Adsorption of Bacterial Surface Polymers to Attachment Substrata

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Extracellular polysaccharide (EP) and lipopolysaccharide (LPS) were isolated from three strains of *Pseudomonas fluorescens* (a wild-type and two mutants) and the adsorption isotherms (relationship between amount of polymer adsorbed and bulk liquid concentration of polymer in solution) of these polymers to hydrophobic, tissue culture treated and sulphonated polystyrene surfaces were measured. The adsorption properties of the polymers were then related to the ability of the three bacterial strains to attach to the polystyrene surfaces in an attempt to elucidate the attachment mechanisms. A Langmuir adsorption isotherm equation was applied to the data, and the mathematical constants thus derived indicated if and at what concentration each surface became polymer-saturated and whether multilayer adsorption occurred. EP isolated from a crenated mutant (strain with the greatest attachment ability) adsorbed at higher concentrations than EP from wild-type and mucoid strains, and the isotherm indicated multilayer adsorption. EP from the mucoid strain (strain with little attachment ability) showed comparatively little adsorption. The isotherm of wild-type LPS was very similar to that of EP from the mucoid strain. Polymer adsorption to the three surface types was different and was generally consistent with the different degrees of bacterial attachment to the surfaces.

INTRODUCTION

The mechanism by which many bacteria attach to solid surfaces is not clear. They appear to adhere through the adsorption of cell-surface components onto the substratum (Fletcher & Marshall, 1982*a*). The attachment abilities of different bacterial species and strains vary considerably (Fletcher, 1980; Pringle & Fletcher, 1983), and this is probably, at least in part, due to their differences in composition and configuration of cell surface polymers. However, there is very little information relating the biochemistry of cell-surface polymers to adhesive ability.

There is evidence that polysaccharides may function in bacterial adhesion to surfaces. Ultrastructural histochemistry has demonstrated polysaccharides which bridge the gap between cell and surface (Fletcher & Floodgate, 1973; Costerton *et al.*, 1978), and the formation of multicellular attached layers, or biofilms, is generally accompanied by the accumulation of large amounts of polysaccharides (Geesey, 1982). Additionally, polysaccharides have been isolated from suspended cultures of bacteria previously isolated from surfaces (Sutherland, 1980). However, there are also data suggesting that surface components other than polysaccharides, e.g. proteins and lipids, may act as adhesives. Proteases disrupt adhesion of certain bacteria (Corpe, 1974; Danielsson *et al.*, 1977; Wood, 1980; Fletcher & Marshall, 1982*b*). Also, many bacteria attach preferentially to hydrophobic surfaces, as compared to hydrophilic ones (Loeb, 1977; Pringle & Fletcher, 1983), and this is inconsistent with a hydrated polysaccharide adhesive. Thus, the biochemical nature of the specific adhesive component(s) is far from clear.

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Abbreviations: EP, extracellular polysaccharide; LPS, lipopolysaccharide; PS, polystyrene; SP, sulphonated polystyrene; TC-PS, tissue culture treated polystyrene.

We have described the adhesion properties of three strains of *Pseudomonas fluorescens* to polystyrene substrata and related this to surface polymers isolated from the cells (Pringle *et al.*, 1983). The wild-type strain attached well to surfaces and produced no extracellular polysaccharide. A mucoid mutant strain had a much reduced attachment ability and produced an alginate exopolymer composed of mannuronic and guluronic acids. The third strain, a mutant which formed a crenated colony, had a slightly increased attachment ability and produced no extracellular polysaccharide. The polysaccharide component of the LPS from all strains contained glucose (25–30%), ribose (21–24%), dideoxyhexosamines (29–33%), rhamnose (11–15%) and glucosamine (4–6%), with the crenated strains having proportionally less polysaccharide in the LPS (13.8–15.4%) than did the wild-type (17.3%) or mucoid strains (19.4–20.2%). There were also some differences in extrinsic membrane protein compositions of the three strains (Pringle *et al.*, 1983).

To determine whether these modifications in exopolysaccharide production and LPS were responsible for the differences in attachment ability of the three strains, it is necessary to evaluate the adsorption properties of the polymers themselves. Thus, this paper describes a study of the adsorption isotherms of polymers isolated from the three strains. The adsorption data were then related to the attachment abilities of the bacteria to elucidate the way in which cell-surface composition might determine cell adhesiveness.

METHODS

Organism. The three strains of a freshwater isolate of *Pseudomonas fluorescens* were H2P2, the wild-type strain H2M2, a mucoid mutant, and H2S1, a mutant which produced a crenated colony; see Pringle *et al.* (1983) for further details of these strains. Bacteria were cultured in a medium containing glucose (1.0 g l⁻¹), NH₄Cl (2.0 g l⁻¹), KH₂PO₄ (5.55 g l⁻¹) and a mineral salts solution (6 ml l⁻¹) containing (g l⁻¹) MgSO₄·7H₂O (10), MnCl₂·4H₂O (1.0), FeSO₄·7H₂O (0.4) and CaCl₂·2H₂O (0.1), adjusted to pH 7.0 with 1 M-NaOH (Williams & Wimpenny, 1976). Batch cultures (100 ml) were grown at 15 °C in an orbital shaken incubator (150 r.p.m.). Growth was followed turbidimetrically and, at mid-to-late exponential phase, 2.5 µCi (92.5 kBq) [¹⁴C]glucose ml⁻¹ was added to label cell-surface polysaccharides. Cells were harvested in late exponential phase by centrifugation.

Isolation of extracellular polysaccharide (EP) and lipopolysaccharide (LPS). Cells were washed by suspension three times in 0.01 M-KH₂PO₄ (adjusted to pH 7.4 with 1 M-NaOH) followed by centrifugation. Radiolabelled extracellular polymer was isolated from supernatants of washed cells. Volumes (100 ml) of the supernatant were dialysed against distilled water at 4 °C using dialysis tubing that had been prewashed with 0.01 M-EDTA in 1% (w/v) Na₂CO₃ solution (Williams, 1974), followed by washing in distilled water until contaminating carbohydrate material was undetectable by the phenol/sulphuric acid method (Dubois *et al.*, 1956). After dialysis the total hexose content of the exopolysaccharide in the supernatant was measured (corrections were made for any volume changes during dialysis). Total protein was determined by the method of Bradford (1976). For supernatants where exopolymer was undetectable or at very low concentration by these assay methods, the remaining supernatant was concentrated in an Amicon ultrafiltration system (YM10 membrane, exclusion limit = 10 kDa; Diaflo ultrafiltration membranes). Assays for total hexoses and protein were repeated on concentrated samples. In samples where exopolysaccharide was present at total hexose concentrations above 500 µg l⁻¹, a gravimetric method was used to measure exopolymer (Williams & Wimpenny, 1977). This polymer was isolated by ethanol precipitation, dried under reduced pressure and weighed.

LPS was extracted from washed cells with aqueous phenol (Lüderitz *et al.*, 1971). Approximately 1 g (dry weight) of bacteria was suspended in 50 ml water and warmed to 65 °C. An equal volume of 90% (w/v) aqueous phenol at the same temperature was added and the mixture stirred vigorously for 5 min. The aqueous and phenol phases were separated by centrifugation (3000 g, 30 min, 0 °C). The aqueous layer was collected and dialysed for 48 h against running tap water to remove phenol. LPS was isolated by ultracentrifugation (100000 g, 4 h), purified by Sepharose CL-4B (Pharmacia) gel filtration (Romanowska, 1970) and dried under reduced pressure over P₂O₅.

The specific activity of each polymer was determined by relating liquid scintillation counts to total hexose concentration, as determined by the phenol/sulphuric acid method.

¹⁴C-Labelled polymer adsorption assays. Methods similar to those of Baszkin & Lyman (1980) were used. Polystyrene (PS; Sterilin), tissue culture treated polystyrene (TC-PS; Costar) and sulphonated polystyrene (SP) Petri dishes (3 cm) were used as substrata. The SP Petri dishes (which have more negative surface charge than PS) were prepared by treating PS Petri dishes with 10 ml concentrated sulphuric acid for 24 h, followed by repeated rinsing in sterile distilled water and air-drying. The Petri dishes were filled with 0.01 M-potassium phosphate buffer, pH 7.4 (5 ml), containing the required amount of ¹⁴C-labelled polymer to give a final range of polymer

Fig. 1. Adsorption isotherms of EP isolated from *P. fluorescens* wild-type H2P2 (a), mucoid mutant H2M2 (b) and crenated mutant H2S1 (c). Adsorption isotherm of LPS isolated from *P. fluorescens* wild-type H2P2 (d). Adsorption (polymer adsorbed in $\mu\text{g cm}^{-2}$) to the three substrata PS (○), TC-PS (●) and SP (□) was plotted against the bulk liquid concentration of each polymer in solution (mg ml^{-1}).

concentrations between 1.0 and 0.01 mg ml^{-1} . The adsorption experiment was continued for 3 h at 20 °C to ensure an equilibrium value of polymer adsorption (Baszkin & Lyman, 1980). Each Petri dish was then rinsed with 300 ml potassium phosphate buffer and allowed to dry at room temperature. The concentration of adsorbed polymer was then determined by liquid scintillation counting.

Experiments were repeated and samples duplicated for each test substratum, with good reproducibility.

Measurement of adsorption by scintillation counting. The Petri dishes were broken into small pieces suitable for insertion into a scintillation vial. Glass scintillation vials (20 ml) containing the broken dishes were then filled with 5 ml toluene and left overnight at room temperature, during which time the dishes dissolved. The vials were then counted after the addition of an emulsifier containing a liquid scintillation cocktail of toluene/Triton X-100 (1:2, v/v) with the scintillator butyl PBD (12 g l^{-1}) to a total volume of 10 ml. Quenching due to the added polystyrene was minimal and was determined by H number using a Beckman Instruments liquid scintillation system LS-7000.

RESULTS

The adsorption isotherms of EP isolated from the wild-type (strain H2P2), crenated mutant (strain H2S1) and mucoid mutant (H2M2) are shown in Fig. 1. These graphs show the isotherms for three substrata, PS, TC-PS and SP, and are plots of the amount of polymer adsorbed in $\mu\text{g cm}^{-2}$ against the bulk liquid concentration of each polymer in solution (mg ml^{-1}). The term EP as used here refers to the radiolabelled alcohol-precipitated material that was released into the culture medium during growth. Unlike the mucoid mutant, the wild-type and crenated cultures did not produce a distinguishable extracellular polysaccharide; EP from these strains contained a complex mixture of extracellular products. The EP produced by the mucoid mutant was further purified by gel filtration chromatography and was adsorbed in its pure form.

The isotherms varied both in the concentration range over which the adsorbed polymer was measured and in the preference of each polymer for the three substrata. For wild-type EP (Fig. 1*a*), both PS and TC-PS isotherms were in the range $0.1\text{--}1.0\ \mu\text{g cm}^{-2}$, with greater adsorption on the PS which levelled off at $0.9\ \mu\text{g polymer adsorbed cm}^{-2}$ at a bulk concentration of $0.1\ \text{mg ml}^{-1}$ or above. The TC-PS isotherm also had a plateau at $0.6\ \mu\text{g cm}^{-2}$ at a bulk concentration of $0.1\ \text{mg ml}^{-1}$ or above. The SP isotherm, on the other hand, was in the range $0.01\text{--}0.3\ \mu\text{g cm}^{-2}$ and did not reach a plateau.

The isotherms from the EP isolated from the mucoid mutant (Fig. 1*b*) ranged from 0.01 to $0.25\ \mu\text{g cm}^{-2}$ for all three substrata tested. There was greater adsorption on the TC-PS surface compared to the PS and SP substrata, and all three substrata failed to form a plateau within the bulk concentration range examined.

Isotherms of the EP isolated from the crenated mutant (Fig. 1*c*) spanned a higher range of adsorbed polymer concentration: $0.02\text{--}2.5\ \mu\text{g cm}^{-2}$. The TC-PS and PS isotherms were identical within experimental error, while the SP isotherm showed less adsorption. These isotherms also failed to form a plateau within the bulk concentration range examined.

Isotherms for the wild-type LPS are shown in Fig. 1(*d*); these curves were similar to those for the mucoid EP (Fig. 1*b*), having the same concentration range for adsorbed polymer and the same preference for the TC-PS substratum.

DISCUSSION

The surface adsorption of surface polymers isolated from the three *P. fluorescens* strains differed and may be related to the different degrees of attachment of these strains to corresponding substrata. With all strains, attachment numbers were higher with PS than with TC-PS (Pringle *et al.*, 1983), although those of the mucoid mutant were much lower with both surfaces. Attachment of only the wild-type strain to SP has been measured, and was at lower numbers than those obtained with PS and TC-PS (Pringle, 1984). Surface energy values of the substrata, calculated from water contact angle data, were 28 , 53 and $73\ \text{mN m}^{-1}$ for PS, TC-PS and SP, respectively (Pringle, 1984).

The results of adsorption isotherms of EP from wild-type and adhesion mutants of *P. fluorescens* can be redrawn as a linear form of the Langmuir equation, $r/a = KN - Kr$, which yields linear plots of r/a against r , where r is the amount of adsorbed polymer in $\mu\text{g per cm}^2$ of polystyrene, a is the equilibrium solution concentration in $\mu\text{g ml}^{-1}$, N is the maximum possible adsorbable amount of polymer and K is a constant. From the intercept at $r/a = 0$, N is derived, and $-K$ is the slope of relationship. Fig. 2 shows this relationship for EP and LPS isotherms. The plots are similar to adsorption isotherms of human globulin to polystyrene latex particles (Brash & Lyman, 1969), in that a slope, representing the formation of an adsorbed monolayer, was found for each surface and in some cases (Fig 2*b, c*) a second line of lower slope was produced, representing either further adsorption to the original monolayer or the production of a second layer of adsorbed molecules by rearrangement of initially adsorbed molecules.

Table 1 shows values of N for the various polymer and substratum combinations. For EP of wild-type and crenated mutants, N was higher with PS and TC-PS than with SP substrata, indicating that these surfaces accommodate more EP per unit area of surface than do surfaces with increased charge (SP). Also, polymer isolates which tended to contain appreciable low molecular mass components (EP from wild-type and crenated mutant) tended to have higher N values than isolates that contained a greater proportion of large polysaccharide molecules (mucoid EP, LPS), which apparently quickly saturated available sites.

We previously suggested (Pringle *et al.*, 1983) that the mucoid polymer, a negatively charged polymer, composed of mannuronic and guluronic acids, was responsible for reduced attachment of the mucoid mutant. The very low adsorption of the mucoid polymer on PS or SP substrata (Fig. 2*b*) suggested that few adsorption sites were available on these substrata or that the adsorption bonding was weaker than on TC-PS and EP was removed during the washing stage. The level of surface charge on the SP substrata may cause sufficient ionic repulsion to prevent significant levels of adsorption to that surface. By contrast, the hydrophobic PS substrata may

Fig. 2. Adsorption isotherms of EP isolated from wild-type *P. fluorescens* H2P2 (a), mucoid mutant H2M2 (b) and crenated mutant H2S1 (c) and of LPS isolated from *P. fluorescens* H2P2 (d) redrawn as a linear form of the Langmuir equation of r/a against r . r is the amount of adsorbed polymer in $\mu\text{g cm}^{-2}$ and a is the equilibrium solution concentration in $\mu\text{g ml}^{-1}$. The isotherms of the three substrata, PS (○), TC-PS (●) and SP (□), are plotted.

Table 1. *Mathematical functions of EP and LPS adsorption derived from the Langmuir adsorption isotherm*

The results are expressed as $\mu\text{g cm}^{-2}$.

Polymer	Substratum . . . PS		TC-PS		SP	
	N_1	N_2	N_1	N_2	N_1	N_2
H2 wild-type EP	1.28	—	0.72	—	< 0.5	—
H2 mucoid EP	0.005	0.02	0.07	0.285	0.005	0.02
H2 crenated EP	0.75	2.81	0.75	2.81	0.001	—
H2 wild-type LPS	0.19	—	0.121	—	0.08	—

form insufficient bonding with the adsorbing EP to prevent the water/air interface from 'capture' of the EP during washing. This lack of firm adsorption at the surface was seen, as the adsorbed liquid film peeled off the surface and beaded up into droplets, due to surface tension forces. This water/air interface interaction was not observed for the TC-PS or SP surfaces, which were able to remain wetted and covered with liquid during washing. Thus, lack of adhesion of the mucoid mutant to these surfaces (Pringle *et al.*, 1983) may be due to a separate mechanism for each substratum. Adhesion to the TC-PS may be inhibited by the adsorption of mucoid EP, preventing cell-surface contacts by steric stabilization (Rutter & Vincent, 1984), whereas adhesion to the PS and SP substrata may be comparatively less or may take place, only to be lost subsequently due to the weak bonding between the PS surface and water.

The adsorption of EP from the wild-type (Fig. 2*a*) and crenated (Fig. 2*c*) strains produced isotherms with N values typical of protein adsorption to similar weakly charged surfaces, i.e. a range of 0.5 to 1.5 $\mu\text{g cm}^{-2}$ (Brash & Lyman, 1969). Adsorption to the PS substrata of wild-type EP showed a greater binding capacity than that obtained with TC-PS, which is consistent with the greater attachment ability of the strain to PS than to TC-PD (Pringle *et al.*, 1983). However, the EP from the crenated mutant adsorbed to both TC-PS and PS substrata equally and was able to continue to adsorb after initial monolayer formation to a second adsorption maximum. These results indicate that wild-type EP adsorption to both TC-PS and PS substrata inhibited further adsorption when a monolayer of polymer had been adsorbed. However, EP from the crenated mutant did not inhibit further adsorption, which might be related to the comparatively greater attachment ability of the crenated strain and its greater ability to colonize surfaces (Pringle *et al.*, 1983).

Finally, LPS adsorption (Fig. 2*d*) showed similar results to the adsorption of mucoid EP (Fig. 2*b*). The polysaccharide component of the LPS may therefore have dominated the adsorption characteristics, allowing adsorption to wetted surfaces where charge density would be low enough to prevent ionic repulsion (i.e. TC-PD). Both mucoid EP and LPS will have hydrated polysaccharides which, because of their adsorbed water layer, would be expected to interact less strongly with more hydrophobic surfaces, e.g. PS. Therefore, adsorption of LPS from the wild-type EP to the TC-PS (Fig. 2*a*) may have inhibited further protein adsorption, hence the reduction in adsorption concentrations (N_1) for TC-PS, as compared with PS. LPS from *Escherichia coli* serogroup O111 at a concentration of 2.5 mg ml⁻¹ considerably inhibited attachment of *P. fluorescens* H2 to PS and TC-PS (data not shown).

The relative proportions of major extrinsic proteins differed somewhat in the crenated and wild-type strains (Pringle *et al.*, 1983). The adsorption of protein from the EP of the crenated strain may have dominated this isotherm (Fig. 2*c*), in which case it would have been affected to a lesser degree by LPS components, as compared with the wild-type EP. Therefore all three substrata may have been covered by a monolayer of LPS with no consequent reduction in attachment. The high N values of this strain may explain, at least in part, its increased attachment ability (Pringle *et al.*, 1983).

These results show that the interaction of polysaccharides and proteins at the substratum surface probably influences the subsequent bacterial adhesive interaction. Adhesion may be mediated by LPS or other cell-surface components, such as those used in this study. Conversely, sufficient production of polysaccharide, as occurred with the mucoid mutant, could produce an adsorbed molecular layer of polysaccharide which may then inhibit subsequent polymer adsorption and bacterial adhesion.

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