Influence of extracellular polymeric substances on deposition and redeposition of *Pseudomonas aeruginosa* to surfaces

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In this study, the role of extracellular polymeric substances (EPS) in the initial adhesion of EPS-producing *Pseudomonas aeruginosa* SG81 and SG81R1, a non-EPS-producing strain, to substrata with different hydrophobicity was investigated. The release of EPS by SG81 was concurrent with a decrease in surface tension of a bacterial suspension from 70 to 45 mJ m⁻² that was absent for SG81R1. Both strains adhered faster and in higher numbers to a hydrophilic than to a hydrophobic substratum, but the initial deposition rates and numbers of adhering bacteria in a stationary-end point were highest for the non-EPS-producing strain SG81R1, regardless of substratum hydrophobicity. Both strains adhered less to substrata pre-coated with isolated EPS of strain SG81. Furthermore, it was investigated whether bacteria, detached by passing air-bubbles, had left behind 'footprints' with an influence on adhesion of newly redepositing bacteria. Redeposition on glass was highest for non-EPS-producing SG81R1 and decreased linearly with the number of times these cycles of detachment and deposition were repeated to become similar to the redeposition of SG81 after six cycles. This indicates that *P. aeruginosa* SG81 leaves the substratum surface nearly completely covered with EPS after detachment, while SG81R1 releases only minor amounts of surface active EPS, completely covering the substratum after repeated cycles of detachment and adhesion. Atomic force microscopy showed a thick and irregular EPS layer (up to 32 nm) after the first detachment cycle of EPS-producing strain SG81, whereas the putatively non-EPS-producing strain SG81R1 left a 9 nm thin layer after one cycle. X-ray photoelectron spectroscopy indicated that the bacterial footprints consisted of uronic acids, the prevalence of which increased with the number of detachment and deposition cycles.

Keywords: proteins, polysaccharides, bacterial adhesion, footprints, atomic force microscopy

INTRODUCTION

Micro-organisms readily deposit and adhere to all surfaces immersed in an aqueous environment as the onset of a multi-step process, leading to the formation of a complex, adhering microbial community termed

'biofilm'. Although the function and appearance of biofilms in various environments may be different, all biofilms form from the same basic sequence of events (Escher & Characklis, 1990), including (i) the formation of a conditioning film of adsorbed macromolecular components; (ii) transport of micro-organisms towards the substratum surface; (iii) initial, mainly reversible, adhesion of linking film organisms to the substratum surface (Busscher *et al.*, 1995) or alternatively, planktonic micro-organisms may co-adhere to already adhering micro-organisms (Bos *et al.*, 1995); (iv) microbial

Abbreviations: ADSA-P, axisymmetric drop shape analysis by profile; AFM, atomic force microscopy; DDS, dimethyldichlorosilane; EPS, extracellular polymeric substance(s); XPS, X-ray photoelectron spectroscopy.

anchoring yielding irreversible adhesion through the production of extracellular polymeric substances (EPS) (Sutherland, 1977; Neu & Marshall, 1990); (v) growth as the main contributing factor to the accumulation of micro-organisms in a biofilm.

More than 90% of the EPS volume consists of water (Schmitt & Flemming, 1999; Sutherland, 1997), located in pores and with a minor portion bound to the EPS molecules (Schmitt *et al.*, 1997; Christensen & Characklis, 1990). EPS has long been considered to consist only of polysaccharides, but recently considerable amounts of proteins, as well as humic substances, nucleic acids and lipids (Cooksey, 1992; Nielsen *et al.*, 1997; Flemming & Wingender, 2001) have been identified as EPS constituents. However, differences in chemical composition as well as in function of the different kinds of EPS have been reported (Omar *et al.*, 1983; Beech *et al.*, 1999).

A role of EPS in initial adhesion of *Pseudomonas* to surfaces has not yet been established. However, it has been found that attachment to an inert substratum stimulates bacterial EPS synthesis (Vandevivere & Kirchman, 1993; Allison & Sutherland, 1987). EPS components, like polysaccharides (Fletcher & Floodgate, 1973; Costerton *et al.*, 1985; Azeredo *et al.*, 1999; Azeredo & Oliveira, 2000) and proteins (Daniels *et al.*, 1977; Dufrene *et al.*, 1996b), enhance bacterial adhesion, while lipopolysaccharides (Williams & Fletcher, 1996), uronic acids (Pringle *et al.*, 1983) and biosurfactants (Velraeds *et al.*, 1998a; Van Hoogmoed *et al.*, 2000; Heinemann *et al.*, 2000) discourage adhesion.

In most natural environments, cyclic detachment and redeposition of organisms occurs more readily than a single round of deposition and adhesion. Passing air-liquid interfaces have been demonstrated to yield extremely efficient detachment forces, as in the oral cavity during eating, speaking, drinking and swallowing (Busscher *et al.*, 1992), on the eye and on contact lenses caused by blinking (Landa *et al.*, 1998), on rocks and ship hulls in marine environments (Cooksey & Wigglesworth-Cooksey, 1995) and in biodeterioration of monumental buildings by falling rain drops (Urzi *et al.*, 1991). Detached organisms may leave 'footprints' (Neu & Marshall, 1991) behind, that either consist of cell surface fragments left behind (Neu, 1992), excreted biosurfactants (Velraeds *et al.*, 1998b) or EPS. These footprints form a new surface in the redeposition of a new generation of depositing organisms.

The aim of the present study is to investigate the role of EPS in the initial adhesion of *Pseudomonas aeruginosa* to substratum surfaces with different hydrophobicities. To this end, the deposition and redeposition after detachment by the passage of an air-liquid interface of an EPS-producing and a putatively EPS-non-producing strain of *P. aeruginosa* was studied in a parallel plate flow chamber.

METHODS

Bacterial strains and culture conditions. An EPS-producing strain and an isogenic non-mucoid strain of *Pseudomonas aeruginosa* (SG81 and SG81R1, respectively) were used in this study. Both strains were grown aerobically at 37 °C in Nutrient Broth (NB; Oxoid). For each experiment, strains were inoculated from NB agar in batch culture for 24 h. This culture was used to inoculate a second culture, which was grown for 16 h prior to harvesting bacteria by centrifugation (5 min at 10000 g). Bacteria were washed twice with Millipore-Q water and suspended in PBS, pH 7.0 (g l⁻¹: KH₂PO₄, 0.68; K₂HPO₄, 0.86; NaCl, 8.76) supplemented with 5% (v/v) NB (1.25 g l⁻¹) medium at concentrations of 3 × 10⁸ ml⁻¹ or 1 × 10⁹ ml⁻¹ for deposition and redeposition experiments, respectively. NB medium (5%, v/v) was added to enhance metabolic activity of the organisms without stimulating growth (Habash *et al.*, 1997).

EPS production and isolation. Strains were examined for EPS production using axisymmetric drop shape analysis by profile (ADSA-P; Cheng *et al.*, 1990) through effects of EPS production on the surface tensions of bacterial suspensions. Droplets (0.1 ml) containing 1 × 10⁹ cells of both strains ml⁻¹, suspended in PBS (pH 7.0), were placed on a clean piece of fluoroethylenepropylene and the circumference of the suspension droplet was monitored during 2 h in an enclosed chamber at room temperature. Droplet circumferences were recorded twice with a minimal time interval (<0.5 s) between measurements and fitted to the Laplace equation of capillarity, yielding the surface tension of the bacterial suspensions. EPS production was inferred from a decrease in surface tension of the bacterial suspension.

EPS-producing *P. aeruginosa* SG81 cells from a 250 ml culture (containing approximately 2.5 × 10¹¹ cells) were washed three times with 60 ml Millipore-Q water and the supernatants containing the EPS were collected. The supernatants were centrifuged again (5 min, 10000 g), decanted and the EPS-containing supernatant was dialysed for 24 h against Millipore-Q water, using a cellulose membrane with a 14 kDa cut-off (Spectrum). EPS (150 ml) isolated in this manner was mixed with 150 ml PBS for substratum coating in deposition assays.

Physico-chemical characterization of bacterial cell surfaces. Water contact angles (θ_w) on lawns of both *P. aeruginosa* strains were determined using the sessile drop technique (Van Oss & Gillman, 1972; Busscher *et al.*, 1984). Briefly, bacterial cells were layered from Millipore-Q water onto 0.45 µm pore filters (Millipore) using negative pressure. The filters were left to air dry in ambient air until so-called 'plateau contact angles' could be measured.

Electrophoretic mobilities of *P. aeruginosa* strains were measured in PBS supplemented with 5% NB medium with a Lazer Zee Meter 501 (PenKem), which uses scattering of incident laser light to enable detection of bacteria at relatively low magnifications. Aliquots of the bacterial suspensions were diluted to a density of approximately 1 × 10⁸ cells ml⁻¹ in PBS (pH 7.0) prior to the measurements. The voltage difference across the electrophoresis chamber was set to 150 V and the electrophoretic mobilities measured were converted to apparent zeta potentials (ξ) through the use of the Von Smoluchowski equation (Hiemenz, 1977).

X-ray photoelectron spectroscopy (XPS) was applied to obtain the overall chemical composition of the microbial cells. After culturing and washing, the bacterial pellets were transferred to stainless steel troughs and frozen in liquid nitrogen. The

samples were subsequently freeze-dried and the resulting bacterial powder was pressed in small stainless steel cups and inserted into the XPS chamber (S-Probe; Surface Science Instruments). X-rays (10 kV, 22 mA) at a spot size of $250 \times 1000 \mu\text{m}$ were produced using an aluminium anode. A scan of the overall spectrum in the binding energy range of 1–1200 eV at low resolution (pass energy 150 V) was recorded, followed by scans over a 20 eV binding energy range at high resolution (pass energy 50 eV) for C_{1s} (carbon), O_{1s} (oxygen), N_{1s} (nitrogen) and P_{2p} (phosphorus). The area under a peak, after linear background subtraction, was used to calculate peak intensities, yielding elemental surface concentration ratios for nitrogen, oxygen and phosphorus to carbon, after correction with sensitivity factors provided by the manufacturer. Bacterial cell surface characterizations were carried out in triplicate on three different bacterial cultures.

Substratum surfaces. Glass microscope slides ($76 \times 25 \times 1.5 \text{ mm}$) were used as substratum surfaces. Glass surfaces were cleaned with 2% (w/v) RBS 35 detergent (Omnilabo International) and alternately rinsed in water, methanol, water again and finally demineralized water. Cleaned glass plates were used as a negatively charged, hydrophilic substratum surface, while hydrophobic, negatively charged surfaces were prepared from cleaned glass by first drying in an oven at 80°C , followed by silanization in 0.05% (w/v) dimethyldichlorosilane (DDS) in trichloroethylene. After silanization for 15 min, the surfaces were rinsed with trichloroethylene, methanol and Millipore-Q water.

Physico-chemical characterization of substratum surfaces. Water contact angles (θ_w) of glass and DDS-coated glass, as well as of EPS-coated glass were measured by the sessile drop technique, as described above. Zeta potentials (ξ) of bare glass and DDS-coated glass surfaces in PBS (pH 7.0) were measured by streaming potentials in a parallel plate flow chamber (Van Wagenen & Andrade, 1980).

In redeposition experiments, the chemical composition of the substrata after air-bubble-induced detachment of adhering bacteria was determined by XPS after the first and second deposition cycle to detect potential footprints. However, since both glass surfaces and EPS are rich in oxygen, complicating the interpretation of XPS data, redeposition experiments were also carried out on gold-coated glass slides to uniquely identify potential EPS material left behind after bacterial detachment. In addition, potential footprints on glass were sought by atomic force microscopy (AFM). AFM images and force-distance measurements were taken under water at room temperature using an optical lever microscope (Nanoscope IIIa Dimension 3100; Digital Instruments). Contact mode topographic images were recorded in both height and deflection modes. Height images provided quantitative topographic information from which the mean roughness (R_a) of the surfaces was calculated, while deflection images revealed finer surface details. The imaging force was kept below 1 nN and the scan rate was in the range of 2–4 Hz. A Si_3N_4 cantilever tip of 0.06 N m^{-1} spring constant was used.

Parallel plate flow chamber and image analysis. Adhesion experiments were carried out in a parallel plate flow chamber containing three basic modules: a flow loop, a syringe pump and image analysis components (Gómez-Suárez *et al.*, 1999). The chamber is made of poly(methyl methacrylate) with channel dimensions $76 \times 38 \times 0.6 \text{ mm}$ ($l \times w \times h$) for deposition assays. For detachment and redeposition experiments the channel of the flow chamber was modified ($76 \times 5 \times 0.6 \text{ mm}$, $l \times w \times h$) to control the passage and velocity of the air bubbles introduced. The top plate was made out of glass and separated from the bottom plate by two Teflon spacers. The

bottom plate was made of glass or DDS-coated glass, depending of the substratum under study. A pulse-free liquid flow was achieved by hydrostatic pressure and recirculated by a roller pump. The volumetric flow was 0.025 ml s^{-1} , yielding to a laminar flow (Reynolds number 0.6).

The entire flow chamber was mounted on the stage of a phase-contrast microscope (Olympus BH-2) equipped with a $\times 40$ ultra-long working distance objective (Olympus ULWD-CD Plan 40 PL). A charged-coupled device camera (CCD-MXR/5010, Adimec Advanced Image Systems) was mounted on the phase-contrast microscope and connected to a PCVision+ video frame grabber interface card (Imaging Technology Inc.) to digitize the video signal. For enumeration of the number of bacteria adhering to the bottom plate before and after the passage of an air bubble, a cell-finder slide ($76 \times 26 \text{ mm}$) attached to the outside of the bottom plate was focused for accurate repositioning ($\pm 2 \mu\text{m}$) of the flow chamber in the enumeration process.

Experimental protocol. All experiments were performed at room temperature in triplicate with suspensions of separate bacterial cultures and newly prepared collector surfaces. Before each experiment, all tubes, flow chamber and syringe pump were filled with PBS and care was taken to remove all air bubbles from the system. The PBS solution was first perfused through the chamber for 30 min. At this stage and when appropriate, the flow was switched to PBS supplemented with isolated EPS for 1 h after which the flow was switched for 30 min to PBS to remove all remnants of EPS components from the tubing and the flow chamber.

In deposition experiments, after perfusion with PBS, bacteria were allowed to adhere on the different substrata up to 5 h from a suspension containing 3×10^8 bacteria ml^{-1} in PBS supplemented with 5% (v/v) NB. Initial deposition rates and numbers of adhering cells after 5 h of both *P. aeruginosa* strains on bare, EPS pre-coated and DDS-coated glass were determined.

In detachment experiments, 4×10^6 bacteria cm^{-2} were allowed to adhere to the substrata from PBS supplemented with 5% (v/v) NB, as enumerated by the image analysis system on ten arbitrarily chosen areas of 0.017 mm^2 distributed over the length of the substratum surface. Subsequently, flow was switched for 30 min to PBS, again to remove all non-adhering bacteria from the flow chamber and the tubing system. At this stage, a $0.1 \pm 0.02 \text{ ml}$ air bubble fully spanning the width of the flow chamber was introduced in the tubing and passed over the substratum surface at a controlled velocity of 5.6 mm s^{-1} with the aid of a syringe pump (Terumo SC512). The syringe pump was connected to the flow chamber by a three-way valve and filled with PBS. The number of bacteria that remained adhering after air bubble passage was enumerated after retrieving the positions used before with the aid of the cell-finder.

In redeposition experiments, *P. aeruginosa* was allowed to adhere to glass from a suspension with 1×10^9 cells ml^{-1} in PBS supplemented with 5% (v/v) NB medium up to 40 min. Thereafter, six air bubbles were allowed to pass over the substratum surface at 5.6 mm s^{-1} , removing all adhering bacteria. Finally, a fresh bacterial suspension was allowed to adhere again to this substratum surface and the number of adhering bacteria enumerated. This process of detachment, deposition and adhesion was repeated for up to six cycles.

The initial increase in the number of adhering bacteria with time was expressed as an initial deposition rate (j_0). This rate was calculated in deposition and redeposition experiments and represents the number of initially adhered bacterial cells

per unit area and time. The number of bacterial cells detached after a passing an air bubble was expressed as the percentage of bacteria caused to detach, relative to the number of adhering bacteria prior to air bubble passage.

Data analysis. Detachment data were analysed by a one-way analysis of variance (ANOVA). ANOVA tests the ratio of the mean squares between groups and within groups, and provides an *F*-ratio (i.e. Fisher distribution) and its corresponding *P* value. If the null hypothesis is true, the *F* ratio should be approximately 1, since both mean squares between and within groups should be about the same. If the ratio is much larger than one, the null hypothesis is false. The shape of the *F* distribution depends on the degrees of freedom within and between groups: the lower the degrees of freedom, the larger the value of *F* needed for significance. A *P* value of less than 5% was considered to indicate a significant difference between groups. The subscript of *F* indicates the value of *F* needed for a hypothesis to be significant at the 5% level. When differences among means were determined, post-hoc Dunnett's range tests and pairwise multiple comparisons were employed.

RESULTS

Table 1 shows the physico-chemical properties of the cell surfaces of *P. aeruginosa* SG81 and SG81R1, as well as those of the pristine, EPS-coated and DDS-coated glass. EPS-producing *P. aeruginosa* SG81 was significantly more hydrophobic ($F_{7,71} = 13.5$, $P = 0.020$) than strain SG81R1, as judged from water contact angles. However, no significant differences in zeta potentials ($F_{7,71} = 3.58$, $P = 0.131$) were found in PBS. Correspondingly, there were no significant differences in elemental surface compositions of both bacterial strains. DDS-coated glass was significantly more hydrophobic than glass ($F_{7,71} = 346.2$, $P = 0.00005$), while EPS coating made glass more hydrophobic as compared to bare glass ($F_{7,71} = 13.6$, $P = 0.021$), but did not significantly affect the hydrophobicity of DDS-coated glass. No significant differences were found in zeta potentials of both substrata, as measured in PBS. Both O/C and N/C

Fig. 1. Liquid surface tension (γ_{lv}) of a suspension of *P. aeruginosa* SG81 (●) and *P. aeruginosa* SG81R1 (○) (1×10^9 cells ml⁻¹ in PBS, pH 7.0) as a function of time as determined by ADSA-P. Data are the means of three experiments performed with separately grown bacterial strains.

elemental surface concentration ratios of glass increased significantly after EPS-coating ($F_{18,51} = 106.2$, $P = 0.017$), but an EPS coating did not significantly affect the elemental surface composition of DDS-coated glass.

A suspension of *P. aeruginosa* SG81 showed a strong decrease in liquid surface tension (γ_{lv}) over time from 70 to 45 mJ m⁻² (Fig. 1), indicating the release of surface-active EPS. *P. aeruginosa* SG81R1 in suspension, however, did not cause a significant change in surface tension over time. EPS-producing *P. aeruginosa* SG81 adhered significantly less well ($F_{4,3} = 29.93$, $P = 0.00001$) than the non-EPS-producing strain SG81R1, as based both on initial deposition rates and numbers of bacteria

Table 1. Water contact angles (θ_w) on the different bacterial strains and substratum surfaces involved in this study, together with their zeta potentials (ξ) in phosphate-buffered solution (pH 7.0), and elemental surface concentration ratios from XPS

All data are the results of three experiments with separately grown bacterial strains and substratum surfaces, yielding a mean SD of around 5° for contact angles, 3 mV for zeta potentials and 0.010 for XPS elemental concentrations. ND, Not determined; BDL, below detection limit.

Strain/substratum		θ_w (°)	ξ (mV)	N/C	O/C	P/C
Bacterial strains						
<i>P. aeruginosa</i> SG81		62	-9	0.083	0.445	0.026
<i>P. aeruginosa</i> SG81R1		47	-16	0.101	0.413	0.017
Substrata						
Bare	Glass	27	-22	0.018	0.422	BDL
	DDS-coated glass	99	-20	0.010	1.234	BDL
	Glass	41	ND	0.086	2.303	BDL
	DDS-coated glass	95	ND	0.031	1.521	BDL

Table 2. Initial deposition rates (j_0), numbers of adhering organisms after 5 h of deposition (n_{5h}) and percentages of adhering organisms detached by a passing air bubble

Deposition was performed from a suspension containing 3×10^8 cells ml^{-1} in PBS supplemented with 5% (v/v) NB to bare or EPS-coated substrata. All data are the results of three experiments with separately grown bacterial strains and substratum surfaces, yielding a mean SD of around 11% in all parameters. ND, Not determined.

Substratum surface	<i>P. aeruginosa</i> strain	j_0 ($\text{cm}^{-2} \text{s}^{-1}$)		$10^{-6} \times n_{5h}$ (cm^{-2})		Detachment (%)	
		Bare	EPS-coated	Bare	EPS-coated	Bare	EPS-coated
Glass	SG81	2250	1547	15.3	7.8	38	ND
	SG81R1	5450	2800	22.5	8.5	28	ND
DDS-coated glass	SG81	2000	1263	11.5	6.1	15	ND
	SG81R1	5100	2550	12.8	9.4	9	ND

adhering after 5 h (Table 2). However, both strains adhered faster (higher deposition rates) and in higher numbers to hydrophilic glass than to hydrophobic DDS-coated glass. Coating of the substrata with isolated EPS prior to bacterial deposition yielded a significant decrease in initial deposition rates ($F_{4,3} = 10.34$, $P = 0.004$) and numbers of bacteria adhering after 5 h ($F_{4,3} = 25.73$, $P = 0.00004$) as compared to pristine substrata for both strains. However, these decreases were less important for the EPS-producing strain. No significant differences were found in the detachment of the strains, as induced by the passage of an air bubble through the flow chamber, but their detachment from hydrophilic glass was significantly higher ($F_{7,71} = 172.1$, $P = 0.0002$) than from hydrophobic DDS-coated glass.

Fig. 2 shows that the initial deposition rate and the number of adhering *P. aeruginosa* SG81 R1 after 40 min ($n_{40\text{min}}$) to glass decrease linearly with the number of detachment and deposition cycles, becoming similar to the results obtained for EPS-producing SG81 after six cycles.

The surface chemical compositions of gold-coated glass after air-bubble-induced detachment of adhering *P. aeruginosa* SG81 and SG81 R1 of the first two cycles of detachment and deposition were determined by XPS, as presented in Table 3. The C_{1s} carbon peak was decomposed in four components and attributed to: carbon bound only to carbon and hydrogen, $\text{C}-(\text{C},\text{H})$, at a binding energy of 284.8 eV; carbon singly bound to oxygen or nitrogen, $\text{C}-(\text{O},\text{N})$, including ether, alcohol, amine and amide bonds, at a binding energy of 286.2 eV; carbon with either two single bonds to oxygen or one double bond, $\text{C}=\text{O}$, including amide, carbonyl, carboxylate, ester, acetal and hemiacetal bonds, at a binding energy of 287.8 eV; and finally, carbon involved in carboxyl groups, $\text{O}=\text{C}-\text{OH}$, at 289.1 eV. The EPS of agar-grown *P. aeruginosa* SG81 has been described (Grobe *et al.*, 1995) to contain uronic acids (UA), proteins (PR) and polysaccharides (PS). Based on these compositional data, the XPS results in Table 3 enable determination of the composition of the EPS left on the gold after detachment, as described by Rouxhet *et al.*

Fig. 2. Adhesion of *P. aeruginosa* SG81 ($\bullet$) and *P. aeruginosa* SG81R1 ($\circ$) from a 5% (v/v) NB medium supplemented with PBS (pH 7.0) to glass as a function of the number of detachment and deposition cycles. (a) Initial deposition rates (j_0); (b) number of adhering of *P. aeruginosa* after 40 min ($n_{40\text{min}}$). Bars indicate the standard deviation of three experiments performed with separately grown bacterial strains and substratum surfaces.

(1994). Accordingly, the $\text{O}=\text{C}-\text{OH}$ peak was attributed to uronic acids yielding:

$$(\text{O}=\text{C}-\text{OH})/\text{C} = \text{C}_{\text{UA}} \quad (1)$$

in which C_{UA} denotes the ratio of carbon due to uronic acids. Similarly, the ratio of carbon due to proteins, C_{PR} , was obtained from the N/C ratio, assuming

Table 3. Chemical composition of gold-coated glass after air-bubble-induced detachment of adhering bacteria after the first two cycles of detachment and deposition, as determined by XPS, and calculated contents of uronic acids (C_{UA}), proteins (C_{PR}), polysaccharides (C_{PS}) and hydrocarbon-like compounds (C_{HC}), according to equations 1–4

Strain	Redeposition cycle	N/C	O/C	(O=C-OH)/C	[C-(O,N)]/C	C_{UA}	C_{PR}	C_{PS}	C_{HC}
<i>P. aeruginosa</i> SG81	1	0.177	0.312	0.032	0.496	0.032	0.634	0.287	0.047
	2	0.150	0.275	0.072	0.457	0.072	0.538	0.235	0.155
<i>P. aeruginosa</i> SG81R1	1	0.189	0.329	0.027	0.500	0.027	0.677	0.284	0.012
	2	0.171	0.320	0.043	0.472	0.043	0.613	0.258	0.086

Fig. 3. Height AFM images recorded under water (area: $1\ \mu\text{m} \times 1\ \mu\text{m}$) of glass after one cycle of air-bubble-induced detachment of adhering *P. aeruginosa*. (a) *P. aeruginosa* SG81; (b) *P. aeruginosa* SG81R1. The arrows indicate the lines along which cross sections, shown in the respective graphs below, were taken.

proteins possess about 0.28 nitrogen atoms per molecule:

$$N/C = 0.28 \times C_{PR} \quad (2)$$

As proteins contain negligible amounts of carbon singly bound to oxygen (Dûfrene & Rouxhet, 1996), the ratio of carbon due to polysaccharides, C_{PS} , can be calculated from:

$$[C-(O,N)] - N/C - C_{UA} = C_{PS} \quad (3)$$

A residual content due to hydrocarbon-like compounds, C_{HC} , subsequently results according to:

$$1 = C_{UA} + C_{PR} + C_{PS} + C_{HC} \quad (4)$$

Based on this analysis, it can be concluded (see also Table 3) that the EPS left after detachment contains up to 68% protein. The amounts of protein (C_{PR}) and polysaccharides (C_{PS}) decreased significantly ($F_{7.71} = 158.4$, $P = 0.002$) after the second deposition cycle for

Table 4. Mean roughness (R_a) of glass prior to and after two cycles of detachment and deposition of *P. aeruginosa* SG81 and SG81R1, as calculated from AFM height images

All data are means $\pm$ SD based on measurements on three different areas of two experiments with separately grown bacterial strains and substratum surfaces.

Substratum	<i>P. aeruginosa</i> strain	R_a (nm)
Bare glass		1.0 $\pm$ 0.1
After redeposition cycle 1	SG81	4.6 $\pm$ 4.0
	SG81R1	1.4 $\pm$ 0.2
After redeposition cycle 2	SG81	13.5 $\pm$ 9.4
	SG81R1	2.5 $\pm$ 0.8

both strains, leaving significantly higher ($F_{7,71} = 162.4$, $P = 0.02$) amounts of uronic acids. However, the increase in C_{UA} was significantly higher for the EPS-producing strain SG81 than for the non-EPS-producing strain SG81R1.

Topographic height AFM images of EPS left on glass after the first air-bubble-induced detachment of adhering *P. aeruginosa* are shown in Fig. 3. Streaks oriented in the direction of the scan can be observed for both SG81 and SG81R1. However, those from the EPS-producing strain were more numerous. A cross-section of those streaks revealed an irregular film of thickness ranging from 3 to 32 nm for the EPS-producing SG81, and from 1.5 to 9 nm for the non-EPS-producing strain SG81R1. The mean surface roughness, R_a , of the substrata after the first deposition cycle of the EPS-producing strain increased significantly ($F_{7,71} = 10.36$, $P = 0.032$), as compared to bare glass substratum (Table 4). No significant difference was found between the roughness of glass prior to and after adhesion and detachment of the non-EPS-producing strain SG81R1, but after the second cycle of deposition and detachment, a significantly elevated ($F_{7,71} = 10.3$, $P = 0.008$) mean roughness was observed for both strains.

DISCUSSION

In this study, the role of EPS in the initial adhesion of an EPS-producing and an EPS-non-producing *P. aeruginosa* strain to inert substrata with different hydrophobicity was investigated. Both strains adhered better to a hydrophilic than to a hydrophobic substratum, while bacterial EPS production, as well as pre-coating a substratum surface with isolated EPS, discouraged bacterial adhesion. Moreover, an anchoring effect of EPS production could not be observed because, despite its EPS production, a slightly higher percentage of strain SG81 cells were detached by a passing air bubble than the non-producing strain SG81R1. Detachment percentages were, however, low for both strains (Gómez-

Suárez *et al.*, 2001) and the strains left EPS behind as footprints on the substratum surface. The height of protuberances in the EPS left behind are consistent with the sizes of globular proteins, such as lysozyme ($4.5 \times 3.0 \times 3.0$ nm) and albumin ($11.6 \times 2.7 \times 2.7$ nm) (Haynes & Norde, 1994).

Most often and contrary to the present findings, EPS is reported to enhance bacterial adhesion to inert substrata (Costerton *et al.*, 1985; Van Loosdrecht *et al.*, 1990). For instance, Dûfrene *et al.* (1996a) found that adhesion of *Azospirillum brasilense* to both hydrophilic and hydrophobic substrata occurred only after stimulated EPS production and correlated it to an increase of protein content of the EPS over time. A similar increase in adhesion of *Pseudomonas fluorescens* associated with high protein contents in EPS has been reported by DeFlaun *et al.* (1990). Recently, Azeredo & Oliveira (2000) found that the presence of surface-active compounds increased the adhesion of *Sphingomonas paucimobilis* to glass.

It is interesting that the non-EPS-producing strain, as shown in this study by a lack of a decrease in surface tension of a suspension of strain SG81R1, has cell surface characteristics similar to those of the EPS-producer SG81. Moreover, after repeated cycles of deposition and detachment, SG81R1 shows similar adhesion behaviour to SG81, and also the substratum characteristics after detachment become alike. This indicates that SG81R1 excretes minor amounts of EPS, undetectable by ADSA-P. The role of this EPS in discouraging bacterial adhesion only becomes evident after several cycles of detachment and deposition. Recently, production of a minor amount of EPS by *P. aeruginosa* SG81R1, growing in a biofilm, has been described by Strathmann *et al.* (2002). Alternatively, Vandevivere & Kirchman (1993) as well as Allison & Sutherland (1987) suggested that attachment stimulates bacterial EPS synthesis, and putatively non-EPS-producing *P. aeruginosa* strains related to cystic fibrosis could through mutation or selection turn into EPS-producing ones during adaptation to different ecological sites, such as stress and growth conditions (Boucher *et al.*, 2000; Grobe *et al.*, 1995).

The EPS excreted by *P. aeruginosa* SG81 is highly surface active, which is probably due to a uronic-acid-containing polymer. Grobe *et al.* (1995) have analysed the chemical composition of EPS excreted by *P. aeruginosa* SG81 grown on Pseudomonas Isolation Agar and found that the crude extracellular material of this strain contained polysaccharides (16.4%), uronic acids (79.3%) and small amounts of proteins (4.3%). The chemical composition obtained by XPS of the footprints left behind by *P. aeruginosa* after air-bubble-induced detachment showed a greater content of uronic acid in the footprints of the EPS-producing strain than of the putatively non-EPS-producing strain. The uronic acid content calculated increased with the number of detachment and redeposition cycles. Uronic acids (i.e. mannuronic and glucuronic acids) excreted by *P. fluor-*

escens strains have been reported to have surface-active properties, preventing their adhesion to surfaces (Pringle *et al.*, 1983), an effect similar to that of the uronic acids excreted by the *P. aeruginosa* strain in this study. These findings agree with earlier studies that showed how the excretion of surface-active material by lactobacilli (McGroarty & Reid, 1988; Velraeds *et al.*, 1998a), *Streptococcus thermophilus* (Busscher *et al.*, 1997) or *Streptococcus mutans* (Van Hoogmoed *et al.*, 2000) either discourage their own adhesion or adhesion of competitors to substrata.

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