Direct evidence for the involvement of extracellular proteins in the adhesion of *Azospirillum brasilense*

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Adhesion of *Azospirillum brasilense* to glass and polystyrene was investigated by bringing the cells into contact with the support by sedimentation. Adhesion depended on time and temperature: lower adhesion densities were observed when the contact time was only 2 h or 6 h, as compared to 24 h, or when the test was performed at 4 °C, as compared to 30 °C. The influence of cell physiology was further demonstrated by the effect of tetracycline, which inhibited adhesion. Scanning electron microscopy showed that cells produced extracellular material when left in contact with a support for 24 h. The surface elemental composition of cells and of polystyrene supports after cell adhesion and subsequent detachment was determined by X-ray photoelectron spectroscopy; this provided information on the relative concentrations of proteins and polysaccharides at the surface. The protein concentration at the surface of a cell sediment increased as a function of time at 30 °C, correlating with an increase of adhesion density. A similar correlation between protein concentration and adhesion density was found when comparing exponential-phase cells with stationary-phase cells. The surface composition of polystyrene supports examined after cell detachment was found to be rich in proteins, indicating that proteins are the major constituent at the support surface. Lowering the contact time, or performing adhesion under unfavourable metabolic conditions (4 °C) or in the presence of tetracycline, resulted in a decrease in protein concentration at the support surface, which was correlated with a decrease in adhesion density. The correlation between protein concentration at the cell surface or at the support surface and adhesion density, under different experimental conditions, provides a direct demonstration of the involvement of extracellular proteins in the adhesion of *A. brasilense* to inert surfaces.

Keywords: *Azospirillum brasilense*, adhesion, extracellular proteins, X-ray photoelectron spectroscopy

INTRODUCTION

Bacterial adhesion is a widespread phenomenon, encountered in natural environments as well as in industrial processes. Surfaces on which adhesion may occur display a great diversity (Marshall, 1991), from inanimate surfaces, such as soil particles, rocks, pipes, heat exchangers and biotechnological reactors, to animate surfaces, such as other microbial cells, plant roots and

leaves, and animal tissues. Bacterial adhesion and biofilm processes have important consequences (Characklis & Marshall, 1990; Melo *et al.*, 1992) which can be either beneficial, such as in biotechnology (wastewater treatment, bioremediation, immobilized cells in reactors), or detrimental, such as in industrial systems (fouling, contamination) and in medicine (accumulation on teeth, implants and prosthetic devices).

The colonization of solid surfaces by micro-organisms can be described as a multi-step process (Rouxhet & Mozes, 1990). In the first step, the cells reach the support

Abbreviations: XPS, X-ray photoelectron spectroscopy.

surface by various transport mechanisms (diffusion, sedimentation, convection, active transport). Then initial adhesion, a process controlled by physicochemical interactions between the cells and the support, may occur. Subsequently, the production of extracellular polymers by the cells may lead to the firm attachment ('anchorage') of the cells to the support. Finally, multiplication of the attached cells may lead to the formation of microcolonies or biofilms.

The initial adhesion of bacterial cells to solid surfaces can be understood in the light of DLVO theory, which accounts for van der Waals attraction and electrostatic interactions between supposedly smooth surfaces (van Loosdrecht *et al.*, 1989; Rouxhet & Mozes, 1990; Rijnaarts *et al.*, 1995); additional interactions (bridging, steric repulsion) may involve macromolecules present at the surfaces (Rijnaarts *et al.*, 1995). An alternative approach is to consider the balance of interfacial free energies between the cell-support interface and the interface of each partner with water (Absolom *et al.*, 1983; Busscher *et al.*, 1984). The influence of cell or support surface hydrophobicity on adhesion has been frequently pointed out (Mozes *et al.*, 1987; van Loosdrecht *et al.*, 1987; Rijnaarts *et al.*, 1995).

However, bacteria are not inert colloids but living cells capable of metabolism, growth and motility. The production of extracellular polymeric substances and of appendages (fimbriae, pili, fibrils and flagella) has been found to play a key role in the adhesion of bacteria to surfaces (Marshall, 1985). Marshall *et al.* (1971) suggested that the production of adhesive polymers during the early adhesion process may result in irreversible adhesion, by bridging across the repulsion barrier. Polymer accumulation between bacteria and surfaces has been directly observed by electron microscopy (Fletcher & Floodgate, 1973; Marshall & Cruickshank, 1973). To date, bacterial polymers have been characterized after isolation from culture supernatants or from the cell surface; however, their relevance to adhesion is not straightforward (Neu & Marshall, 1991). For instance, Allison & Sutherland (1987) proposed that extracellular polymers produced by freshwater bacteria may not be involved in adhesion, but rather in the biofilm matrix formation.

Little information has been obtained concerning the chemical composition of the bridging polymers, because they are available in small amounts and are located at the cell-support interface. Marshall *et al.* (1971) showed by electron microscopy that part of the polymers remains on the support in the form of 'footprints' after removal of the adhering cells. In a later study, footprints remaining on polystyrene after detachment of marine bacteria were examined by scanning and transmission electron microscopy and characterized using lectins combined with a fluorescent protein stain (Neu & Marshall, 1991).

Azospirillum spp. are nitrogen-fixing bacteria that live in close association with plant roots and possess plant-growth-promoting potential (Michiels *et al.*, 1989; Okon, 1994). One important step in the colonization of the rhizosphere by azospirilla is their attachment to plant

roots and soil particles. Attachment to sand particles (Bashan *et al.*, 1991) and to root surfaces (Bashan *et al.*, 1986) was found to be mediated by a network of fibrillar material. Proteins and polysaccharides have been reported to play a role in the adhesion to plant roots (Michiels *et al.*, 1991) but the fundamental mechanisms governing the process are still not fully understood due to the lack of direct surface measurements.

In the present study, the adhesion of *Azospirillum brasilense* to inert surfaces was investigated with the aim of better understanding the role of compounds produced by the cells. The morphology of adhering cells was examined by scanning electron microscopy. The surface chemical composition of cells and of supports was determined by X-ray photoelectron spectroscopy (XPS), which provides an elemental and, to a certain extent, a functional analysis of surfaces, with an analysed depth of 3–5 nm (Ratner & McElroy, 1986; Rouxhet & Genet, 1991; Rouxhet *et al.*, 1994).

METHODS

Bacterial strain and culture conditions. *Azospirillum brasilense* Sp7 (ATCC 29145) was used. It was maintained by restreaking every three weeks on fresh plates of Luria-Bertani rich medium (Sambrook *et al.*, 1989) supplemented with 2.5 mM CaCl₂ and 2.5 mM MgSO₄ (LB*). Bacteria were cultivated at 30 °C in standard Erlenmeyer flasks on a rotary shaker (100 r.p.m.). Precultures were inoculated with material collected from several colonies on a fresh stock plate, and incubated for 10–12 h in 50 ml flasks containing 10 ml LB*. Experimental cultures were grown in 1 litre flasks containing 200 ml LB*, inoculated with 2 ml preculture. During the culture, the cell concentration increased from 6×10^6 to 2×10^9 cells ml⁻¹. Cells were collected 12 h or 24 h after inoculation, corresponding to the exponential phase and the stationary phase, respectively. They were harvested by centrifugation at 13 400 *g* for 10 min, washed three times by resuspension in demineralized water and centrifugation and finally resuspended in water to a concentration of 6×10^8 cells ml⁻¹. The cell concentration of the suspension was determined by direct count and by measuring OD₆₀₀. A comparison of demineralized water and culture medium as the resuspending fluid showed that no loss of viability occurred after brief exposure (2 h) of cells to demineralized water.

Supports. Glass and polystyrene were selected as model supports, representing hydrophilic and hydrophobic surfaces, respectively. Glass samples were microscope slides (Menzel Glaser, Germany); they were cleaned by immersion overnight in sulfochromic mixture, and rinsed with demineralized water prior to the adhesion tests. Polystyrene Petri dishes (Merck) were used as hydrophobic supports; their hydrophobicity was always checked before use (water contact angle around 90°).

Adhesion test. Adhesion experiments were performed in static conditions using a procedure similar to that described previously (Mozes *et al.*, 1987), which brings the cells into contact with the supports (glass and polystyrene) by sedimentation. Cell suspension (30 ml) was poured into a Petri dish containing a glass plate. In certain experiments, tetracycline was added at an inhibitory concentration (200 mg l⁻¹) or a sub-minimal inhibitory concentration (1 mg l⁻¹). The suspension was left undisturbed for a given time (2, 6 or 24 h), at 30 °C or 4 °C. The glass plate was then withdrawn and rinsed either mildly, by vertical immersion with gentle agitation (15 s) in three baths of

demineralized water, or drastically, by sprinkling it with a water jet. The Petri dish was rinsed either mildly by filling three times with demineralized water and gentle stirring, or drastically. The wet support was immediately observed under the microscope, before drying. The entire support surface was examined, to ensure that the adhesion pattern was homogeneous, and three representative photographs were taken. If the pattern was non-homogeneous, which occurred rarely, the sample was not considered for analysis. The number of adhering cells was determined by counting the three photographs for each adhesion experiment.

Scanning electron microscopy. Cells adhering on glass and on polystyrene supports were examined by scanning electron microscopy. A 30 ml portion of a suspension of exponential-phase cells was poured into a Petri dish containing small supports (squares of about 1 cm). After 2 h or 24 h contact time at 30 °C the supports were mildly rinsed as described above. The samples were immediately fixed by immersion for 2 h in a solution of 3% (v/v) glutaraldehyde and 0.1 M sodium cacodylate, and kept overnight in 0.1 M sodium cacodylate. They were then dehydrated by successive 15 min immersions in water/ethanol mixtures at 25, 50, 70, 80, 90, 95 and 100% (v/v) ethanol, and dried in a CO₂ atmosphere in a critical-point dryer (Polaron E3000). Samples were coated with gold by a diode sputtering system (Polaron E5100) and examined using a scanning electron microscope (Philips XL20) at 20 kV.

Sample preparation for XPS analysis. Polystyrene was analysed by XPS after cell adhesion and subsequent detachment by ultrasonication in demineralized water (several consecutive steps of 15 s until optical microscope observation showed that all cells were detached). Examination of several sonicated supports by scanning electron microscopy confirmed cell detachment and did not reveal the presence of cell fragments. The sonicated dishes were held vertically to allow drainage of the liquid film when present, then dried overnight at 30 °C in a room atmosphere, and finally stored at room temperature in a desiccator containing P₂O₅. Before XPS analysis, squares of about 1 cm were cut from the bottom with a hot blade.

Cell sediments were analysed after the following preparation procedure. Cells harvested in the exponential phase were allowed to settle down onto polystyrene plates (squares of about 1 cm), at 4 °C and at 30 °C, and left undisturbed for 24 h. Supports were also examined after sedimentation of cells at 30 °C and mild rinsing. Unrinsed and rinsed supports were immediately immersed in liquid nitrogen and freeze-dried, using a freeze-dryer specially designed by Leybold (P. B. Dengis & P. G. Rouxhet, unpublished). The freeze-dryer shelf was precooled at -50 °C. The temperature was maintained at -50 °C for 3 h, then raised progressively to -5 °C in about 15 h, maintained at -5 °C for 6–12 h, and finally raised to 25 °C. Freeze-dried samples were stored at room temperature in a desiccator containing P₂O₅.

Surface analysis by XPS. The XPS analyses were performed with an SSI X-Probe (SSX-100/206) photoelectron spectrometer from Fisons, interfaced with a Hewlett Packard 9000/310 computer allowing instrument control, data accumulation and data treatment. The pressure during analysis was between 2.5×10^{-6} and 2.5×10^{-7} Pa. The spectrometer used monochromatized Al K_α X-ray radiation (1486.6 eV). The irradiated zone was an elliptical spot, with a shorter axis of 1000 µm. The constant pass energy in the hemispherical analyser was 150 eV and 50 eV for survey analysis and individual peak analysis, respectively. The flood gun energy was set at 10 eV and a nickel grid was placed 3 mm above the surface. The following

sequences of spectra were recorded: C_{1s}, O_{1s}, N_{1s}, survey spectrum and finally C_{1s} again to check for the absence of sample degradation. To assess the level of surface contamination, C_{1s} and O_{1s} peaks and a survey spectrum were recorded on sorbitol, freeze-dried with the samples, and on bare polystyrene.

The binding energies were determined by reference to the C_{1s} component due to carbon bound only to carbon or hydrogen, set at 284.8 eV. The background was subtracted linearly. The data treatment was performed with the ESCA 8.3 D software provided by the spectrometer manufacturer. The atom concentrations relative to carbon (mole/mole) were calculated from the peak areas normalized on the basis of acquisition parameters and of sensitivity factors proposed by the manufacturer [mean free path varying according to the 0.7th power of the photoelectron kinetic energy; Scofield cross-sections (Scofield, 1976); transmission function assumed to be constant]. The C_{1s} and O_{1s} peaks were decomposed by using the software least-squares fitting routine with a Gaussian/Lorentzian ratio of 85/15, following a methodology described below.

RESULTS

Adhesion

Table 1 shows the influence of several factors on the density of *A. brasilense* adhering on glass and polystyrene: culture age, rinsing, contact time, temperature, and presence of tetracycline. The standard deviations calculated on the three photographs indicated that the variability of adhesion densities was satisfactory. A duplicate experiment (data not presented) gave similar results. Adhesion of cells harvested in the exponential phase, after 24 h contact time at 30 °C and mild rinsing (standard conditions), was clearly denser on polystyrene than on glass. Under the same conditions, cells harvested in the stationary phase adhered in higher numbers, with a similar density on both supports.

With cells harvested in the exponential phase, the density of adhering cells was lower on supports drastically rinsed (water jet), as compared with supports mildly rinsed; it

Table 1. Density of *A. brasilense* cells adhering on glass and polystyrene as a function of various parameters

	Cell density (10 ⁴ cells cm ⁻²)*	
	Glass	Polystyrene
Standard conditions†	220 (40)	587 (61)
Variants:		
Stationary-phase cells	1157 (82)	982 (81)
Drastic rinsing	88 (14)	163 (18)
Contact time 6 h	9 (< 1)	< 1
Contact time 2 h	< 1	< 1
Temperature 4 °C	14 (1)	< 1
Tetracycline (200 mg l ⁻¹)	63 (12)	132 (17)

* The results are means of data from three photographs, with the standard deviation in parentheses.

† Standard conditions: exponential-phase cells; mild rinsing; 24 h contact time; temperature 30 °C.

Fig. 1. Scanning electron micrographs of *A. brasilense* cells adhering on glass after 2 h (a) and 24 h (b) contact time at 30 °C; cells harvested in the exponential phase; mild rinsing. Bars, 1 µm.

Fig. 2. Representative C_{1s} (right) and O_{1s} (left) peaks of bare polystyrene (a), of cell sediments (exponential-phase cells, 24 h contact time) prepared at 30 °C (b) and at 4 °C (c), and of polystyrene after 24 h contact time with exponential-phase cells at 30 °C and subsequent detachment of the adhering cells (d).

was very low when the contact time was 6 h or 2 h instead of 24 h, or when the test was performed at 4 °C.

Addition of tetracycline at 200 mg l⁻¹ (inhibitory concentration) caused a substantial decrease in adhesion density. Similar results (not presented) were obtained with a sub-minimal inhibitory concentration of tetracycline (1 mg l⁻¹).

Scanning electron microscopy of adhering cells

Fig. 1 shows scanning electron micrographs of *A. brasilense* cells harvested in the exponential phase, adhering on glass after 2 h and 24 h contact time at 30 °C followed by mild rinsing. Cells adhering after 2 h contact time appeared as single and well-defined vibrioids (Fig. 1a), whereas 24-h-old cells were characterized by a smaller size and a rod-like shape, and appeared to be surrounded by small particles which may be attributed to collapsed extracellular material (Fig. 1b). Examination of cells adhering on polystyrene failed to give satisfactory results due to the quick fracture of the gold coating upon irradiation.

Surface chemical composition

Fig. 2(a-c) shows C_{1s} and O_{1s} XPS peaks of bare polystyrene and of cell sediments (exponential-phase cells, 24 h contact time) obtained at 30 °C and 4 °C. The carbon peaks were decomposed into four components: (i) a component set at 284.8 eV, due to carbon bound only to carbon and hydrogen $\underline{C}-(C,H)$; (ii) a component at 286.8 eV due to carbon singly bound to oxygen or nitrogen $\underline{C}-(O,N)$, including ether, alcohol, amine and amide; (iii) a component at 288.4 eV due to carbon making one double bond or two single bonds with oxygen, including amide, carbonyl, carboxylate, ester, acetal and hemiacetal – for the sake of simplicity this is represented below by $\underline{C}=\underline{O}$; (iv) a shake-up component at 291.4 eV, due to an energy loss attributed to a $\pi \rightarrow \pi^*$ transition, characteristic of the aryl moiety in polystyrene. The broad oxygen peak was tentatively decomposed into two components: (i) a component at 531.9 eV attributed to oxygen making a double bond with carbon ($\underline{O}=\underline{C}$), in carboxylic acid, carboxylate, ester, carbonyl or amide; (ii) a component at 533.3 eV, attributed to hydroxide ($\underline{C}-\underline{O}H$), acetal and hemiacetal ($\underline{C}-\underline{O}-\underline{C}-\underline{O}-\underline{C}$). The C_{1s} and O_{1s} peaks were decomposed in two steps. In a first fitting, the initial binding energy, full width at half-maximum and intensity of the components were obtained without imposing constraints. A second fitting of the C_{1s} peak was performed by imposing the same full width at half-maximum for the three components of the cell sediments at 30 °C, or for the second and the third components of other samples, and selecting the mean value obtained for these components in the first fitting; for the O_{1s} peak, the full width at half-maximum of the two components was set equal to the mean value obtained with the first fitting. Nitrogen appeared at 400.4 eV, attributable to unprotonated amine or amide functions (Rouxhet & Genet, 1991; Gerin *et al.*, 1995).

Table 2 (top) presents the surface elemental compositions, expressed as atomic concentration ratios with respect to total carbon, determined by XPS on pure polystyrene, on cell sediments obtained at 30 °C and 4 °C (exponential-phase cells, 24 h contact time), and on polystyrene after sedimentation of cells (exponential-phase cells, 24 h contact time) and mild rinsing. Good agreement was obtained between two sets of independent experiments using different cultures.

Table 2. Surface composition determined by XPS on polystyrene, *A. brasilense* cell sediments, and polystyrene after cell adhesion under different conditions and subsequent detachment

The data, expressed as atomic concentration ratios with respect to total carbon, are from two independent sets of determinations with different bacterial cultures. Exp, cells harvested in the exponential phase; Stat, cells harvested in the stationary phase.

	C_{1s}				O_{1s}		N_{1s}
	$\underline{C}-(C,H)/C$	$\underline{C}-(O,N)/C$	$\underline{C}=O/C$	C_{sh}^*/C	$\underline{O}=C/C$	$(\underline{C}-\underline{O}-C, C-\underline{OH})/C$	N/C
Bare polystyrene							
	0.93	0.00	0.00	0.07	0.00	0.00	0.00
	0.94	0.00	0.00	0.06	0.00	0.00	0.00
Cell sediment (Exp; 24 h contact time)							
30 °C	0.45	0.35	0.19	0.01	0.09	0.23	0.11
	0.50	0.33	0.15	0.02	0.12	0.25	0.10
4 °C	0.58	0.29	0.10	0.03	0.08	0.23	0.05
	0.62	0.25	0.09	0.03	0.07	0.21	0.05
Polystyrene after cell sedimentation and mild rinsing (Exp; 24 h contact time at 30 °C)							
	0.67	0.20	0.08	0.04	0.08	0.13	0.06
	0.71	0.17	0.08	0.04	0.07	0.11	0.06
Polystyrene after detachment of adhering cells							
Culture age (24 h contact time at 30 °C)							
Exp	0.77	0.13	0.06	0.04	0.07	0.03	0.07
	0.76	0.14	0.07	0.03	0.07	0.03	0.08
Stat	0.74	0.14	0.08	0.04	0.09	0.03	0.08
	0.77	0.13	0.07	0.03	0.08	0.04	0.08
Contact time (Exp, 30 °C)							
2 h	0.90	0.04	0.00	0.06	0.00	0.04	0.01
	0.92	0.06	0.01	0.01	0.02	0.01	0.01
6 h	0.82	0.10	0.04	0.04	0.05	0.02	0.05
	0.83	0.09	0.03	0.05	0.05	0.03	0.05
Temperature (Exp, 24 h contact time)							
4 °C	0.85	0.09	0.02	0.04	0.03	0.02	0.03
	0.89	0.06	0.01	0.04	0.03	0.02	0.03
Presence of tetracycline (Exp, 24 h contact time at 30 °C)							
	0.80	0.12	0.04	0.04	0.03	0.06	0.03
	0.82	0.11	0.03	0.04	0.04	0.05	0.03

* C_{sh} , shake-up component of the carbon peak.

The results obtained on cell sediments prepared at 4 °C show a contribution of the polystyrene support revealed by the shake-up peak in Fig. 2(c), indicating the presence of cracks in the freeze-dried cell sediment. As compared with those obtained at 30 °C (Table 2) they are characterized by lower N/O, $\underline{C}=O/\underline{C}-(O,N)$ and $\underline{O}=C/(\underline{C}-\underline{OH}, \underline{C}-\underline{O}-C)$ surface concentration ratios. It is interesting to note that a similar difference was observed between exponential-phase cells and stationary-phase cells (Y. F. Dufrêne & P. G. Rouxhet, unpublished).

Fig. 2(d) shows XPS spectra of polystyrene examined after adhesion of *A. brasilense* (standard conditions: exponential-phase cells, 24 h contact time at 30 °C), rinsing and subsequent detachment. The C_{1s} and O_{1s}

peaks were decomposed as described above. The binding energy of some components was slightly shifted, compared to those of cell sediments: the $\underline{C}-(O,N)$ and $\underline{C}=O$ components of the carbon peak were found respectively at 286.2 and 288.3 eV, the $\underline{O}=C$ and $(\underline{C}-\underline{OH}, \underline{C}-\underline{O}-C)$ components of the oxygen peak were found at 531.7 and 532.8 eV respectively, and nitrogen appeared at 400.3 eV.

Table 2 (bottom) shows the surface elemental compositions determined on polystyrene after detachment of cells that had previously adhered under various conditions. After adhesion and detachment of stationary-phase cells kept in contact with the support for 24 h at 30 °C, the surface composition of the supports was rich in oxygen, nitrogen and oxidized carbon, reflecting the presence of biochemical compounds. As a corollary to the

appearance of the $\underline{\text{C}}-(\text{O}, \text{N})$ and $\underline{\text{C}}=\text{O}$ components, the relative intensity of the C_{1s} shake-up component, characteristic of polystyrene, decreased due to screening by adsorbed compounds. For cells harvested in the exponential phase, the surface composition observed after cell-support contact of 24 h and subsequent cell detachment was similar to that observed with stationary-phase cells. Lower $\underline{\text{C}}=\text{O}/\text{C}$, $\underline{\text{O}}=\text{C}/\text{C}$ and N/C surface concentration ratios were observed when the contact time was only 2 h or 6 h, and when adhesion was performed at 4 °C or in the presence of tetracycline (200 mg l⁻¹).

DISCUSSION

Factors influencing adhesion of microbial cells

The results in Table 1 show that the trends concerning the influence of experimental conditions on cell adhesion are the same for glass and polystyrene. The density of adhering cells is strongly reduced by drastic rinsing, indicating reversibility of adhesion with respect to shear. It has been reported that adhesion of *A. brasilense* Cd cells to quartz sand (Bashan & Levanony, 1988) or wheat root surfaces (Bashan *et al.*, 1986) is relatively weak. Reversibility with respect to shear has also been found for other bacteria (van Loosdrecht *et al.*, 1989).

Adhesion of *A. brasilense* depends on contact time and on temperature: the adhesion density is lower when the contact time is only 2 h or 6 h compared to 24 h, or when adhesion is performed at 4 °C compared to 30 °C. Two interpretations may account for this effect. On the one hand, it may be attributed to a slow kinetics: it is expected that, as the cells are approaching the support, they encounter an important potential energy barrier (hundreds of kT) due to electrostatic repulsions between the two surfaces (Rijnaarts *et al.*, 1995). Therefore, an increase in time and temperature should increase the number of cells which cross the potential barrier. On the other hand, the influence of time and temperature may reflect the influence of cell physiology, and in particular of the synthesis of adhesive macromolecules. The electron micrographs in Fig. 1 indicate that *A. brasilense* cells produce extracellular material when left in contact with a support for 24 h. Marshall *et al.* (1971) showed that adhesion of marine bacteria is a two-stage process, an instantaneous reversible step being followed by a time-dependent irreversible step. Polymeric fibrils observed by electron microscopy were suggested to be involved in the irreversible adhesion step. Also for *Rhizobium* bacteria (Smit *et al.*, 1992) and for *A. brasilense* (Michiels *et al.*, 1991) a two-step mechanism was proposed for the attachment to plant roots.

The influence of cell metabolism on adhesion is demonstrated by the action of tetracycline, an antibiotic known to inhibit protein synthesis. The fact that adhesion is reduced even in the presence of sub-minimal inhibitory concentrations indicates that protein synthesis is required. Tetracycline may inhibit either the synthesis of attachment proteins or the production of enzymes necessary for the

synthesis of macromolecules, like polysaccharides, involved in adhesion. Fletcher (1980) demonstrated, by using a range of metabolic inhibitors, that both energy production and protein synthesis may be required for attachment of marine bacteria to solid surfaces. Growth of *Actinomyces viscosus* and *Bacteroides gingivalis* in the presence of sublethal concentrations of tetracycline was found to reduce fimbriation, cell hydrophobicity (Peros *et al.*, 1985) and attachment to experimental salivary pellicles (Peros & Gibbons, 1982). The influence of chloramphenicol on the aggregation of *A. brasilense* Cd was taken as evidence for the involvement of proteins (Madi & Henis, 1989).

Observations supporting the notion that active bacterial metabolism is required for attachment of *A. brasilense* to roots and inert surfaces are accumulating. Killing bacteria by γ -irradiation or by heat inhibited attachment to plant roots (Bashan *et al.*, 1986; Gafny *et al.*, 1986; Eysers *et al.*, 1988) and to sand particles (Bashan & Levanony, 1988). However, these treatments may also alter the cell surface properties. The anchoring of *A. brasilense* to polystyrene and wheat roots was found to be reduced by several bacterial-inhibitory substances (Bashan & Holguin, 1993).

Surface composition of the cells and of the supports

The binding energy of the N_{1s} peak (400.4 eV) is characteristic of unprotonated amine or amide. No protonated amine was detected at 401.3 eV. Fig. 3 is a plot of the atomic concentration ratios, with respect to total carbon, of oxygen making a double bond with carbon $\underline{\text{O}}=\text{C}/\text{C}$ (open symbols) and of carbon making a double bond or two single bonds with oxygen $\underline{\text{C}}=\text{O}/\text{C}$ (filled symbols), as a function of the N/C ratio. With polystyrene after detachment of adhering cells or after cell sedimentation and rinsing, the regression has a slope close to unity with a small intercept. This shows that the concentrations of $\underline{\text{O}}=\text{C}$, $\underline{\text{C}}=\text{O}$ and N are close to a 1:1:1 ratio, which is the ratio expected for the amide function ($\text{O}=\text{C}-\text{N}$), in other words that nitrogen is mainly present in amide functions, which is compatible with the presence of proteins. For cell sediments, $\underline{\text{C}}=\text{O}/\text{C}$ is systematically higher than $\underline{\text{O}}=\text{C}/\text{C}$ and N/C . This was also observed for the surface of cells (Y. F. Dufrène & P. G. Rouxhet, unpublished) and was attributed to acetal functions present in polysaccharides, the latter being revealed by the high $(\text{C}-\underline{\text{O}}-\text{C}, \text{C}-\underline{\text{O}}\text{H})/\text{C}$ ratios.

The XPS data are consistent with the presence of polystyrene, proteins and polysaccharides. For ease of comparison the discussion will be based on molecular composition rather than on direct elemental or functional data. The molecular composition of the analysed surfaces has been considered to comprise four model constituents: proteins (Pr), polysaccharides (PS), hydrocarbon-like compounds typical of biological material (HCbio) and of the polystyrene support (sup). The chemical composition of the model constituents is given in Table 3; it corresponds to $\text{C}_6\text{H}_{10}\text{O}_5$ for polysaccharides, CH_2 for biological hydrocarbon-like compounds and C_8H_8 for polystyrene. The amino acid composition considered for

Fig. 3. Atomic concentration ratios, with respect to total carbon, of oxygen making a double bond with carbon ($O=C$, open symbols) and of carbon making a double bond or two single bonds with oxygen ($C=O$, filled symbols) as a function of the N/C concentration ratio (data from Table 2); $\circ$, $\bullet$, Cell sediments at 30 °C and at 4 °C; $\triangle$, $\blacktriangle$, polystyrene after cell sedimentation at 30 °C and mild rinsing; $\square$, $\blacksquare$, polystyrene after cell adhesion under different conditions and detachment. Dashed line, relation expected for amide function.

the surface proteins and used to calculate the elemental composition is that of the major outer-membrane protein of *Pseudomonas fluorescens* OE 28.3 (De Mot *et al.*, 1992). In order to assess the proportion of carbon associated with polystyrene (C_{sup}/C), the relative intensity of the shake-up component observed on the sample, $(C_{sh}/C)_{obs}$, was divided by the ratio $(C_{sh}/C)_{bare}$ obtained on pure polystyrene.

Considering the average composition of the constituents, the proportion of carbon associated with each molecular constituent, (C_{Pr}/C) , (C_{PS}/C) , (C_{HCbio}/C) and (C_{sup}/C) was obtained by solving the following system of equations:

$$\begin{aligned} (N/C)_{obs} &= 0.279 (C_{Pr}/C) \\ (O/C)_{obs} &= 0.325 (C_{Pr}/C) + 0.833 (C_{PS}/C) \\ (C/C)_{obs} &= (C_{Pr}/C) + (C_{PS}/C) + (C_{HCbio}/C) + (C_{sup}/C) = 1 \\ (C_{sh}/C)_{obs} &= (C_{sup}/C) (C_{sh}/C)_{bare} \end{aligned}$$

These proportions could then be converted into weight fractions of the model constituents using the carbon content of each constituent given in Table 3.

Fig. 4(a) shows the protein and polysaccharide concentration (weight %) determined on exponential- and stationary-phase cells of *A. brasilense* (Y. F. Dufrène & P. G. Rouxhet, unpublished). The data presented in Table 2 (mean values) were used for Fig. 4(b, c), which shows the surface composition determined on cell sediments, on polystyrene after cell sedimentation and mild rinsing, and on polystyrene after cell adhesion under various conditions and subsequent detachment; the balance is constituted of hydrocarbon-like compounds originating from both support and bacteria.

The protein to polysaccharide ratio of the cell sediment prepared at 4 °C (Fig. 4b, top) is similar to that observed on exponential-phase cells (Fig. 4a, top), the major constituent being polysaccharide. However, when the cell sediment is prepared at 30 °C, a relative increase of the surface protein content is observed; a similar increase is noted for stationary-phase cells as compared with exponential-phase cells. This observation, deduced from elemental composition, is consistent with the shape of C_{1s} and O_{1s} peaks as quantified by the data presented in Table 2: the cell sediment prepared at 30 °C shows higher $C=O/C-(O,N)$ and $O=C/(C-O-C, C-OH)$ ratios compared to 4 °C. It appears therefore that the surface of sedimented cells becomes richer in proteins when metabolism is favoured.

The supports analysed after sedimentation at 30 °C and mild rinsing have a protein to polysaccharide ratio similar to that of cell sediment. The fact that lower protein and polysaccharide concentrations are observed, as compared to the cell sediment, is not surprising since the rinsing procedure removes all non-adhering cells, and thus several layers of cells, from the support.

The surface composition of the supports examined after detachment of adhering cells, harvested either in the exponential phase or in the stationary phase, after 24 h of contact at 30 °C, is very different from that of free cells and of cell sediments: apart from the presence of hydrocarbon-like compounds due to the original poly-

Table 3. Chemical composition of the model constituents considered for deduction of the molecular composition of surfaces

Constituent	$C-(C,H)/C$	$C-(O,N)/C$	$C=O/C$	O/C	N/C	g constituent (mol C) ⁻¹
Protein*	0.428	0.293	0.279	0.325	0.279	23.0
Polysaccharide	0.000	0.833	0.167	0.833	0.000	27.0
Hydrocarbon	1.000	0.000	0.000	0.000	0.000	14.0
Polystyrene	1.000	0.000	0.000	0.000	0.000	13.0

* Data computed from the amino acid analysis of the major outer-membrane protein of *Pseudomonas fluorescens* OE 28.3 (De Mot *et al.*, 1992).

Fig. 4. Surface molecular composition deduced from XPS data, expressed in terms of protein (Pr) and polysaccharide (PS) concentration (weight %): (a) *A. brasilense* cells harvested in the exponential phase and in the stationary phase (Y. F. Dufrène & P. G. Rouxhet, unpublished); (b) cell sediments prepared at 4 °C and at 30 °C, and polystyrene after cell sedimentation at 30 °C and mild rinsing; (c) polystyrene after cell adhesion under different conditions and detachment: exponential-phase cells, 24 h contact time at 30 °C; stationary-phase cells, 24 h contact time at 30 °C; exponential-phase cells, 6 h and 2 h contact time at 30 °C; exponential-phase cells, 24 h contact time at 4 °C, and at 30 °C in the presence of tetracycline.

styrene and possibly to some biochemical compounds, it is composed essentially of proteins, the polysaccharide content being negligible (Fig. 4c, top). This observation, deduced from elemental composition, is in agreement with the shape of the O_{1s} peak (Fig. 2): for cells, the prevailing O_{1s} component is due to $C-OH + C-O-C$, which reflects essentially the presence of polysaccharides, whereas for the supports analysed after cell detachment the major component is due to $C=O$, which reflects the presence of proteins. The two independent observations show that polysaccharides are characteristic of cells, whereas proteins are the major constituent at the support surface.

The contrast of protein/polysaccharide ratio between the support surface after cell detachment and the surface of

cells, even those with the highest protein concentration (cell sediment, 24 h, 30 °C), indicates that the material remaining on the support is not made of cell surface fragments. It is also not compatible with shear occurring within the cell walls, which should lead to exposure of polysaccharides (peptidoglycan, lipopolysaccharides). Scanning electron microscope observation indeed confirmed that no cell debris remained on the support surface after sonication. The material remaining on the support surface is attributed to extracellular proteins that accumulated at the cell-support interface, the shear taking place within the layer of proteins or between this layer and the cells.

When the contact time is lowered to 6 h or 2 h, when adhesion is carried out at 4 °C or when tetracycline is added, a smaller amount of biological compounds is detected at the support surface. Note that, due to the low concentrations, the relative proportion between proteins and polysaccharides is poorly significant.

Biomolecules involved in the adhesion process

The above discussion shows that changes of experimental conditions which affect the protein concentration at the cell surface (growth phase, temperature) or at the support surface (time, temperature, addition of tetracycline) influence the density of adhering cells in the same direction. This is illustrated in Fig. 5, which shows the relationship between the adhesion density and the N/C atomic concentration ratio determined by XPS on polystyrene supports, after detachment of adhering cells of *A. brasilense*. Clearly, two groups of data are distinguished: one characterized by very low adhesion densities and by low or intermediate N/C ratios, corresponding to surface protein concentrations ranging from 5% to 30%, the other characterized by high adhesion densities and high protein concentrations (40%). This provides direct evidence that extracellular proteins play a role in the adhesion of *A. brasilense* to an inert surface.

Fig. 5. Density of *A. brasilense* cells adhering on polystyrene under various conditions, as a function of the N/C atomic concentration ratio determined by XPS on the supports after detachment of adhering cells.

These results may be related to biochemical and genetic studies, which suggested the involvement of surface proteins in the attachment of rhizobacteria, including *Azospirillum*, to plant roots or inert surfaces. Attachment of *Klebsiella* (Korhonen *et al.*, 1983) and *Enterobacter* (Hahtela *et al.*, 1985) strains to plant roots was clearly shown to be promoted by fimbriae. Pili have been found to be involved in the attachment of *Bradyrhizobium japonicum* to hydrophobic plastic surfaces and to soybean roots (Vesper & Bauer, 1986). Vesper (1987) showed that the presence of pili on *P. fluorescens*, produced abundantly in low-nutrient medium, was quantitatively correlated with attachment to polystyrene, haemagglutination, and attachment to corn roots. Members of the *Rhizobiaceae* family, including *Rhizobium*, *Bradyrhizobium* and *Agrobacterium* spp., synthesize a rhicadhesin which promotes initial adhesion to plant surfaces (Smit *et al.*, 1992). DeFlaun *et al.* (1990) showed that flagellin, a major outer-membrane protein, is important for the adhesion of a *P. fluorescens* strain to sand. The major outer-membrane protein from the rhizosphere isolate *P. fluorescens* OE 28.3 adsorbed strongly and selectively to plant roots, indicating a potential role in root colonization (De Mot & Vanderleyden, 1991).

Attachment of *A. brasilense* Cd to sand particles was found to be actively mediated by a network of fibrillar material, presumably proteinaceous (Bashan & Levanony, 1988). Fibrillar material was also found to anchor the cells to root surfaces (Bashan *et al.*, 1986). Michiels *et al.* (1991) showed, by the use of protease treatments, that adsorption of *A. brasilense* Sp7 to wheat roots was possibly mediated by proteinaceous compounds. Furthermore, a mutant deficient in expression of both polar and lateral flagella (Croes *et al.*, 1991) failed to adsorb to wheat roots (Michiels *et al.*, 1991). Croes *et al.* (1993) demonstrated that adsorption of *A. brasilense* to wheat roots is mediated by the polar flagellum. Madi *et al.* (1988) observed the formation of lateral flagella and fimbriae-like structures by *A. brasilense* Cd in static liquid culture and suggested that these filaments may play a role in cell-to-cell adhesion resulting in pellicle formation.

Surface polysaccharides are thought to be involved in attachment to plant roots (Michiels *et al.*, 1991). At first glance, it may therefore be surprising that in the present study, the material remaining on the support after cell detachment was very poor in polysaccharides. Three interpretations may be taken into consideration. First, the attachment to plant roots was determined in a medium containing a carbon source which is known to favour polysaccharide production (Michiels *et al.*, 1991). Second, extracellular polysaccharides may be involved not directly in the initial steps of the adhesion process, but rather in the subsequent stages of biofilm formation, as proposed for freshwater bacteria (Allison & Sutherland, 1987). Third, bacterial surface polysaccharides may be involved only in the specific *Azospirillum*-plant root interaction, and not in the adhesion to inert surfaces. Specific interactions between bacterial saccharide receptors and plant root lectins have been suggested for *Azospirillum*

associations (Umali-Garcia *et al.*, 1980; Del Gallo *et al.*, 1989; Zaady & Okon, 1990).

Further work is needed to elucidate the mechanism(s) by which proteins influence adhesion: on the one hand, they may be released into the aqueous solution and adsorb on the support to create a conditioning film which in turn may influence adhesion; on the other hand, they may result from the *in situ* secretion of adhesive surface compounds by the adhering cells and thus lead to firm anchorage.

The possible action of the support surface in inducing the secretion of proteins by the cells may also be speculated. It is known that surface growth of *A. brasilense* induces the formation of lateral flagella (Hall & Krieg, 1983; Moens *et al.*, 1995). The same phenomenon is also known for *Vibrio parahaemolyticus*, where lateral flagella are believed to be involved in the adhesion to surfaces (Belas & Colwell, 1982).

Conclusions

Adhesion of *A. brasilense* to glass and polystyrene depends on time and temperature, lower adhesion densities being observed when the contact time is only 2 h or 6 h, as compared to 24 h, or when the test is performed at 4 °C, as compared to 30 °C. This points to the influence of cell physiology, which is demonstrated by the inhibition of adhesion as a result of the addition of tetracycline. Electron microscope observations show that *A. brasilense* cells produce extracellular material when they are left in contact with a support for 24 h. The XPS analysis of cells and polystyrene supports has permitted an evaluation of the surface composition in terms of model molecular constituents. Proteins are the major constituent at the support surface after cell detachment. The comparison of the surface compositions of cell sediments prepared at 30 °C and at 4 °C indicates that the cell surface becomes richer in proteins when metabolism is favoured (30 °C); this may be related to the increase of cell surface protein concentration and cell adhesiveness observed during growth. Changes of experimental conditions (growth phase, temperature, time and addition of tetracycline) that affect the protein concentration at the cell surface or at the support surface have a corresponding influence on the adhesion density. This provides direct evidence for the involvement of extracellular proteins in the adhesion of *A. brasilense* to inert surfaces.

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