azospirillum* (Reinhold *et al.*, 1985), and strain-specific variations in the outer membrane of Gram-negative bacteria do occur and have been used as a basis of subtyping of species (Barenkamp *et al.*, 1981), the significantly different profile of K67 from the typical *A. brasilense* profile, and the previous difficulty in classifying this strain (Franche & Elmerich, 1981), make it difficult to reconcile with the idea that the strain merely belongs to a different subgroup. Among the new isolates of the genus *Azospirillum*, however, subtyping of species along with species identification may

become important and outer membrane protein profiles could be an aid to a rapid taxonomic grouping. Furthermore, comparison of the protein profiles of different groups and subgroups could reveal interesting information regarding their association with roots of different plants.

Outer membrane proteins of the phytopathogen *Erwinia chrysanthemi* induced during iron-limitation have been implicated in the plant pathogenic activity of this bacterium (Expert & Toussaint, 1985). Moreover, phytopathogenic microbial species are, in general, siderophore producers (Neilands & Leong, 1986). The four new outer membrane proteins induced under iron-limited growth of *A. brasilense* RG probably act as receptors for iron-containing compounds (accompanying paper: Bachhawat & Ghosh, 1987) but whether these proteins play any significant role in 'associative symbiosis' remains to be investigated.

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