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Biofouling, 1998, Vol 13(3), pp 213–231
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CELL SURFACE PHYSICOCHEMISTRY ALTERS BIOFILM DEVELOPMENT OF *PSEUDOMONAS AERUGINOSA* LIPOPOLYSACCHARIDE MUTANTS

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(Received 23 April 1998; in final form 9 July 1998)

The hydrophobic and electrostatic characteristics of bacterial cell surfaces were compared with attachment proclivity and biomass accumulation over time between wildtype *Pseudomonas aeruginosa* serotype O6 (possesses A and B band LPS), and three LPS-deficient mutants, *viz.* A28 (A^+B^-), R5 (A^+B^-), and Gt700 (A^-B^-). The hydrophobic character of each serotype was determined by hydrophobic interaction chromatography and salt-aggregation, and strains were ranked similarly by each method, *viz.* R5 $\geq$ A28 > Gt700 > O6. The anionic characteristics of cell-surfaces were determined by electrostatic interaction chromatography and by zeta-potential measurements, and ranked R5 > A28 $\geq$ Gt700 > O6. Adhesion and biofilm accumulation on stainless steel were significantly different between strains, following the order R5 > A28 $\gg$ O6 > Gt700. Biofilm rankings were similar on glass, a second hydrophilic substratum. The mutant strains with a strongly hydrophobic character (R5 and A28) demonstrated a significantly greater capacity to form biofilms. These adherent mutants also appeared to have a more anionic cell surface, which may have played a role in biofilm formation on the hydrophilic substrata.

Keywords: hydrophobicity; biofilms; zeta potential; *Pseudomonas aeruginosa*; attachment; lipopolysaccharides

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INTRODUCTION

Bacterial attachment and biofilm formation is considered a primary step in surface colonization by micro- and macroorganisms in the biofouling process (Characklis, 1990). Bacterial fouling of surfaces is a well documented process resulting in serious health problems, such as dental decay (Lappin-Scott & Costerton, 1989; Cowan *et al.*, 1992), and economic problems such as corrosion of submerged structures and reduced fuel efficiency of ships (Lappin-Scott & Costerton, 1989; Characklis, 1990; Bohlander, 1991; Alberte *et al.*, 1992). In contrast, a functional biofilm is central to many biotechnological processes such as water and wastewater treatment (Bryers, 1990; Bryers & Characklis, 1990). Therefore, elucidation of bacterial attachment mechanisms is an important aid in the development of effective and economical fouling-control strategies.

Cell attachment has been related to overall electrokinetic or thermodynamic properties (Fletcher, 1977), such as electrostatic charge (Pedersen, 1980; Fletcher, 1987), hydrophobicity (Magnusson & Johansson, 1977; Dahlbäck *et al.*, 1981; Clark *et al.*, 1985; Van Loosdrecht *et al.*, 1987a; Allison *et al.*, 1990; VanHaecke *et al.*, 1990) and surface free energy (Van Loosdrecht *et al.*, 1987b; Van der Mei *et al.*, 1992). Several cell surface polymers play a significant role in physicochemistry and adhesion, including surface proteins (Tylewska *et al.*, 1979; Paul & Jeffrey, 1985; Montgomery & Kirchman, 1993), lipopolysaccharide (Magnusson & Johansson, 1977; Hermansson *et al.*, 1982; Beech & Gaylarde, 1989; Paradis *et al.*, 1994; Williams & Fletcher, 1996), capsular polysaccharide (Lappin-Scott & Costerton, 1989; Shea *et al.*, 1991), and surface fimbriae or fibrils (Smyth *et al.*, 1978; Hermansson *et al.*, 1982; Van der Mei *et al.*, 1992). Very few studies, however, have simultaneously examined the relationships between and the physicochemical characteristics of microorganisms, cell surface biomolecular structure, and adhesion capability (*e.g.* Makin & Beveridge, 1996a; Hermansson *et al.*, 1982).

Laboratory bioadhesion studies range from the examination of initial adsorption in static systems over a few hours (Clark *et al.*, 1985; Shea *et al.*, 1991; Montgomery & Kirchman, 1993; Makin & Beveridge, 1996a), to biofilm development over a number of days in flow-through systems (Mittelman *et al.*, 1992; Vassilakos *et al.*, 1993; Arrage *et al.*, 1995; Palmer & Caldwell, 1995). The mass transport dynamics (diffusion, sedimentation, convection, shear forces) of these two approaches differ greatly (Sjollema *et al.*, 1989; Mittelman *et al.*, 1992), as do the growth conditions and physiological stresses presented to bacteria. Flow systems are advantageous

because data comparisons can be more easily made between systems differing in size and design (Sjollema *et al.*, 1989) and they more closely approximate most natural environments. On-line systems for monitoring biofilm development offer additional advantages because they are non-destructive and examine processes in real-time (Arrage *et al.*, 1995; Nivens *et al.*, 1995; Palmer & Caldwell, 1995; White *et al.*, 1996).

Pseudomonas aeruginosa, a Gram-negative bacterium, was chosen for the present study because it is ubiquitous and well-characterized mutants are available (Lam *et al.*, 1992; Dasgupta *et al.*, 1994; Makin & Beveridge, 1996a). Most *P. aeruginosa* strains produce two chemically and antigenically distinct types of O-polysaccharide, *viz.* low molecular weight "A-band" (common antigen) and high molecular weight "B-band" (O-antigen; Dasgupta *et al.*, 1994). A-band polysaccharide is composed of neutral D-rhamnose (Arsenault *et al.*, 1991) and carries a low anionic charge; B-band is composed of reactive amino sugars and carries an anionic charge (Knirel *et al.*, 1988). O-polysaccharides (*i.e.* O-antigen) allow Gram-negative bacteria to evade host immune systems (Dasgupta *et al.*, 1994) and rapidly alter their surface (*i.e.* monosaccharide composition and O-side chain length) to adapt to changing environmental conditions (Makin & Beveridge, 1996b; Beveridge *et al.*, 1997).

Three LPS-deficient mutants of *P. aeruginosa* serotype O6 (Table I) were used in this study to investigate the relationship between O-specific heteropolysaccharide moieties (A and B-band) and cell physicochemical and adhesion on hydrophilic surfaces. The relative hydrophobic character of the cell surfaces was estimated using hydrophobic interaction chromatography (HIC) and a modified salt aggregation test. Cell surface electrostatic character (net negativity) was examined by electrostatic interaction chromatography (ESIC) and by electrophoretic mobility. Attachment and biofilm development on hydrophilic substrata were examined non-destructively in laminar flowcells using on-line monitoring of biomass as tryptophan fluorescence (TF).

TABLE I Summary of derivational and structural information for *P. aeruginosa* serotype O6 and LPS-deficient mutants from Dasgupta *et al.* (1994)

Strain	*Mutant selection rationale*	*O-Polysaccharide*	*Core sugars*
O6	Wild type ATCC#33354	A^+B^+	Complete[a]
R5	Resistance to smooth-LPS phage E79	A^+B^-	Deletions[b]
A28	Transposon Tn*5-751* induced	A^+B^-	Complete[a]
Gt700	Gentamicin resistance (700 μg ml^{-1})	A^-B^-	Deletions[c]

[a] contains D-glucose, L-rhamnose, 2-amino-2-deoxy-D-galactose, L-glycero-D-manno-heptose, L-alanine, and KDO; [b] contains less D-glucose and no L-rhamnose; [c] truncated core oligosaccharides; not further elucidated chemically

MATERIALS AND METHODS

Bacterial Strains

P. aeruginosa ATCC 33354 wild type (IATS serotype O6) and its LPS-deficient mutants described in Table I were obtained from J S Lam, University of Guelph, Ontario, Canada. Examination of cell cultures by light microscopy showed O6 and Gt700 to be motile; motility-plate assays (0.3% tryptic soy agar stabs) showed A28 and R5 mutants to be non-motile. Transmission electron microscopy revealed that R5 did not possess flagella (data not shown).

Maintenance and Growth

Strains were maintained on tryptic soy agar (TSA) slants (Difco Laboratories, Detroit, MI) at room temperature, and sub-cultured monthly. Strain Gt700 was maintained on agar supplemented with 700 $\mu g\,ml^{-1}$ gentamicin (Sigma Chemical Company, St. Louis, MO); A28 was maintained on agar of kanamycin/trimethoprim (Sigma; 100 $\mu g\,ml^{-1}$ each) agar (Dasgupta *et al.*, 1994). Batch cultures were grown for 14 h at 25°C with shaking (100 rpm) in 250 ml flasks containing 50 ml tryptic soy broth (TSB; Difco). Strain Gt700 was grown for 18 to 20 h because its growth rate was lower. For attachment studies a minimal medium was used containing ($mg\,l^{-1}$): 12.5 NH_4Cl, 2.5 $CaCl_2$, 12.5 $MgSO_4$, 175.0 K_2HPO_4, 50.0 D-glucose (unless otherwise indicated), and 0.63 $ml\,l^{-1}$ of Wolfe's minerals (Balch *et al.*, 1979). For inoculation of flowcells, *P. aeruginosa* strains were grown for at least 36 h in 2 l chemostats (dilution rate 0.1 h^{-1}) in minimal medium containing a final concentration of 550 $mg\,l^{-1}$ glucose. Chemostats were vented and aerated by constant stirring at 200–250 rpm.

Radiolabeling and Harvesting of Cells

Cells were grown as described earlier but in 250 ml center-well flasks (Bellco Biotechnology, Vineland, NJ) with 5 ml of 2 N NaOH as a $^{14}CO_2$ trap. Sodium [1-^{14}C]-acetate (56.8 mCi $mmol^{-1}$; New England Nuclear Research Products-DuPont, Wilmington, DE) was added with inoculum at 0.75 $\mu Ci\,ml^{-1}$ yielding specific activities of $\sim 4 \times 10^{-4}$ dpm $cell^{-1}$ in 14 h batch cultures. Specific activities were determined by filtering triplicate aliquots of bacterial culture through 0.2 μm pore size clear polycarbonate filters (Poretics Corporation, Livermore, CA) for liquid scintillation counting and through similar blackened filters for acridine orange direct microscopic

TABLE II Properties of buffered solutions used for hydrophobic interaction chromatography (HIC), electrostatic interaction chromatography (ESIC), zeta-potential measurements (ZP), and salt-aggregation experiments (SA)

Solution[a]	*Concentration* (M)	pH	μ[b] (M)	*Experiment*
Adsorption	0.01	7.2	0.01	HIC, SA
Buffer[c] (AB)	0.01	8.0	0.01	ESIC
HEPES (Na salt)	0.10	8.0	0.09	ESIC
K Phosphate	0.10	8.0	0.28	ESIC
	0.20	8.0	0.55	ESIC
K Phosphate[d]	0.01	7.0	0.03	ZP
	0.01	8.0	0.04	ZP
	0.01	9.0	0.05	ZP
Buffered NaCl[e]	3.0	7.2	3.01	HIC, SA
1% Tween 80 (v/v)	0.08	7.2	0.01	HIC, SA

[a] solutions were prepared in AB except as indicated and pre-filtered (0.2-μm); [b] ionic strengths (μ) were calculated as described by Skoog and West (1980); [c] AB (Clark *et al.*, 1985): 1 mM K phosphate, 5 mM KCl, 1 mM $CaCl_2$, and 0.01 mM $MgCl_2$; [d] Phosphate buffer used for ZP studies was prepared in distilled water; [e] prepared in AB with an additional 10 mM K phosphate

counting (AODC; Arrage *et al.*, 1995). The detection limit was 5×10^5 cells (*i.e.* 200 cpm). Culture density was determined at 600 nm (Shimadzu UV-120-01 Specrophotometer, Shimadzu Corporation, Japan) and cultures were harvested by centrifugation for 30 min at approximately $4000 \times g$ (Dynac benchtop centrifuge, Beckton and Dickinson and Company, Parsippany, NJ), and resuspended in filtered buffer solution (Table II) to $OD_{600nm} \sim 4.0$ (approximately 10^{10} cell ml^{-1}).

Hydrophobic and Electrostatic Interaction Chromatography (HIC and ESIC)

Glass Pasteur pipettes (14.6×0.5 cm) were used as chromatographic columns (Smyth *et al.*, 1978) fitted with seven-cm lengths of medical grade silicone tubing (1.58 mm ID) with plastic clamps serving as flow regulators. Octyl-substituted or unsubstituted CL-4B (4% cross-linked agarose) were resins used for HIC, and cationic diethylaminoethyl (DEAE)-substituted or unsubstituted CL-6B (6% cross-linked agarose) were resins used for ESIC (Sigma). Packed columns with a mean bedvolume of 0.5 ± 0.05 ml were pre-washed with 5–10 ml of pre-filtered eluent (Table II). ^{14}C-labeled bacterial suspensions (0.1 ml; approximately 10^9 cells) were applied directly to resin beds using extended pipette tips (Molecular Bio-Products, San Diego, CA); 0.1 ml aliquots were also placed directly into vials for scintillation counting. Bacteria were drained onto columns and equilibrated 20 min before elution with 1 ml (HIC) or 5 ml (ESIC) of eluent (Table II).

The total effluent from each column was collected onto 0.2 μm polycarbonate filters for liquid scintillation counting. Leakage of ^{14}C-label from adsorbed cells was consistently below 6% in 3 M salt and phosphate buffers, and below 3% in other buffers.

As a check for non-specific retention, anionic carboxylated-latex particles (1.2 ± 0.012 μM diameter Fluoresbrite YG beads, Polysciences Incorporated, Warrington, PA) were chromatographed. Approximately 10^6 fluorescent beads were eluted with 5 ml eluents and chromatographic recoveries measured by fluorometry at emission/excitation wavelengths of 458/540 nm. Microscopic examination of beads in the chromatographic buffers showed that clumping occurred rarely and if so, in aggregates of less than 5 beads.

Liquid Scintillation Counting

Six ml plastic vials containing filter discs or aqueous sample (volume $\leq$ 1 ml) were filled with 5 ml of scintillation cocktail (Insta-gel XF; Canberra-Packard, Meriden, CT) and counted for 3 min (1212 RackBeta Liquid Scintillation counter, LKB Instrument Company, Gaithersburg, MD) at energy levels specified in operators manual. Counting efficiency was $87.7\% \pm 0.2\%$ ($n = 10$) against an external standard.

Zeta Potential Measurements

Particulate microelectrophoresis was performed on late log-phase bacterial cells in 10 mM phosphate buffer adjusted to pH 7.0, 8.0 and 9.0 as described by Van der Mei *et al.* (1992). Mean zeta potentials were calculated from mobility of at least 200 individual cells. Triplicate experiments were performed.

Cell Attachment and Biofilm Formation in Flowcells

Attachment and biofilm development experiments were performed in triplicate in laminar flowcells (Arrage *et al.*, 1995). Glass or polished 316L stainless steel coupons ($35 \times 70 \times 3$ mm; Metal Samples, Munford, AL) were used as test surfaces. Coupons were washed in detergent, rinsed in ultrapure (17 MΩ) water, then rinsed in analytical grade acetone. Prior to inoculation, medium flow was initiated at 4 ml min^{-1} and background fluorescence was measured at $\lambda_{exc}/\lambda_{em}$ of 295/342 nm, wavelengths used to monitor biomass *via* tryptophan fluorescence (TF). Fluorescence measurements were taken with a bifurcated fiber-optic cable (Arrage *et al.*, 1995) using a Fluorolog II fluorometer (Spex Industries Incorporated, Edison, NJ). Flowcells were inoculated with a 4 h pulse of chemostat culture (OD 0.05; approximately

10^7 cells ml^{-1}) delivered into a stream of medium at an equal flow rate. As described by Arrage *et al.* (1995), cells were removed from coupons by sonication and enumerated by AODC.

Statistical Analyses

Standard deviations (SD), 95% confidence intervals, one-tailed F-tests, and 2-tailed Students *t*-Tests ($\alpha = 0.05$) were calculated using Excel (Microsoft Corporation, Redmond, WA). Error values for data quotients (*e.g.*% cells eluted) were calculated from individual errors using statistical error propagation (Skoog & West, 1980).

RESULTS

Hydrophobic Interaction Chromatography (HIC) and Salt-aggregation Tests

Figure 1 illustrates the percentage of radiolabeled cells eluted from hydrophobic (octyl-substituted) or control (unsubstituted) columns with adsorption buffer (AB), 3 M NaCl and 1% Tween 80 (bars left to right). The affinity of O6 cells for the hydrophobic resin decreased slightly with increased ionic strength (μ) of eluent, indicating a hydrophilic character, while Gt700, R5 and A28 cells showed significant increase in affinity to hydrophobic resin under conditions of high salt (*i.e.* hydrophobic character). The affinity of strains for octyl columns in 3 M NaCl followed the order A28 > R5 > Gt700 $\gg$ O6 (Figure 1). The recovery of R5 and A28 cells in the control columns (in the absence of hydrophobic ligand) was low when eluted with 3 M NaCl (0.7%) and also low with Tween 80 detergent (40%). The control column data illustrate that non-specific (*i.e.* non-hydrophobic) forces such as physical trapping were partially responsible for the retention of bacterial cells in agarose columns, particularly that of R5 and A28 (Figure 1). Elution with Tween 80 resulted in a significant reduction in the affinity of R5, A28 and Gt700 (but not O6) to octyl resins, illustrating a degree of hydrophobic interactions of these serotypes.

The data in Figure 1 have been corrected to reflect hydrophobic ligand-affinity by calculating hydrophobic indices (HI; Clark *et al.*, 1985) in Table III. The HI represents the fraction of the eluted cell population that was retained in the hydrophobic column due to hydrophobic interactions alone. A strongly hydrophobic character is reflected by HI values approaching 1.0. Under conditions of low μ both R5 and A28 were strongly hydrophobic

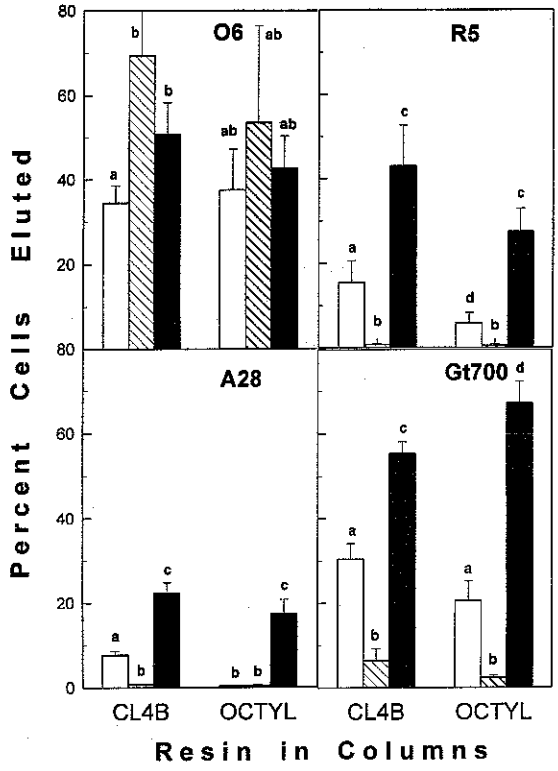

FIGURE 1 Percentage *P. aeruginosa* cells eluted from control (CL-4B) and hydrophobic (octyl-substituted) agarose columns by hydrophobic interaction chromatography, for strains O6, Gt700, A28 and R5 using eluents at pH 7.2. □ = AB; ▨ = 3 M buffered NaCl; ■ = 1% Tween 80. Error bars = 95% confidence limits. Different letters denote significant differences ($\alpha = 0.05$) between percentages eluted within an individual serotype.

relative to O6 and Gt700 cells. In buffered 3 M NaCl, high retention of R5, A28 and Gt700, but not of O6, was observed (Figure 1; Table III). HI values were reduced by Tween for O6, Gt700 and A28, but not for R5. These data demonstrate that O6 exhibited strongly hydrophilic behavior, whereas R5 and A28 were highly hydrophobic. Gt700 appeared to be intermediate in hydrophobicity. The relative affinity of these strains to hydrophobic resin were A28 > R5 > Gt700 > O6 (Figure 1 and Table III).

Salt Aggregation Test and Latex Sphere Elution

Cell aggregate formation was observed microscopically in eluents at different μ and strains were arranged in Table IV, in the order in which they were

TABLE III Hydrophobic indices (HI) and charge indices (CI) calculated[a] for *P. aeruginosa* strains in various eluents

Index	*Eluent*	*P. aeruginosa strains*			
		O6	R5	A28	Gt700
HI	AB[b]	0[c]	0.62	0.95	0.32
	3 M NaCl	0.23	0.35	0.42	0.64
	1% Tween 80	0.16	0.36	0.23	0
CI	AB	0.96	0.97	0.97	0.99
	0.1 M HEPES	0.74	0.96	0.97	0.98
	0.1 M Phosphate	0.50	0.97	0.96	n.d.[d]
	0.2 M Phosphate	0.05	0.97	0.44	0.18

[a] HI (and CI) = % cells eluted from control columns minus % cells eluted from reactive column, divided by % cells eluted from control column (Clark *et al.*, 1985); [b] Adsorption Buffer (see Table II); [c] zero denotes cell recovery from reactive columns $\geq$ that from control columns; [d] not determined

TABLE IV Salt-aggregation of *P. aeruginosa* strains after suspension in various eluents

Eluent	*P. aeruginosa strain*			
	R5	A28	Gt700	O6
AB[a]	*	—	—	*
1% Tween 80	—	—	—	—
0.1 M HEPES	—	—	—	—
0.1 M Phosphate	*	*	—	—
0.2 M Phosphate	**	**	*	—
3 M NaCl	***	***	*	—

[a] Adsorption Buffer; — = no microscopically visible cell clumps in unstained wet mounts 20 min after resuspension; * = small clumps (estimated to be 5 to 50 cells in size); ** = intermediate clumps (50 to 100 s of cells); *** = large clumps (100 to 1000 s of cells)

salted-out from the aqueous phase (a modified salt-aggregation test; Lindahl *et al.*, 1981). The same hydrophobicity sequence was obtained by salting-out suspended cells as by HIC, *viz.* R5 $\geq$ A28 > Gt700 > O6 (Table IV). Cell clumping may be responsible for the high retention of R5, A28 and Gt700 in agarose columns, as aggregate size correlated with non-specific cell retention in control resin columns. For example, cell recovery was much lower for R5 and A28 under high-salt conditions that caused flocculation (Figure 1). Non-aggregated O6 cells were also retained in control columns by up to 30% (Figure 1).

To investigate trapping of individual cells, fluorescent latex spheres were aliquotted on columns with sequential elution using 5 ml AB followed by 5 ml of 1% Tween 80 (v/v). Bead recovery was 87.3 (±14.9)% and 75.8 (±4.8; $n = 3$)% from unsubstituted CL-4B and CL-6B, respectively, and 40.6 (±10.2)% from octyl-substituted columns.

Electrostatic Interaction Chromatography

The relative affinities for cation-exchange resin were compared between strains by ESIC and expressed as the percentage of cells eluted from anionic or unsubstituted columns at increasing μ (Figure 2). Due to substantial non-specific retention of cells in control columns (Figure 2) the data were corrected to reflect electrostatic interactions by calculating charge indices (CI) (Table III). CI values approaching 1.0 indicate cells with a high degree of electrostatic affinity to DEAE columns. CI values were eluent-dependent and were equal for all strains in environments with low μ. As μ increased strain O6 was readily eluted from DEAE columns in roughly direct proportion to μ (Figure 2), whereas R5 affinity remained unchanged with

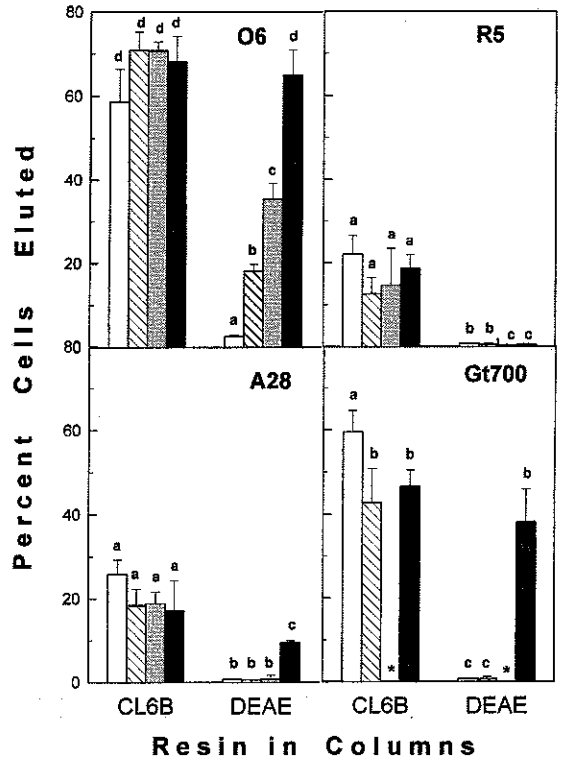

FIGURE 2 Percentage *P. aeruginosa* cells eluted from control (CL-6B) and anionic (DEAE-substituted) agarose columns by electrostatic interaction chromatography, for strains O6, Gt700, A28 and R5 using eluents of increasing ionic strength (at pH 8.0). □ = AB; ▨ = HEPES; ▩ = 0.1 M phosphate; ■ = 0.2 M phosphate buffer. Error bars = 95% confidence limits. Asterisks = experiments not performed. Different letters denote significant differences ($\alpha = 0.05$) between percentages eluted within an individual serotype.

increased μ (Table III). The electrostatic affinity of O6 was disrupted by 0.1 M phosphate, of Gt700 and A28 by 0.2 M, and of R5 not at all. The relative strengths of cell surface electrostatic attraction to cationic resin, at high μ, were R5 > A28 > Gt700 > O6. These data indicate that R5 is highly charged, Gt700 and A28 are intermediate, and O6 is uncharged. R5 did not elute at the highest μ suggesting that retention in DEAE columns may have been due to factors other than electrostatic attraction, such as clumping (as shown in Table IV) and possibly hydrophobic interactions.

Zeta Potential Measurements

Zeta potentials were determined by the particulate microelectrophoretic mobility of individual cells in suspension at various pHs. Data are shown in Figure 3 for one representative experiment; triplicate experiments revealed the same ranking of zeta potentials between strains but the absolute values

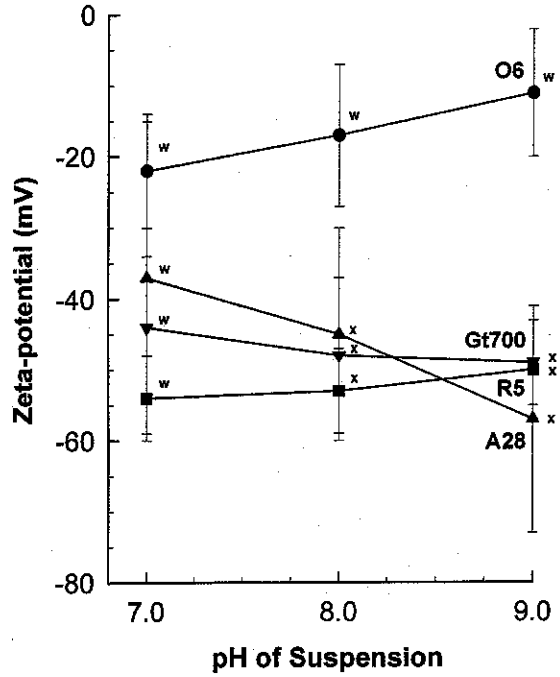

FIGURE 3 Mean zeta potentials of *P. aeruginosa* wildtype, O6 (●), and its LPS-deficient mutants R5 (■), A28 (▼) and Gt700 (▲) at different pH values. Error bars = SD from at least 200 cells. Different letters denote significant differences (at 95% confidence) between serotypes at a given pH.

shifted slightly. Strain O6 had a significantly lower zeta potential (−11 to −22 mV) than the three LPS mutants (−37 to −57 mV) at 95% confidence ($n = 200$). Standard deviations were large in the case of O6 and Gt700, indicating some degree of heterogeneity within cell populations. Even more pronounced heterogeneity was seen with A28; the electrophoretic mobilities of A28 cells revealed two distinct sub-populations with mean zeta potentials at approximately −45 mV and −3 mV. At neutral pH and under the pH conditions of ESIC experiments (*i.e.* pH 8) the relative ranking of zeta potential was in the order R5 ≥ Gt700 ≥ A28 > O6. This ranking order was very similar to that obtained by ESIC.

Cell Attachment and Biofilm Development

Attachment and biofilm development of *P. aeruginosa* strains on stainless steel coupons were monitored in flowcells by on-line tryptophan fluorescence (Figure 4A), and by enumeration of attached cells (Figure 4B). Initial attachment (6 h) of R5 and A28 was statistically similar, and was significantly greater than that of O6 and Gt700 (Figure 4A). The accumulated biomass was significantly different for each strain after 24 h, following the order R5 > A28 > O6 > Gt700 (Figure 4A). The cell numbers of O6 and R5 cm^{-2} in biofilms (Figure 4B) corroborated the TF data on stainless steel, *i.e.* R5 cells cm^{-2} was initially 9-fold higher than that of O6 and remained substantially higher over time. TF is linearly related to cell numbers over the range of biofilm densities reported here ($\sim 10^5$ to 10^7 cells cm^{-2}; Angell *et al.*, 1993). On glass, initial attachment of R5 and O6 was similar, but biofilm development of R5 was 15-fold greater than that of O6 after 24 h (Figure 4C). Biomass accumulation on stainless steel coupons was 2- to 20-fold greater than on glass coupons (*cf.* Figure 4A and C), but similar trends were observed between strains.

DISCUSSION

It is necessary to perform a number of assays to determine physicochemical properties of cell surfaces, since reliance on one assay is inadequate (Mozes & Rouxhet, 1987; Dillon *et al.*, 1996). Good agreement between HIC and salt-aggregation was demonstrated for these strains, even for strains with an intermediate hydrophobic character. Each method has its shortcomings, such as cell density effects in salting-out or high variation in HIC (Dillon *et al.*, 1996). In chromatographic techniques, non-specific retention of

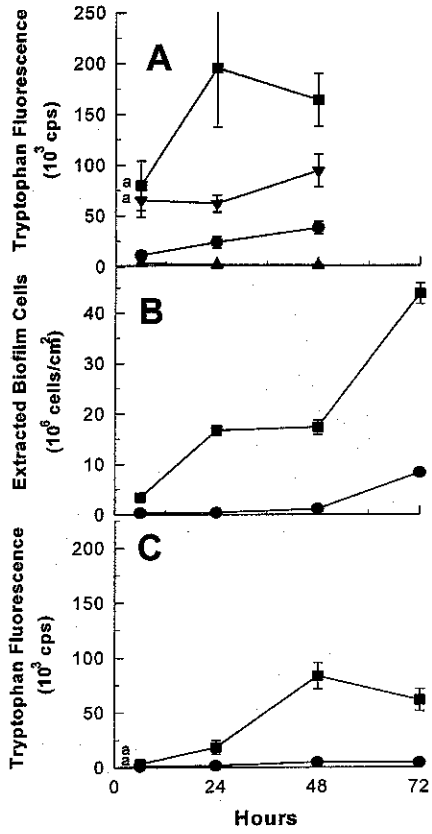

FIGURE 4 Biomass accumulation of *P. aeruginosa* O6 (●), R5 (■), A28 (▼), and Gt700 (▲) over time in laminar flowcells. A = monitored on stainless steel coupons by on-line tryptophan fluorescence; B = by direct cell counts after extraction; C = on glass coupons by tryptophan fluorescence. Error bars = 95% confidence limits. Similar letters beside data points denote no statistical difference ($\alpha = 0.05$).

bacterial cells in agarose columns can create a problem. This was overlooked originally (Smyth *et al.*, 1978; Pedersen, 1980), found to be negligible by some investigators (Makin & Beveridge, 1996a), but has been documented by numerous authors (Kjelleberg & Hermannson, 1984; Clark *et al.*, 1985) and is generally ascribed to aggregation of hydrophobic cells. The present results demonstrate that 25% of individual bacterium-sized hydrophilic latex spheres were lost in control columns and up to 60% in octyl-substituted columns. Strict use of controls is required in these chromatography methods to avoid erroneous interpretation of affinity data.

The ESIC data compared well to electrophoretic mobility in this study. Assuming cells with lower anionic surface charge were more readily

displaced from anion-exchange columns, the ranking for anionic character from each method was $O6(A^+B^+) < Gt700(A^-B^-) \leq A28(A^+B^-) \leq R5$. Makin and Beveridge (1996a) also observed low affinity of an A^+B^+ strain of *P. aeruginosa* PAO1 and high affinity of B^- strains to anion-exchange columns in ESIC in 0.2 M phosphate buffer. Zeta potential measurements have been employed since the early 1930s to examine cell surface electrokinetic properties and to supply information on the nature of cell surface constituents (Dyar & Ordal, 1946); electrophoretic mobility is therefore better understood than the more recent ESIC technique (Pedersen, 1980). In addition to cell trapping, the high degree of non-specific affinity observed in ESIC columns may have resulted from hydrophobic interactions. Amine sites on packed DEAE resin (130–170 μEq ml^{-1}; Sigma) were presumably saturated by 0.2 M phosphate buffer (~370 mEq ml^{-1} at pH 8.0; pK_a 7.2); after neutralization the resin may have acquired a hydrophobic character due to diethyl groups. Kjelleberg and Hermansson (1984) also noted that cell retention in ESIC columns reflected cell hydrophobicity and attributed it to hydrophobic interactions within the DEAE matrix.

LPS has been demonstrated to affect cell surface physicochemistry in a number of bacteria. Magnusson and Johansson (1977) showed that rough mutants of *Salmonella typhimurium* had greater hydrophobicity than the wild type. Hermansson *et al.* (1982) showed that truncation of O-side-chain and core oligosaccharide in rough mutants of *Serratia marcescens* and *S. typhimurium* were related to increased hydrophobicity. Makin and Beveridge (1996a) also observed greater hydrophobic and electronegative character for rough mutants of *P. aeruginosa* serotype O5. In the present study, mutants R5 and A28 that expressed A- but not B-band O-polysaccharide (A^+B^-) had more hydrophobic and electronegative physicochemical surface properties than the wildtype O6 (complete LPS, A^+B^+). R5 and A28 (A^+B^-) were also strongly adherent to glass and to stainless steel relative to both O6 (A^+B^+) and Gt700 (A^-B^-). The severely truncated LPS of strain Gt700 (A^-B^-) yielded a cell surface of higher hydrophobicity in 3 M NaCl than A-band-containing counterparts, yet the hydrophobic interactions of Gt700 were completely disrupted by detergent. This suggests that the chemical basis for those interactions is exposed and accessible in aqueous suspension. Core oligosaccharide is normally highly charged and hydrophilic, and Gt700 should theoretically have a highly hydrophilic and charged cell surface because its core LPS is not masked by A or B band O-polysaccharide. However, Gt700 has an incomplete core; the HIC, ESIC and zeta-potential results from this study support the prediction that Gt700 has lost charged regions of its core, thereby rendering it intermediate

in charge and hydrophobicity between the hydrophilic wild type and the highly hydrophobic A28 and R5 strains.

In contrast to the results of the present adhesion studies, Makin and Beveridge (1996a) showed that hydrophilic wildtype *P. aeruginosa* PAO1 (serotype O5; phenotype A^+B^+) was much more adherent on glass surfaces than its hydrophobic A^+B^- mutant. Differences in relative adhesion rankings among the mutant lineages of PAO1 or O6 may be due in part to differences in density, distribution and/or composition of the B-band LPS on cell surfaces. Lam *et al.* (1992) demonstrated by TEM that B-band polysaccharide was patchy on O6 cells but dense on PAO1. Furthermore, the B-band polysaccharide of PAO1 has different reactive monosaccharide components from that of O6 (Wilkinson, 1983; Knirel *et al.*, 1988). Adhesion differences seen between these two studies may also have resulted from the different transport dynamics (*e.g.* convection, desorption; Sjollema *et al.*, 1989) of batch conditions (as in PAO1 study; Makin and Beveridge, 1996a) *vs* flowing systems (as in this study), different surface conditioning films in different media, differing cell motility (Korber *et al.*, 1989; DeFlaun *et al.*, 1994) or perhaps cell age or other factors (Fletcher, 1977).

It appears that the long chain B-band polysaccharide masked the charge of the core region from bulk solution; O6 cells, with B-band, had a net neutral zeta potential but strains without B-band O-polysaccharide (R5, A28, and Gt700) possessed negative zeta potentials. Electronegativity and hydrophobicity correlated with adherence of the strains in this study except in the case of Gt700. Gt700 may be atypical because it has deletions of charged core oligosaccharides (Dasgupta *et al.*, 1994) from its completely exposed core. The core oligosaccharide is usually highly charged with upwards of 10 phosphate residues (Wilkinson, 1983). Core deletions may contribute to the intermediate anionic charge between its wildtype and the A^+B^- mutants. Magnusson and Johansson (1977) also showed an increase in cell surface charge in *S. typhimurium* with truncation of LPS from an uncharged S-form to negatively charged R-mutants.

Bacterial attachment and biofilm formation were profoundly altered in LPS-deficient *P. aeruginosa* mutants. The results presented here, and those from other studies (Magnasson & Johansson, 1977; Beech & Gaylarde, 1989; Paradis *et al.*, 1994; Makin & Beveridge, 1996a; Williams & Fletcher, 1996) demonstrate that the presence and composition of LPS greatly contributes to bacterial adhesion and cell surface physicochemical properties. It is suggested that hydrophobicity played the major role in attachment of these strains, and surface charge played a less important role. Hydrophobicity and cell surface charge are interrelated however and cannot

be entirely differentiated. For example, hydrophobicity of *Cryptosporidium* oocysts varies with the ionic strength and pH of the suspending medium (Drozd & Schwartzbrod, 1996) and bacterial electrokinetic potentials correlate with hydrophobicity (Van Loosdrecht *et al.*, 1987). Hydrophobic interactions are not entirely responsible for cell adhesion since bacterial attachment occurs in the presence of surfactants (Clark *et al.*, 1985; Paul & Jeffrey, 1985). The attachment mechanisms of bacteria also differ between hydrophilic or hydrophobic substrata even within a single bacterium (Paul & Jeffrey, 1985).

The composition of LPS expressed by *P. aeruginosa* can be altered in hours (Makin & Beveridge, 1996b) or days (Beveridge *et al.*, 1997) in response to changes in growth temperature or biofilm aging. It is tempting to suggest that the differences in cell wall composition (LPS chemistry) control development of the biofilm. However, other factors should not be dismissed. Motility generally appears to promote initial attachment and recolonization of surfaces in flowing systems (Korber *et al.*, 1989; DeFlaun *et al.*, 1994). The present strongly adherent strain R5 lacked flagella and was non-motile. The presence of flagella did not appear to influence either hydrophobicity or cell surface anionic character as seen in the similar rankings of R5 (no flagella) and A28 (possessing flagella). Further examination of the effects of cell motility on attachment is recommended for these strains since a negative correlation has been loosely demonstrated in these flow cell studies.

The *P. aeruginosa* O6 rough mutants displayed a wide phenotypic range of physicochemical surface characteristics in conjunction with defined alterations of LPS configuration. Adhesion to hydrophilic substrata was markedly affected by the bacterial cell surfaces. This series and other well described suites of rough mutants could therefore be used effectively to test antifouling strategies from paints, to additives, to ablative and mechanical methods to discourage fouling, or to test surfaces which may enhance biofilm formation in biotechnological applications.

Acknowledgements

This work was funded by grant N00014-93-1-1317 from the US Office of Naval Research to DCW. Excellent technical assistance was provided by K Harter and T Anderson. We are grateful to J S Lam for the bacterial strains and also thank JSL, T J Beveridge, S A Makin, and H J Busscher for thoughtful discussions throughout this work.

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