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Starvation-induced changes in the cell surface of *Azospirillum lipoferum*

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Abstract

Three starvation regimes (a deficient culture medium, a saline buffer solution and distilled water) were evaluated for their possible effect on cell surface characteristics of *Azospirillum lipoferum* 1842 related to the initial adsorption of the bacterium to surfaces. The bacteria survived for 7 days in all media although they did not multiply. Upon transfer from a rich growth medium (nutrient agar) to starvation conditions, cell surface hydrophobicity dropped sharply but recovered its initial value within 24 to 48 h, except in phosphate-buffered saline, the length of the recovery period depending on the starvation medium. Starvation affected the sugar affinity of the *A. lipoferum* cell surface mainly towards *p*-aminophenyl-α-D-mannopyranoside, to a lesser extent to glucose, but not to other monosaccharides tested. Starvation changed the concentration of several cell surface proteins but did not induce the synthesis of new ones. The cell surface hydrophobic protein (43 kDa) of *A. lipoferum* 1842 was unaffected by any starvation treatment for a period of up to 48 h, but later disappeared. These data showed that starvation is not a major factor in inducing changes in the cell surface which lead to the primary phase of attachment of *Azospirillum* to surfaces.

Keywords: *Azospirillum*; Starvation; Cell surface protein; Plant growth-promoting rhizobacteria

1. Introduction

In undisturbed or untreated soil without growing plants, most bacteria persist under nutrient-limited conditions [1]. Bacteria have evolved mechanisms allowing survival under starvation, rapidly restarting growth once nutrients become available. For example, bacilli and clostridia undergo differentiation leading to the formation of highly resistant endospores [2]. Similarly, under the influence of toxic metals, culture conditions, aging or water stress, *Azospirillum* cells can be transformed into cysts [3–9] with long survival times [10], that can rapidly reform vegetative cells when favorable conditions return [11].

The physiological responses to starvation, like protein synthesis and cell culturability, have been studied in detail in *Escherichia coli*, *Salmonella* and in marine vibrios. The adaptation to starvation conditions is often accompanied by the synthesis of proteins essential for long-term survival [12–18]. Limited information is available for soil bacteria such as *Pseudomonas* sp. [19,20], and for plant-associated bacteria such as *Rhizobium leguminosarum* bv. *phaseoli* [18] and *Azospirillum brasilense* [21].

Azospirillum sp. are plant growth-promoting bacteria (PGPB) surviving in the rhizosphere of numerous plant species for prolonged periods [22,23], but usually poorly in semiarid soils [24]. Without root exudates, the *Azospirillum* cells are in a permanent state of starvation. It is unknown whether starvation helps or impairs the capacity of a bacterium inoculated into the soil to reach the rhizosphere of the target plant.

Attachment of *Azospirillum* to surfaces is an essential part of the life cycle of the bacterium. *Azospirillum* can attach to soil particles [25], to root surfaces [26], to inert surfaces such as alginate beads [27] or hydrophobic polystyrene [28]. On roots, the general attachment process is divided into two phases, adsorption and anchoring, probably governed by different mechanisms [29,30]. Since the effects of starvation on the primary adsorption capacity of *Azospirillum* cells is poorly known, we studied several starvation-induced changes in *Azospirillum lipoferum* 1842. We

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specifically monitored cell surface changes in hydrophobicity, protein composition and the expression of lectin-like proteins, which are related to the initial adsorption of the cells to surfaces.

2. Materials and methods

2.1. Bacterial strain and starvation conditions

A. lipoferum 1842 (DSM 1842, Braunschweig, Germany) was used in this study. Under unstarved conditions, the bacteria were grown in nutrient agar (NA; Merck, Darmstadt, Germany) at 30 ± 2°C for 48 h in Petri dishes.

Bacterial cells were harvested from the surface of five Petri dishes with a microbiological loop, resuspended and diluted in phosphate-buffered saline (PBS) (0.015 M K_2HPO_4 and 0.015 M KH_2PO_4 supplemented with 0.15 M NaCl, pH 7.2) to a concentration of approximately 10^{10} CFU ml^{-1} and then were diluted 1:1000 in one of the following media to a final volume of 100 ml: (1) nitrogen-free malate medium (Nfm) [31], serving as a control of a minimal full medium; (2) C-free Nfm medium, supplemented with a nitrogen source (NH_4Cl, 0.2%); (3) PBS; and (4) distilled water. The bacteria were incubated in a horizontal shaker at 100 rpm at 30 ± 2°C. Aliquots (2 ml) from each medium were taken at 1, 3, 5, 8, 24, 48, 72, 96 and 120 h; the optical density (OD) was measured at 540 nm and the samples were then set aside for a count of the CFU by the plate count method on NA. The remaining 98 ml of each medium were centrifuged at 4000 × *g* for 10 min, the pellet was resuspended in 2 ml of PBS from which 200 μl were used to address cell surface hydrophobicity (CSH) and lectin activity by particle agglutination assays (PAA). The remaining 1.8 ml of bacterial suspension was centrifuged at 5000 × *g* for 5 min and resuspended in 6 M urea to obtain the cell surface protein extract.

2.2. CSH assay

CSH was determined by the aqueous, two-polymer phase-partitioning assay of Johansson [32]. Results were expressed as $\Delta \log G$ as described in detail previously [33].

2.3. Sugar-conjugated latex beads

The preparation of neoglycoprotein-coated latex beads (0.813 mm diameter; Seradyn, IN, USA) was done according to Naidu et al. [34]. The latex bead suspension was divided into 200-μl aliquots and each aliquot was mixed with 100 μg of the following neoglycoproteins (0.5 mg ml^{-1}) separately: bovine serum albumin (BSA)–fucosylamide, BSA–fructosamine (glycated) (Sigma # A8426), BSA–galactosamide, BSA–glucosamide, BSA–*p*-aminophenyl-α-D-mannopyranoside, BSA–*p*-amino-*N*-acetyl-β-D-galactosaminide and BSA–*p*-aminophenyl-*N*-acetyl-β-D-glucosaminide (Sigma, St. Louis, MO, USA). These mixtures were incubated at 30 ± 2°C for 12 h in a horizontal shaker at 50 rpm to ensure covering of the latex beads with each of the neoglycoproteins. The mixtures were then centrifuged at 9200 × *g* for 5 min at 20 ± 2°C and the supernatants discarded. The pellets containing latex beads conjugated with neoglycoproteins were resuspended in 2 ml of PBS and kept at 4 ± 1°C until used.

2.4. PAA

The PAA was done as described by Naidu et al. [34]. Briefly, 20-μl aliquots of the sugar-conjugated latex beads were placed on a glass slide and 20 μl of bacterial cell suspension were added and mixed. The reactions were scored using the index 0, no agglutination; 1, weak agglutination; 2, apparent agglutination; and 3, strong agglutination, where almost all cells were agglutinated. The controls for autoaggregation were (i) 20 μl of bacterial cell suspension mixed with 20 μl of PBS buffer, (ii) 20 μl of bacterial cell suspension mixed with 20 μl of 1 mg ml^{-1} BSA, (iii) 20 μl of bacterial cell suspension mixed with 20 μl of latex bead suspension and (iv) 20 μl of bacterial cell suspension mixed with 20 μl of latex beads coated with BSA.

2.5. Extraction of cell surface proteins

This was done by minor modifications of the method of Chagnaud et al. [35]. One ml of starved *A. lipoferum* 1842 cells in PBS from each medium after 1, 3, 5, 8, 24, 48, 72, 96 and 120 h were centrifuged at 3000 × *g* for 5 min at 25 ± 1°C. Cell pellets were resuspended in 1 ml of 6 M urea (Merck, Darmstadt, Germany) for 1 h at 25 ± 1°C. The extracted cell suspension was centrifuged at 8000 × *g* for 10 min at 10 ± 1°C. The pellet was discarded and the supernatant precipitated with 80% $(NH_4)_2SO_4$ at 4 ± 1°C overnight. The precipitate was dissolved in 2 ml distilled water and dialyzed against 2 l of 0.01 M ammonium bicarbonate, pH 8.0 for 48 h at 4 ± 1°C with several replacements of the buffer. The protein concentration in the extracts was determined according to Bradford [36]. Extracts of cell surface proteins from unstarved cells were obtained by the same method as in our previous study [33].

2.6. Antibody preparation

Partially purified 43-kDa cell surface hydrophobic protein of *A. lipoferum* 1842 with affinity for several monosaccharides [37] was used to immunize 2-month-old BALB-cAnM mice (Instituto de Investigaciones Biomedicas, UNAM, Mexico City) by four intraperitoneal injections at 2-week intervals. One mg ml^{-1} of the partially purified protein was run in a preparative gel [38] and the band cut into 1-cm fragments. Each fragment was emulsified in a syringe with 1 ml of complete Freund's adjuvant

for the first immunization and incomplete Freund's adjuvant for the subsequent three boosters. The mice were bled after 50 days. Antibody titers and specificity were determined by enzyme-linked immunosorbent assay. Antisera were stored at −20°C.

2.7. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and 2D SDS–PAGE

The cell surface protein extracts of the three starvation treatments and an extract from unstarved bacteria were compared by SDS–PAGE using 12.5% polyacrylamide slab gels according to Laemmli [38]. Concentrations of 0.1 mg ml^{-1} of protein were loaded in each lane of each gel. In Western blots, the cell surface protein extracts were also analyzed by 2D SDS–PAGE using isoelectric focusing gels as the first dimension and SDS–PAGE as the second dimension [39]. Briefly, the first dimension was run in 1.5-mm inner diameter gas tubes, filled with a gel solution containing: urea, 9 M; acrylamide/bis-acrylamide, 40% T 5% C_{bis}; Np40 (Igepal), 10% (Sigma); ampholites 5–7, 40%; ampholites 3–10, 10% (Bio-Rad); TEMED; ammonium persulfate, 10%; and distilled water. The gel was polymerized for about 3 h. After the polymerization the tubes were placed in the electrophoresis chamber adding 5 μl of sample overlay (9 M urea, 2% ampholytes). The gel was prerun for 15 min at 200 V, 30 min at 300 V and 30 min at 400 V. Then a sample was added onto the top of each tube, in sample buffer (9.5 M urea, 2% NP-40, 2% ampholytes, 5% 2-mercaptoethanol) and the gel was run for 16 h at a constant 400 V. After the run, the gel was removed from the tube and placed horizontally onto the top of a polymerized slab gel for the second dimension electrophoresis, and run for 2 h at 100 mA. The gels were stained by polychromatic silver staining [40].

2.8. Immunoblotting

The separated proteins from 2D SDS–PAGE were electrophoretically transferred to a nitrocellulose membrane (Bio-Rad, Richmond, CA, USA) in a semidry trans-blot cell (Owl, Woburn, MA, USA) for 2 h at 200 mA. They were then blocked with 3% BSA in PBS for 1 h at 50 ± 1°C. The membranes were then incubated for 1 h at 25 ± 1°C in a 1:20 dilution of the antibody preparations. After three rinses (15 min each, in 0.05% Triton X-100 (Sigma) in PBS (PBST)), the membranes were incubated with a 1:5000 dilution of alkaline phosphatase-conjugated secondary mouse antibody in PBST (Boehringer-Mannheim, Indianapolis, IN, USA) for 1 h at 25 ± 1°C. The membranes were rinsed three times for 15 min each with PBST and once with PBS. Bands were visualized after incubating the membranes for 5–10 min in a solution containing 66 μl nitro-blue tetrazolium and 33 μl 5-bromo-4-chloro-5-indolyl phosphate (Boehringer-Mannheim, Indianapolis, IN, USA) in 10 ml of a solution containing 100 mM NaCl, 5 mM of $MgCl_2$ and 100 mM diethanolamine (Boehringer-Mannheim, Indianapolis, IN, USA) [41].

2.9. Experimental design and statistical analysis

Experiments were repeated at least twice for different cultures and every determination was done in duplicate. A replicate consisted of two partitioning assays for the hydrophobicity assays. The data from both repetitions were combined and analyzed by one-way analysis of variance (ANOVA) at $P \leq 0.05$ using SigmaPlot (Jandel, San Rafael, CA, USA) and GraphPad Prism (San Diego, CA, USA) software. Bacterial growth measurements in the three starvation media were repeated four times. Data for a representative experiment are shown. The PAA data shown are the averages of two repetitions. The SDS–PAGE was done three times and the 2D SDS–PAGE gels and immunoblotting were done four times. The results shown are from a representative run.

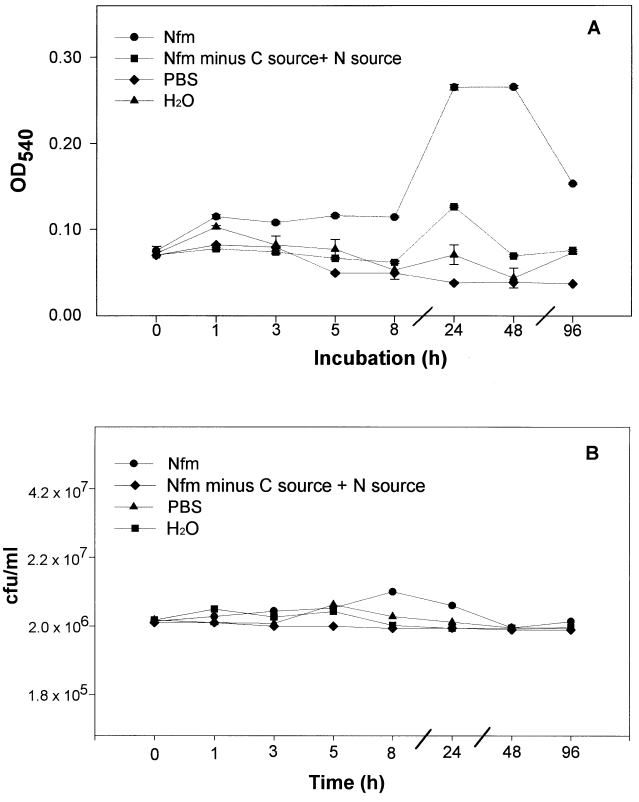

Fig. 1. Variations in culture characteristics of *A. lipoferum* 1842 during three starvation regimes. A: Cell mass determined as OD at 540 nm. B: Number of culturable cell CFU ml^{-1}. Bars represent S.E.M. The absence of S.E.M. indicates that the S.E.M. is smaller than the point.

3. Results

3.1. Survival of *A. lipoferum* under starvation conditions

Cultures of *A. lipoferum* 1842 grew during the first 24 h in the control Nfm medium and slightly in Nfm C-free medium, but not in PBS and water. A slight decrease in OD occurred during carbon starvation with the OD being lowest in PBS and H_2O (Fig. 1A).

Cell viability and culturability showed no significant changes during the experiment and ranged from 1×10^6 to 1×10^7 CFU ml^{-1} (Fig. 1B). Cells of *A. lipoferum* 1842 remained viable after a month in Nfm growth medium (control) and in the three starvation media at a level of 1×10^6 CFU ml^{-1} (data not shown).

3.2. Hydrophobicity of *A. lipoferum* under starvation conditions

The three starvation regimes displayed different patterns of CSH to cells growing in Nfm control medium. Upon transferring the cells from the rich NA medium on which they grew, to poorer (Nfm minimal medium) or starvation media (PBS, C-free Nfm and H_2O), the CSH sharply decreased in the first 4–5 h, but later showed an increase at about 8 and 24 h in the Nfm and Nfm medium plus N source, this increase is higher and quicker in PBS and there is no increase in H_2O. After 40–48 h the hydrophobicity of the cells recovered except in the PBS treatment (Fig. 2A–D).

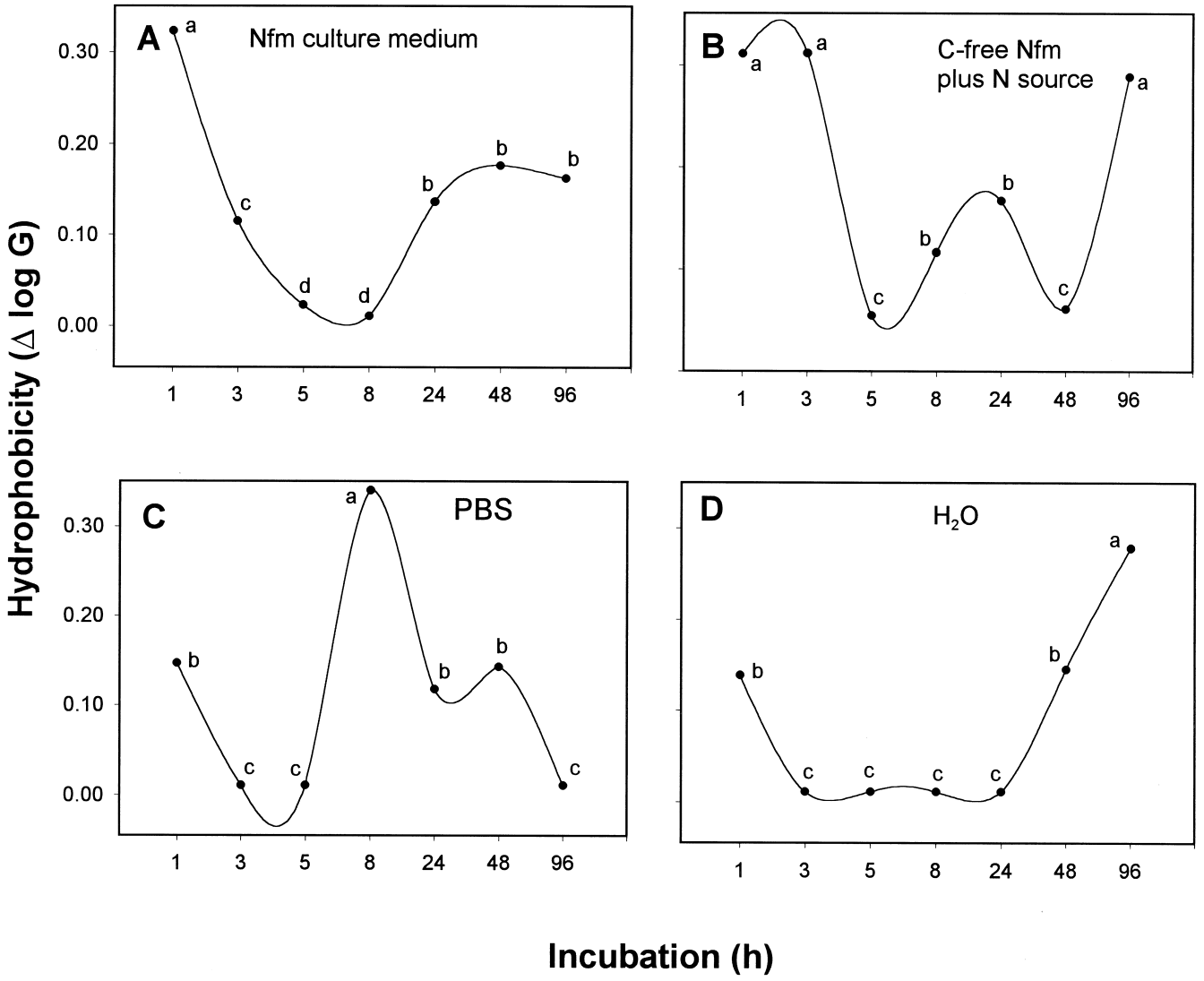

Fig. 2. CSH (expressed as $\Delta\log G$) of *A. lipoferum* 1842 under starvation. Points denoted with different letter at each subfigure separately differ significantly at $P\leq0.05$ using one-way ANOVA.

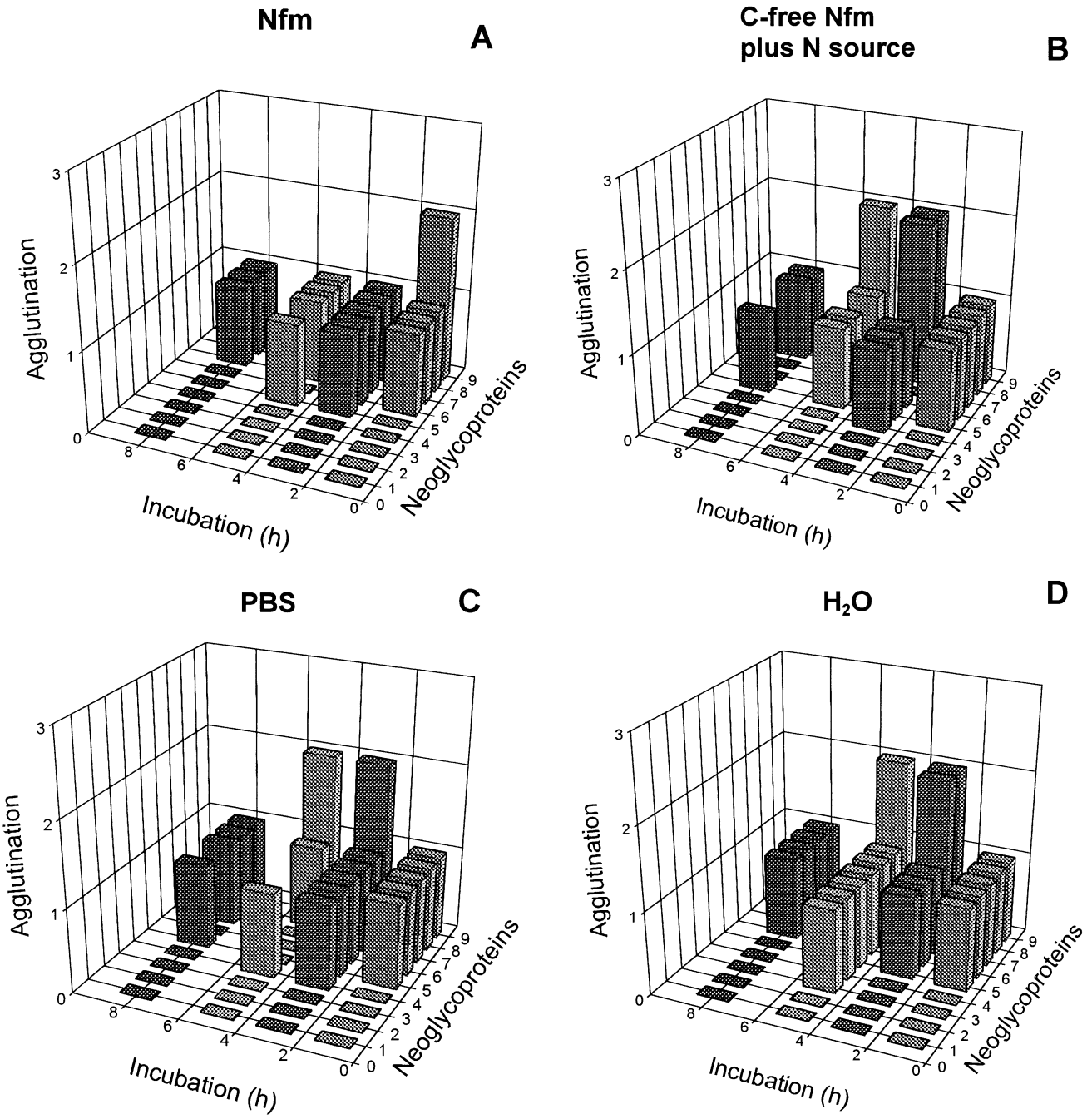

Fig. 3. PAA of *A. lipoferum* 1842 exposed to starvation in: A: Nfm medium as a control, B: C-free Nfm with nitrogen source, C: PBS, and D: H_2O. Lane 1, BSA; lane 2, PBS; lane 3, BSA–fructosamine (glycated); lane 4, BSA–fucosylamide; lane 5, BSA–galactosamide; lane 6, BSA–*p*-aminophenyl-*N*-acetyl-β-D-glucosaminide; lane 7, BSA–*p*-amino-*N*-acetyl-β-D-galactosaminide; lane 8, BSA–glucosamide; lane 9, BSA–*p*-aminophenyl-α-D-mannopyranoside.

3.3. Carbohydrate-binding activity

The PAA showed a general slow decrease in affinity of *A. lipoferum* 1842 cells for monosaccharides with time, both in starved and unstarved conditions (Fig. 3A–D). Expression of the highest carbohydrate-binding affinity was towards *p*-aminophenyl-α-D-mannopyranoside both under starved and unstarved treatments (Fig. 3A–D, lane 9). In starvation regimes using H_2O and C-free Nfm medium, glucosamide was highly expressed (Fig. 3B,D, lane 8). BSA and PBS (as controls) and the neoglycoprotein BSA–fructosamine caused no agglutination in all starvation regimes. Confirmation of the affinity of several cell surface proteins towards *p*-aminophenyl-α-D-mannopyranoside done by Western blot demonstrated that in Nfm control medium and in starvation in H_2O the affinity to-

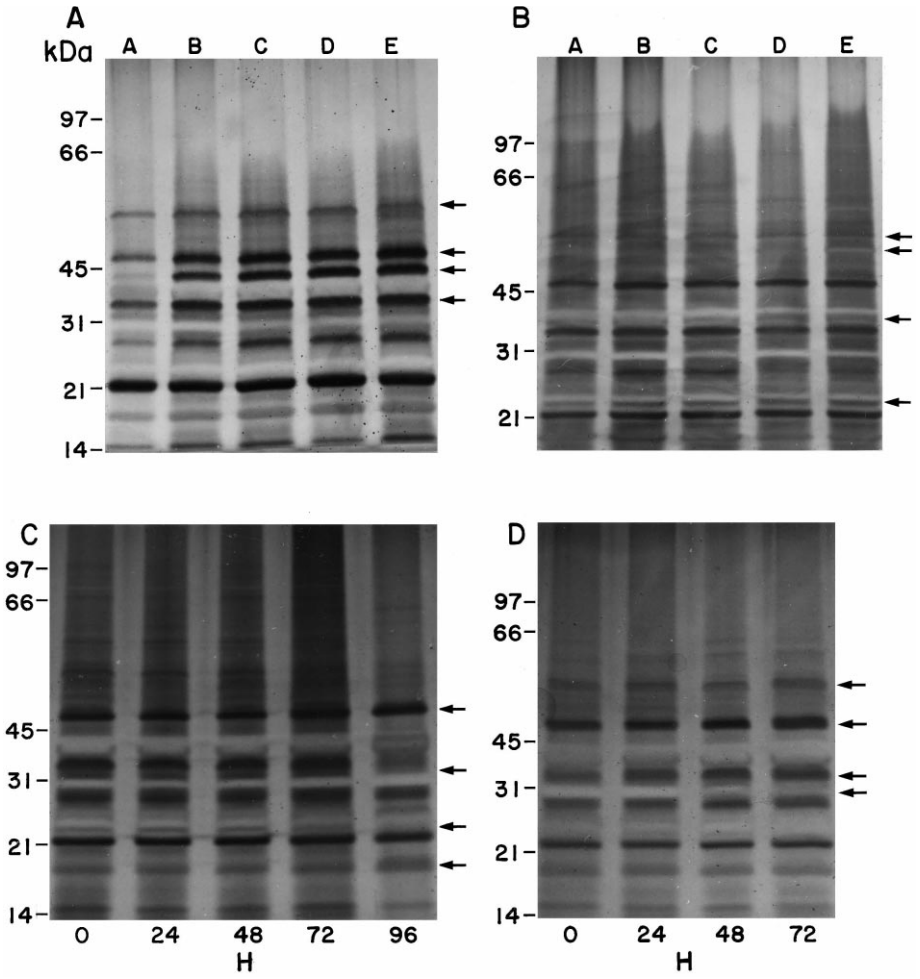

Fig. 4. SDS–PAGE pattern of cell surface proteins of *A. lipoferum* 1842 exposed to starvation regimes in: A: Nfm medium as a control, B: C-free Nfm with a nitrogen source, C: PBS, and D: H_2O. Starvation-changed proteins are marked by arrowheads. For clarity, the migration of molecular mass standard proteins is not shown but is indicated in the left margin. Lanes A–E indicate sampling time after 0, 24, 48, 72, 96 h respectively, of starvation.

wards the sugar remained for 48 h while in C-free Nfm medium and in PBS, it diminished with time (data not shown).

3.4. SDS–PAGE analysis of cell surface proteins

Changes in cell surface proteins were analyzed in SDS–PAGE after O, 1, 3, 5, 8, 12, 24, 48, 72, and 96 h of starvation (Fig. 4). No changes were observed during the first 24 h. No formation of new proteins occurred during the starvation regimes, but they were changed in quantity. The major cell surface proteins present under standard growth conditions were all maintained during starvation regardless of the medium (Fig. 4A–D). A protein of about 44 kDa present in the Nfm which increased within hours, decreased and almost disappeared during the other starvation treatments. Changes in the concentration of various proteins occurred. Bacteria cultured in C-free Nfm medium showed an increase in concentration of one protein of 40 kDa and one of 22 kDa (Fig. 4B, lanes A–E). Bacterial cells kept in PBS showed an increased concentration of two proteins, 47 and 18 kDa, and an decreased concentration in two others, 23, and 33 kDa (Fig. 4C, lanes A–E). Cells starved in H_2O showed an increase in abundance of two proteins of 47 and 28 kDa and a decrease in concentration in one of 30 kDa (Fig. 4D, lanes A–D). Two proteins of 39 and 48 kDa increased in abundance in all starvation treatments with time. A protein of 55 kDa is present in similar quantities in Nfm C-free medium and in H_2O while decreasing in the other starvation media. Similar patterns were observed in the four independent repetitions.

3.5. 2D PAGE analysis of cell surface proteins

When cell surface extracts were analyzed first by 2D PAGE followed by Western blot using antibodies raised against the 43-kDa hydrophobic protein from the cell wall of *A. lipoferum* 1842 [33], three spots were detected from bacteria grown in C-free Nfm medium and in PBS and H_2O. These represent isoforms of the same protein (Fig. 5A–C). These isoforms changed in concentration depend-

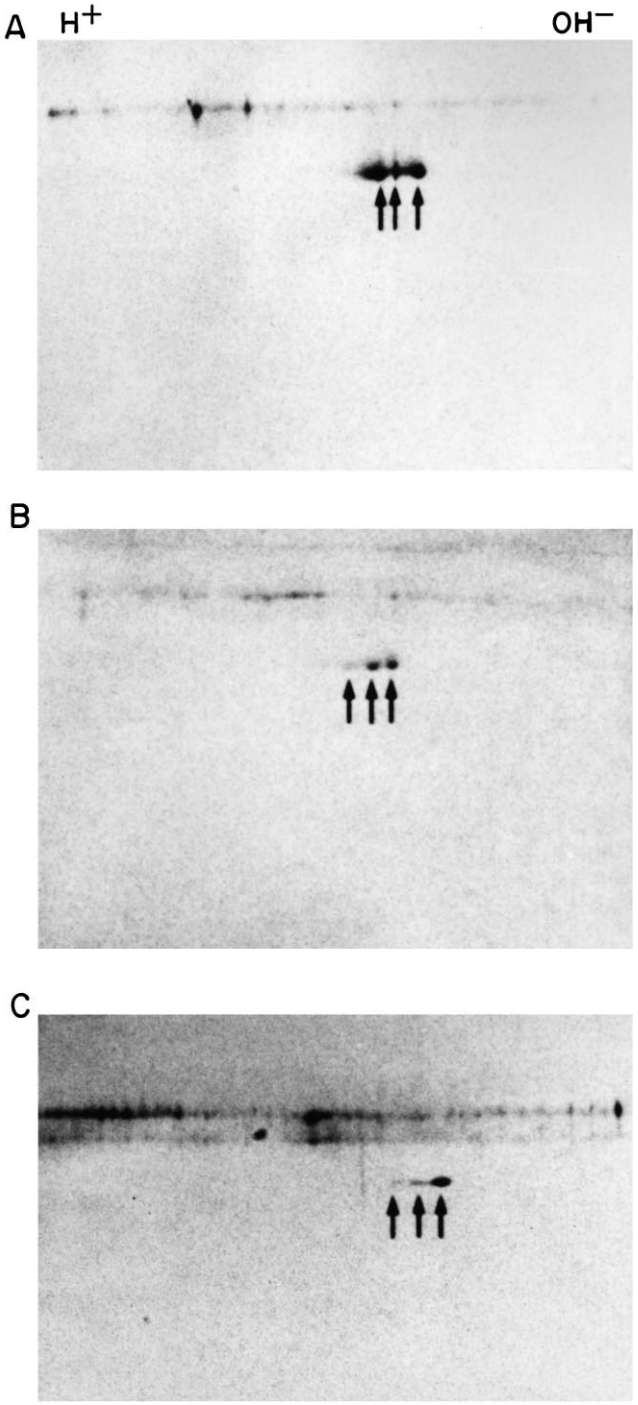

Fig. 5. Western blot of a 2D PAGE of cell surface proteins of *A. lipoferum* 1842 after 48 h of starvation. A: C-free Nfm with a nitrogen source, B: PBS, and C: H_2O. Isoforms are marked by arrowheads.

ing on the starvation type up to 48 h incubation (data not presented). No isoforms were detected after 72 h incubation in all starvation regimes.

4. Discussion

The process of inoculating plants with the PGPB *Azospirillum* sp. starts with culturing of the bacteria in a rich medium either in a laboratory flask or in a plant fermenter and then a solid or liquid inoculant is elaborated in a rich medium [42]. Immediately after seed or soil inoculation, the bacteria, repleted with poly-β-hydroxybutyrate (PHB) [43] undergo a progressive starvation process. It can be short (a few days) if the seed germinates quickly and the new rhizosphere starts to support the bacterial population, or long (up to several weeks) when the seed germinates slowly or when the inoculant is inoculated onto unirrigated crop seeds awaiting rain.

Cells containing high levels of PHB make a better inoculant than those lacking PHB [44]. Nevertheless, regardless of the cell history, both types (with and without PHB) must attach to the root surface. We did not examine PHB effects during starvation because the goal of this study was to analyze proteins of the cell surface. Of the two attachment phases of *Azospirillum* to roots [29], the absorption phase is relatively unknown compared to the anchoring phase in which fibrillar material is involved [11,45]. Recent findings suggested that changes in cell surface characteristics such as CSH and charge [33], changes in adhesion of the cell [46], and expression of the bacterial cell surface lectins [37] occur during this period. How the cell surface is changed during the imposed starvation of the bacteria is unknown.

In this work, the apparent growth in Nfm during the first 24 h could be because *A. lipoferum* develops predominantly into pleomorphic cells within 48 h, mainly as an elongated S or helical shape. This may be related to alkalinization of the malate in the medium [47].

Four patterns of starvation survival when microorganisms are stressed were proposed by Morita [48]: (1) increase in the number of cells when starved, (2) a rapid die-off, (3) increase in the number of cells followed by maintenance of a constant level of viable cells, and (4) organisms that neither die nor increase cell numbers. According to these criteria, *A. lipoferum* 1842 could be considered a type-four bacterium. While the first three patterns have been observed in marine *Vibrio* [49], marine *Pseudomonas* sp. [50], and in freshly isolated open ocean bacterial strains [51], type four is more common in bacteria from an energy-poor environment [48].

In *E. coli*, at the onset of starvation, there is a formation of 20 to 40 new proteins [52,53]. In contrast, in *A. brasilense* cells growing in iron-limited conditions, only four new proteins in the outer membrane were synthesized [54]. Similarly, our starvation regimes yielded no new proteins but only a change in the concentration of existing proteins. One 39-kDa protein increased in concentration under most of the nutritional stresses. The formation of *E. coli* starvation proteins could be detected either early or late during starvation [52]. In *A. lipoferum* 1842, the changes in the existing proteins occurred in the first 24 h of starvation.

Western blot showed that the cell surface hydrophobic

protein [33], which has a lectin-like affinity [37], could not be detected after 72 h of starvation. This was in accordance with the gradual loss of affinity for monosaccharides that we measured by PAAs. The explanation for this could be: (i) during starvation the protein content is very small and we failed to detect it, (ii) the protein may have been transformed, explaining why we detected three spots of isoforms, or (iii) the protein was completely degraded during starvation.

Adhesion to surfaces is a possible survival strategy for starved bacteria [55]. In PGPB, the increase in cell surface protein concentration and CSH during growth of *A. brasilense* was correlated with an increase of cell adhesion to glass and polystyrene, but only in rich media [47]. In *R. leguminosarum*, nutrient limitation coincides with optimal attachment. The type of starvation determined whether host lectins participated in the attachment process [56]. Our results with *A. lipoferum* 1842 showed a decreased activity of the lectin-like proteins and only small changes in the quantity of cell surface proteins. There is still a need to show that these changes might affect the adsorption of *A. lipoferum* to roots.

From the data we have so far, it appears that starvation is not a main cause of changes in the cell surface properties that lead to the primary phase of attachment of *Azospirillum* to surfaces.

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References

[1] Kjelleberg, S. (1993) Starvation in Bacteria. Plenum Press, New York.
[2] Errington, J. (1993) *Bacillus subtilis* sporulation: regulation of gene expression and control of morphogenesis. Microbiol. Rev. 57, 1–33.
[3] Arunakumari, A., Lamm, R.B. and Neyra-Estens, C.A. (1992) Changes in cell surface properties of Azospirilla in relation to cell pleomorphism and aggregation. Symbiosis 13, 291–305.
[4] Bashan, Y., Levanony, H. and Whitmoyer, R.E. (1991) Root surface colonization of non-cereal crop plants by pleomorphic *Azospirillum brasilense* Cd. J. Gen. Microbiol. 137, 187–196.
[5] Bleakly, B.H., Gaskin, M.H., Hubbell, D.H. and Zam, S.G. (1988) Floc formation by *Azospirillum lipoferum* grown on poly-β-hydroxybutyrate. Appl. Environ. Microbiol. 54, 2986–2995.
[6] Gowri, P.M. and Srivastava, S. (1996) Encapsulation as a response of *Azospirillum brasilense* Sp7 to zinc stress. World J. Microbiol. Biotechnol. 12, 319–322.
[7] Oliveira, R.G.B. and de Souza, M.L. (1991) Partial characterization of a brown pigment produced by a *Azospirillum lipoferum*. Rev. Microbiol. (Braz.) 22, 340–344.
[8] Sadasivan, L. and Neyra, C.A. (1985) Flocculation in *Azospirillum brasilense* and *Azospirillum lipoferum*: exopolysaccharides and cyst formation. J. Bacteriol. 163, 716–723.
[9] Sadasivan, L. and Neyra, C.A. (1987) Cyst production and brown pigment formation in aging cultures of *Azospirillum brasilense* ATCC 29145. J. Bacteriol. 169, 1670–1677.
[10] Assmus, B., Hutzler, P., Kirchhof, G., Amann, R., Lawrence, J.R. and Hartmann, A. (1995) In situ localization of *Azospirillum brasilense* in the rhizosphere of wheat with fluorescently labeled rRNA-targeted oligonucleotide probes and scanning with confocal laser microscopy. Appl. Environ. Microbiol. 61, 1013–1019.
[11] Bashan, Y., Levanony, H. and Whitmoyer, R.E. (1991) Root surface colonization of non-cereal crop plants by pleomorphic *Azospirillum brasilense* Cd. J. Gen. Microbiol. 137, 187–196.
[12] Foster, J.W. and Spector, M.P. (1995) How *Salmonella* survive against the odds. Annu. Rev. Microbiol. 49, 145–174.
[13] Morton, D.S. and Oliver, J.D. (1994) Induction of carbon starvation-induced proteins in *Vibrio vulnificus*. Appl. Environ. Microbiol. 60, 3653–3659.
[14] Nyström, T. (1995) Glucose starvation stimulation of *Escherichia coli*: Role of integration host factor in starvation survival and growth phase-dependent protein synthesis. J. Bacteriol. 177, 5707–5710.
[15] Nyström, T., Albertson, N. and Kjelleberg, S. (1988) Synthesis of membrane and periplasmic proteins during starvation of a marine vibrio sp. J. Gen Microbiol. 134, 1645–1651.
[16] Nyström, T., Olsson, R.M. and Kjelleberg, S. (1992) Survival, stress resistance, and alterations in protein expression in the marine *Vibrio* sp. strain S14 during starvation for different individual nutrients. Appl. Environ. Microbiol. 58, 55–65.
[17] Spector, M.P. and Foster, J.W. (1993) Starvation-stress response (ssr) of *Salmonella typhimurium*: gene expression and survival during nutrient starvation. In: Starvation in Bacteria (Kjelleberg, S., Ed.), pp. 201–224. Plenum, New York.
[18] Thorne, S.H. and Williams, H.D. (1997) Adaptation to nutrient starvation in *Rhizobium leguminosarum* bv. phaseoli: Analysis of survival, stress resistance, and changes in macromolecular synthesis during entry to and exit from stationary phase. J. Bacteriol. 179, 6894–6901.
[19] Kragelund, L. and Nybroe, O. (1994) Culturability and expression of outer membrane proteins during carbon, nitrogen, or phosphorus starvation of *Pseudomonas fluorescens* DF57 and *Pseudomonas putida* DF14. Appl. Environ. Microbiol. 60, 2944–2948.
[20] Van Overbeek, L.S., Eberl, L., Bivskov, M., Molin, S. and Van Elsas, J.D. (1995) Survival of induced stress resistance in carbon-starved *Pseudomonas fluorescens* cells residing in soil. Appl. Environ. Microbiol. 61, 4202–4208.
[21] Tal, S., Smirnoff, P. and Okon, Y. (1990) The regulation of poly-β-hydroxybutyrate metabolism in *Azospirillum brasilense* during balanced growth and starvation. J. Gen. Microbiol. 136, 1191–1196.
[22] Bashan, Y. and Holguin, G. (1997) *Azospirillum*-plant relationships: environmental and physiological advances (1990–1996). Can. J. Microbiol. 43, 103–121.
[23] Bashan, Y. and Holguin, G. (1997) Short- and medium-term avenues for *Azospirillum* inoculation. In: Plant Growth-Promoting Rhizobacteria. Present Status and Future Prospects (Ogoshi, A., Kobayashi, K., Homma, Y., Kodama, F., Kondo, N. and Akino, S., Eds.), pp. 130–149. Faculty of Agriculture, Hokkaido University, Sapporo.
[24] Bashan, Y., Puente, M.E., Rodriguez-Mendoza, M.N., Toledo, G., Holguin, G., Ferrera-Cerrato, R. and Pedrin, S. (1995) Survival of *Azospirillum brasilense* in the bulk soil and rhizosphere of 23 soil types. Appl. Environ. Microbiol. 61, 1938–1945.
[25] Bashan, Y., Mitiku, G., Whitmoyer, R.E. and Levanony, H. (1991)

Evidence that fibrillar anchoring is essential for *Azospirillum brasilense* Cd attachment to sand. Plant Soil 132, 73–83.
[26] Vande Broek, A., Michiels, J., Van Gool, A. and Vanderlayden, J. (1993) Spatial-temporal colonization patterns of *Azospirillum brasilense* on the wheat root surface and expression of the bacterial *nifH* gene during association. Mol. Plant-Microbe Interact. 6, 592–600.
[27] Bashan, Y. (1986) Alginate beads as synthetic inoculant carriers for slow release of bacteria that affect plant growth. Appl. Environ. Microbiol. 51, 1089–1098.
[28] Bashan, Y. and Holguin, G. (1993) Anchoring of *Azospirillum brasilense* to hydrophobic polystyrene and wheat roots. J. Gen. Microbiol. 139, 379–385.
[29] Michiels, K.W., Croes, C.L. and Vanderleyden, J. (1991) Two different modes of attachment of *Azospirillum brasilense* Sp7 to wheat roots. J. Gen. Microbiol. 137, 2241–2246.
[30] Michiels, K., Verreth, C. and Vanderleyden, J. (1990) *Azospirillum lipoferum* and *Azospirillum brasilense* surface polysaccharide mutants that are affected in flocculation. J. Appl. Bacteriol. 69, 705–711.
[31] Reinhold, B., Hurek, T., Fendrik, I., Pot, B., Gillis, M., Kersters, K., Thielemans, S. and De Ley, J. (1987) *Azospirillum halopraeferens* sp. Nov., a nitrogen-fixing organism associated with roots of kallar grass (*Leptochloa fusca* (L.) Kunth). Int. J. Syst. Bacteriol. 37, 43–51.
[32] Johansson, G. (1974) Partition of proteins and micro-organisms in aqueous biphasic systems. Mol. Cell. Biochem. 4, 169–180.
[33] Castellanos, T., Ascencio, F. and Bashan, Y. (1997) cell surface hydrophobicity and cell surface charge of the *Azospirillum* spp. FEMS Microbiol. Ecol. 24, 159–172.
[34] Naidu, A.S., Paulsson, M. and Wadström, T. (1988) Particle agglutination assay for rapid detection of fibronectin, fibrinogen, and collagen receptors on *Staphylococcus aureus*. J. Clin. Microbiol. 26, 1549–1554.
[35] Chagnaud, P., Jenkinson, H.F. and Tannock, G.W. (1992) Cell surface-associated proteins of gastrointestinal strains of *lactobacilli*. Microb. Ecol. Health Dis. 5, 121–131.
[36] Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.
[37] Castellanos, T., Ascencio, F. and Bashan, Y. (1998) cell surface lectins of *Azospirillum* spp. Curr. Microbiol. 36, 241–244.
[38] Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.
[39] O'Farrell, P.H. (1975) High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. 250, 4007–4021.
[40] Hames, B.D. (1990) One dimensional polyacrylamide gel electrophoresis. In: Gel Electrophoresis of Proteins. A Practical Approach (Hames, B.D. and Rickwood, D., Eds.), pp. 1–147. IRL Press, Oxford.
[41] Harlow, E. and Lane, D. (1988) Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[42] Bashan, Y. (1998) Inoculants of plant growth-promoting bacteria for use in agriculture. Biotechnol. Adv. 16, 729–770.
[43] Okon, Y. and Itzigsohn, R. (1992) Poly-β-hydroxybutyrate metabolism in *Azospirillum brasilense* and the ecological role of PHB in the rhizosphere. FEMS Microbiol. Lett. 103, 131–139.
[44] Fallik, E. and Okon, Y. (1996) Inoculants of *Azospirillum brasilense*: biomass production, survival and growth promotion of *Setalia italica* and *Zea mays*. Soil Biol. Biochem. 28, 123–126.
[45] Levanony, H., Bashan, Y., Romano, B. and Klein, E. (1989) Ultrastructural localization and identification of *Azospirillum brasilense* Cd on and within root by immuno-gold labeling. Plant Soil 117, 207–218.
[46] Dufrêne, Y.F. and Rouxhet, P.G. (1996) Surface composition, surface properties, and adhesiveness of *Azospirillum brasilense* - variations during growth. Can. J. Microbiol. 42, 548–556.
[47] Tarrand, J.J., Krieg, N.R. and Döbereiner, J. (1978) A taxonomic study of the *Spirillum lipoferum* group, with description of a new genus, *Azospirillum* gen. Nov. and two species, *Azospirillum lipoferum* (Beijerinck) comb. Nov. and *Azospirillum brasilense* sp. Nov.. Can. J. Microbiol. 24, 967–980.
[48] Morita, R.Y. (1985) Starvation and miniaturisation of heterotrophs, with special emphasis on maintenance of the starved viable state. In: Bacteria in Their Natural Environments (Fletcher, M. and Floodgate, G.D., Eds.), Special Publications of the Society for General Microbiology Vol. 16, pp. 111–130. Academic Press, New York.
[49] Kjelleberg, S., Humphrey, B.A. and Marshall, K.C. (1982) The effect of interfaces on small starved marine bacteria. Appl. Environ. Microbiol. 43, 1166–1172.
[50] Kurath, G. and Morita, R.Y. (1983) Starvation-survival physiological studies of a marine *Pseudomonas* sp.. Appl. Environ. Microbiol. 45, 1206–1211.
[51] Amy, P.S. and Morita, R.Y. (1983) Starvation-survival patterns of sixteen freshly isolated open ocean bacteria. Appl. Environ. Microbiol. 45, 1109–1115.
[52] Groat, G.R., Schultz, J.E., Zychlinsky, E., Bockman, A. and Matin, A. (1986) Starvation proteins in *Escherichia coli*: kinetics of synthesis and role in starvation survival. J. Bacteriol. 168, 486–493.
[53] Matin, A. (1990) Molecular analysis of the starvation stress in *Escherichia coli*. FEMS Microbiol. Ecol. 74, 185–196.
[54] Bachhawat, A.K. and Ghosh, S. (1987) Isolation and characterization of the outer membrane proteins of *Azospirillum brasilense*. J. Gen. Microbiol. 133, 1751–1758.
[55] Dawson, M.P., Humphrey, B.A. and Marshall, K.C. (1981) Adhesion: A tactic in the survival strategy of marine *Vibrio* during starvation. Curr. Microbiol. 6, 195–199.
[56] Smit, G., Kijne, J.W. and Lugtenberg, B.J.J. (1986) Correlation between extracellular fibrils and attachment of *Rhizobium leguminosarum* to pea root hair tips. J. Bacteriol. 168, 821–827.