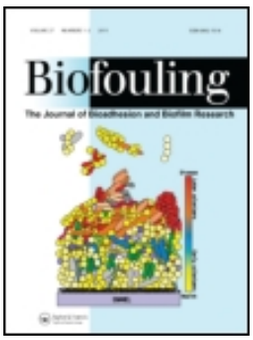

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INFLUENCE OF PROTEIN CONDITIONING FILMS ON BINDING OF A BACTERIAL POLYSACCHARIDE ADHESIN FROM *HYPHOMONAS* MHS-3

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A putative polysaccharide adhesin which mediates non-specific attachment of *Hyphomonas* MHS-3 (MHS-3) to hydrophilic substrata has been isolated and partially characterized. A polysaccharide-enriched portion of the extracellular polymeric substance (EPS_p) from MHS-3 was separated into four fractions using high performance size exclusion chromatography (HPSEC). Comparison of chromatograms of EPS_p from MHS-3 and a reduced adhesion strain (MHS-3 rad) suggested that one EPS_p fraction, which consisted of carbohydrate, served as an adhesin. Adsorption of this fraction to germanium (Ge) was investigated using attenuated total reflection Fourier transform infrared (ATR/FT-IR) spectrometry. Binding curves indicated that the isolated fraction had a relatively high affinity for Ge when ranked against an adhesive protein from *Mytilis edulis*, mussel adhesive protein (MAP) and an acidic polysaccharide (alginate from *Macrocystis pyrifera*). Spectral features were used to identify the fraction as a polysaccharide previously reported to adsorb preferentially out of the EPS_p mixture. Conditioning the Ge substratum with either bovine serum albumin (BSA) or MAP decreased the adsorption of the adhesive polysaccharide significantly. Conditioning Ge with these proteins also decreased adhesion of whole cells.

KEYWORDS: extracellular polymeric substances, adhesion, marine bacterium, conditioning films

INTRODUCTION

Bacterial colonization of surfaces can be engineered for useful purposes, *e.g.* in wastewater treatment (Bryers & Characklis, 1990), or can lead to deleterious consequences, *e.g.* pathogenesis (Evans *et al.*, 1993), persistent implant-associated infections (Gristina, 1987), or fouling of industrial machinery (Väisänen *et al.*, 1994). Specific protein/ligand interactions have been found to mediate adhesion in some specialized ecosystems. Examples include adhesion of pathogens to host tissues (Jann & Jann, 1990), of oral bacteria to components of the pellicle (Clark *et al.*, 1989), and of cellulolytic bacteria to cellulose (Salamitou *et al.*, 1994). It seems reasonable to suppose that, in general, adhesion of bacteria to inert surfaces is a more generic phenomenon, *i.e.* not likely to involve interaction between ligands and customized binding pockets. However, there is evidence that specialized biomolecules have evolved to serve as primary (non-specific) adhesins to inert surfaces (Rosenberg *et al.*, 1982; Bar-ness *et al.*, 1988; Bashan & Levanony, 1988; Zottola, 1991; Yun *et al.*, 1994) and that the molecular architecture of extracellular components may be fashioned for adhesion to (Shea &

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Smith-Somerville, 1994), or detachment from, inert surfaces (Pringle *et al.*, 1983; Wrangstadh *et al.*, 1986).

Various approaches have been taken in order to investigate the biochemistry of adhesion to inert (or inanimate) surfaces including inhibition assays (Merker & Smit, 1988; Quintero & Weiner, 1995), footprint analysis (Nue, 1992), enzyme treatment (Paul & Jeffrey, 1985) and adsorption assays (Pringle & Fletcher, 1986; Bidle *et al.*, 1993). Adsorption of organic compounds probably begins immediately after a material is placed in any natural water (Baier *et al.*, 1983). Interactions between this initial "conditioning film" and compounds proximal to the interface continue as the fouling process progresses (Baty *et al.*, 1996b). Many of these compounds will be biopolymers introduced by members of the fouling community. The more adhesive compounds may become part of the adsorbed film which eventually mediates the adhesive bond anchoring the fouling community to the substratum. Adsorption studies can help elucidate which factors determine the composition and binding properties of this interfacial film.

Surface associated microorganisms (biofilms) are typically embedded in a matrix of extracellular polymeric substance(s) (EPS) (Costerton *et al.*, 1987; Cooksey, 1992). The EPS matrix is often composed primarily, but not exclusively, of polysaccharides. It typically contains a mixture of biomolecules including a significant protein fraction (Humphrey *et al.*, 1979; Abu *et al.*, 1991; Vincent *et al.*, 1994). Therefore, the EPS components from one organism can present a complex set of interaction possibilities at an interface. It has been found that the EPS from *Hyphomonas* MHS-3 (MHS-3) contains at least one polysaccharide component which binds strongly to a clean Ge surface, and that this binding appeared to be blocked by conditioning the surface with intrinsic EPS proteins (Suci *et al.*, 1995). In order to further characterize these interactions, the most adhesive polysaccharide EPS component has been isolated using high performance size exclusion chromatography (HPSEC) and its adsorption behavior has been studied on Ge, and Ge conditioned with proteins expected to confer widely different surface properties to the substratum *viz.* a globular blood protein (bovine serum albumin) (BSA) and proteins from the adhesive plaque of *Mytilis edulis* (mussel adhesive protein) (MAP) (Waite, 1990). BSA inhibits cell adhesion in many cases and is used to block non-specific binding sites (Goding, 1983). In contrast, MAP promotes adhesion of a variety of cell types (Benedict & Picciano, 1987; Notter, 1988) and has been shown to enhance alginate adsorption in seawater (Suci & Geesey, 1995). The adhesion behavior of MHS-3 whole cells has also been characterized on these surfaces.

MATERIALS AND METHODS

Bacterial Strains and Cell Culturing

MHS-3 was isolated from shallow water sediments in Puget Sound, WA. A reduced adhesion strain (MHS-3 rad) was isolated on the basis of distinct colony morphology when cultured on marine agar plates (Quintero & Weiner, 1995). Cultures of MHS-3 and MHS-3 rad were grown in Marine Broth 2216 (37.4 g l^{-1}) (Difco Laboratories, Detroit, MI) at 25°C on a rotating shaker at 100 revs min^{-1}. Teflon™ mesh was introduced into the culture vessels to provide greater surface area for attached growth (mesh opening, 1.8 mm, thread diameter, 0.5 mm, Tetko, Incorporated, Briarcliff, NY). The attached cells and associated EPS were harvested from a culture in which the suspended cell population had just entered stationary phase. The medium was poured off and the biofilm was removed from the teflon mesh and the sides of the culture vessel walls by scraping.

EPS Extraction

The EPS was extracted in two steps. The cell suspension was centrifuged for 20 min at 16,000xg, and the "loosely bound" EPS in the supernatant was precipitated with 4 volumes of ice cold 2-propanol. The more "tightly bound" EPS was extracted from the cell pellet. The cell pellet was blended in a Waring blender in a minimum volume of 10 mM EDTA, 3% NaCl for 1 min at 4°C. The suspension was centrifuged for 15 min at 16000xg and the EPS from the supernatant was precipitated using 2-propanol as described above.

EPS Purification

A crude EPS preparation was obtained by pooling the two EPS fractions and resuspending in a minimum volume of distilled water (dH_2O). They were then dialyzed for 12 h against dH_2O and lyophilized. Polysaccharide-enriched EPS (EPS_p) was prepared by a published protocol (Read & Costerton, 1987). EPS was dissolved in a minimum volume of 0.1 M $MgCl_2$, and DNase and RNase were added to a final concentration of 0.1mg ml^{-1}, followed by incubation at 37°C for 4 h. Protease K was added to 0.1 mg ml^{-1} and incubated at 37°C overnight. Residual protein was removed with a hot phenol extraction, followed by a chloroform extraction. The preparation was dialyzed for 12 h against dH_2O and lyophilized. The EPS_p preparations were stored desiccated at room temperature.

HPSEC fractionation of EPS_p was performed using a Hewlet Packard 1090 liquid chromatograph equipped with an on-line diode array UV-VIS detector (Shodex OHpack B-2004 column). The mobile phase was 0.5 M NaCl, 0.05 M Na_2HPO_4 at pH 7.0 in dH_2O. EPS_p was dissolved in mobile phase at a concentration of 1 mg ml^{-1} and filtered through a 0.22 μm millipore filter. The injected sample volume was 1 ml. The column was run at room temperature at a flow rate of 0.9 ml min^{-1}. Separated EPS_p fractions were collected post column and concentrated in 0.01 M NaCl using ultrafiltration (Amicon 8010 stirred ultrafiltration cell, Amicon 5YM5 membrane). The concentrated fractions were stored at –40°C.

Chemical Analysis of EPS

Neutral hexoses were determined using the phenol sulfuric acid assay with glucose as the standard (Dubois *et al.*, 1956). Proteins were estimated using the Lowry procedure with BSA as standard (Lowry *et al.*, 1951). Total organic carbon (TOC) was analyzed using a Dohrmann DC 80 total organic carbon analyzer.

MHS-3 lipopolysaccharide (LPS) was identified using a monoclonal antibody (Busch, 1993) and Western blots. Polyacrylamide gels were run on a "Mighty Small II SE 250" mini-gel apparatus from Hoeffer Scientific with a 8% Tris/glycine separating gel at pH 8.8 and 4% Tris/glycine stacking gel at pH 6.8 (acrylamide/bisacrylamide ratio was 37.5:1). Running buffer was 3.0 g l^{-1} Tris Base, 14.4 g l^{-1} glycine and 1 g l^{-1} SDS in dH_2O. Sample buffer was 1.52 g Tris Base, 20 ml glycerol, 2.0 g SDS, 1.0 mg bromphenol blue and 80 ml dH_2O (pH 6.8). Aliquots (10 μl) of sample were mixed with an equal volume of sample buffer. The mixture was loaded on individual wells and electrophoresis carried out at 30 mA/gel for 1.5 h. Electroblotting was for 2 h onto a nitrocellulose filter (2117 multiphor II electrophoresis unit from Pharmacia) in buffer consisting of 2.93 g glycine, 5.81 g Tris Base, 0.375 g SDS and 200 ml methanol dissolved in 800 ml dH_2O. After washing in 20 ml of 5% skim milk for 0.5 h, the nitrocellulose was incubated at 25°C overnight with anti-LPS monoclonal antibody

diluted 1:1000 in 20 ml skim milk. The nitrocellulose was washed three times in a PBS-Tween solution (8.0 g l^{-1} NaCl, 2 g l^{-1} KCl, 1.15 gl^{-1} Na_2HPO_4, 0.2 g l^{-1} KH_2PO_4 and 0.5 ml l^{-1} Tween-20 at pH 7.4), then incubated at 25°C for 2 h with a 1:1000 diluted horseradish peroxidase labelled goat anti-mouse IgG antiserum. The nitrocellulose was again washed three times in PBS-Tween and placed in a developing solution containing 1.0 ml Tris buffer at pH 8.0, 20 mg 4-choro-1-napthol and 5 µl 50% H_2O_2.

Surface Preparation

Single crystal, cylindrical germanium (Ge) internal reflection elements (IRE) (Spectra Tech, Stamford, CT) were cleaned by ultrasonication in a base bath (saturated KOH in isopropyl alcohol) for 10 min. Following the base bath were two rinses in ultrapure water followed by a gentle scrubbing with undiluted Micro™ cleaning solution using cotton swabs. The cleaning solution was flushed off in a hard stream of ultrapure water. The IRE was then subjected to the following rinses, which consisted of a 10 min ultrasonication in each liquid: ultrapure water (2×), ethyl alcohol, chloroform and dichloromethane. The advancing contact angle of Ge surfaces cleaned by this protocol is between 10° and 20°, and Auger electron spectroscopy (Phi 595 scanning Auger microprobe) indicated that the elemental composition of the first few monolayers was 9.11 ± 1.38 carbon, 6.51 ± 2.01 oxygen and 83.92 ± 2.24 Ge.

Adsorption Protocol

All adsorption experiments were conducted using synthetic seawater as the aqueous solvent (w/v), *viz.* 2.3% NaCl, 0.024% Na_2CO_3, 0.033% KCl, 0.4% MgCl. $6H_2O$, 0.066% $CaCl_2$. $2H_2O$, pH adjusted to 8.0 with HCl. For seawater lacking divalent cations the NaCl was increased to 3.29% to elevate its ionic strength to that of the seawater containing divalent cations. This preparation was used for adsorption experiments with alginate, which is not soluble in the presence of divalent cations.

For adsorption experiments the cylindrical IRE was positioned within a stainless steel flow chamber (Circle Cell™, Spectra Tech). Details have been described elsewhere (Suci & Geesey, 1995). A simple flow through system was used to deliver solutions into the flow chamber. Teflon valves (Cole-Parmer, Niles, IL) served to shuttle the appropriate solution into tubing leading to the flow chamber. All tubing leading into the flow chamber as well as the fittings were teflon (0.08cm I D).

Before each adsorption experiment the surface was exposed to flowing synthetic seawater for 20 min. A vial containing approximately 1 ml of the appropriate substance was inserted into the flow system and the contents immediately pumped through a short section of leader tubing and through the flow chamber for 105 s. Flow was then stopped to allow adsorption. Adsorption was performed under these static conditions to conserve purified polysaccharide. Flow was then resumed and the surface was rinsed with synthetic seawater. Binding curves were obtained as step isotherms (Hlady *et al.*, 1985). At each bulk concentration the adsorption reaction was preformed for 60 min under quiescent conditions followed by a 30 min rinse with synthetic seawater. For conditioning with proteins, adsorption was for 60 min under quiescent conditions at 0.1 mg ml^{-1} for MAP and 0.5mg ml^{-1} for BSA followed by a 60 min rinse as above.

FT-IR Spectrometry

During the course of each experiment infrared (IR) spectra were acquired periodically using a Perkin Elmer Model 1800 Fourier transform infrared (FT-IR) spectrophotometer.

Experimental details are described elsewhere (Suci & Geesey, 1995). FT-IR measurements were made in a temperature controlled room (25 ± 1°C). For experiments in which the substratum was conditioned with BSA or MAP, the spectrum acquired immediately before the initiation of second adsorption reaction was used as the background.

Langmuir Fit

The binding curves were fit to a Langmuir model. The equation describing Langmuir adsorption is:

$$A = A_s\,[((1/Kc_b)+1)^{-1}] \qquad \text{Eqn 1}$$

where K is the binding (association) constant, C_b is the concentration of the substance in bulk solution, A is the absorbance (or band area in abs cm^{-1}) and A_s is the (estimated) saturation value of the absorbance (projected plateau for large bulk concentrations). Best fits for the parameters K and A_s were obtained by nonlinear regression using the software provided with the SigmaPlot™ application (Jandell Scientific, Corte Madera, CA). A_s is then converted to surface coverage (Γ) as described below.

Theoretical justification for application of the Langmuir model relies on demonstration of reversible adsorption. In the present case the adsorbed components analyzed are essentially irreversibly bound. There is no rigorous theoretical model which applies to the case of irreversible adsorption (Andrade, 1985). The use of the Langmuir model in the present context is intended to be empirical; it serves to quantify the data in terms of affinity of the adsorbate for the surface for comparison purposes.

Estimation of Surface Coverage

Surface coverage of the protein was estimated using a published correlation based on the area of the amide II band (Pitt & Cooper, 1988). Surface coverage of polysaccharide was estimated by comparing absorbances obtained in transmission mode with those obtained in the ATR mode using a previously published expression (Suci *et al.*, 1995). For the MHS-3 adhesive polysaccharide this estimate was based on transmission spectra of EPS_p, since concentrated quantities of the purified polysaccharide component sufficient to obtain transmission spectra were not available. Using the mass of the EPS_p for this estimate gives values of Γ which are maximum. By assuming that the K^{-1} value of the purified polysaccharide is the same as that of the purified EPS_p component, the proportion of EPS_p which is contributed by the purified polysaccharide can be estimated, and thereby a minimum estimate for Γ can be calculated.

Cell Adhesion Assay

Aliquots (6 ml) of an early stationary phase culture were centrifuged for 10 min at 1000 ×g in order to remove cell aggregates. The supernatant was centrifuged for 10 min at 12000 ×g and the cell pellet was resuspended in synthetic seawater. Ge fragments were cleaned using the protocol described above. Fragments conditioned with BSA or MAP were prepared by adsorption under stagnant conditions for 60 min (0.1 mg ml^{-1} protein) followed by a rinse in synthetic seawater. Ge fragments were exposed to the cell suspension for 30 min and then gently rinsed using liquid displacement. For each

experiment 3 fragments (Ge, Ge conditioned with BSA and Ge conditioned with MAP) were placed in the same cell suspension. Cells remaining on the surface were fixed for 30 min in 5% glutaraldehyde and stained with 0.1 mg ml^{-1} DAPI (4, 6 -diaminodino-2-phenylindole). Attached cells were counted using epifluoresence microscopy using an Olympus BX60 microscope.

RESULTS

The chromatogram of EPS_p reveals four baseline separated peaks. The associated fractions will be referred to as fractions 1 through 4 in order of elution (Fig. 1, Table 1). Chromatographic variations between batches were primarily in fractions 3 and 4. A peak position was not assigned for fraction 3, since it split clearly into at least two components in some chromatograms.

Chemical analysis of the four eluted fractions is presented in Table 1. Fractions 1 and 2 contain primarily polysaccharides, with negligible protein. Monoclonal antibody labelling of Western blots indicated that fraction 1 contains MHS-3 lipopolysaccharide (LPS). Fraction 3 contains a mixture of polysaccharides and proteins. Fraction 4 contains negligible amounts of both polysaccharides and proteins. The TOC content for this fraction was barely above background detection, despite the large relative area of the associated chromatographic peak, indicating that it is composed primarily of salts.

A comparison of chromatograms of EPS_p from MHS-3 and MHS-3 rad (Fig. 1) suggested that fraction 2 contained the capsular polysaccharide which was previously reported to serve as an adhesin (Quintero & Weiner, 1995). The chromatograms closely resemble each other in terms of both number of baseline separated peaks and peak positions. However, the ratio of fraction 2 to fraction 1 was considerably reduced for MHS-3 rad (0.25) compared to the wild type (1.85+/–1.23).

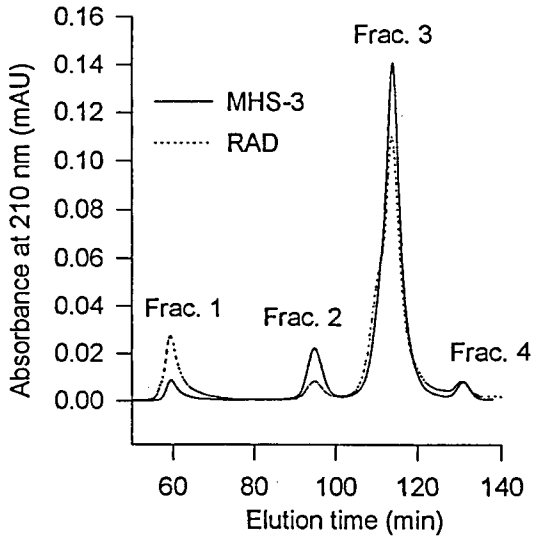

Fig. 1 HPSEC chromatograms of EPS_p from *Hyphomonas* MHS-3 (—) and *Hyphomonas* MHS-3 rad (....).

Table 1 Chemical composition of *Hyphomonas* MHS-3 EPS_p HPSEC fractions

	Peak Position[a] (min)	TOC (mg l^{-1})	Neutral Hexoses (mg l^{-1})	Protein (mg l^{-1})	Mab[b]
Fraction 1	59.4 ± 0.4[c]	5.3 ± 0.2	10.6 ± 0.1	0.0 ± 0.0	+
Fraction 2	94.8 ± 0.2	10.2 ± 0.1	27.6 ± 1.7	0.7 ± 0.0	–
Fraction 3	na[d]	19.5 ± 0.5	6.3 ± 1.7	19.2 ± 1.0	–
Fraction 4	130.3 ± 1.1	3.3 ± 0.3	0.9 ± 0.5	1.2 ± 0.8	nt[e]

[a] = from 4 different EPS_p batches; [b] = monoclonal antibody reactivity; [c] = standard deviation; [d] = not assigned; [e] = not tested.

It had been previously reported that EPS_p contained at least one adhesive polysaccharide which adsorbed preferentially out of the mixture (Suci *et al.*, 1995). Figure 2 shows ATR/FT-IR spectra of the carbohydrate C-O stretch region from this polysaccharide component of EPS_p and from fraction 2 polysaccharide (fr2PS), each adsorbed to Ge. The spectra exhibit very similar features with primary or secondary maxima at 972, 1033, 1064, 1080 and 1132 cm^{-1}. This strongly suggests that the component which adsorbs preferentially out of EPS_p has been isolated in fraction 2. Since it adsorbs preferentially out of the mixture this implicates it as the most adhesive component in the EPS_p matrix with respect to a Ge substratum.

Figure 3 shows the kinetics of adsorption of fr2PS onto the Ge substratum. The process was followed by computing areas of the spectral feature shown in Figure 2. It can be seen that the adsorption was essentially complete after 60 min. The rinse was begun at 100 min. There is a small amount of desorption during the rinse period, especially during

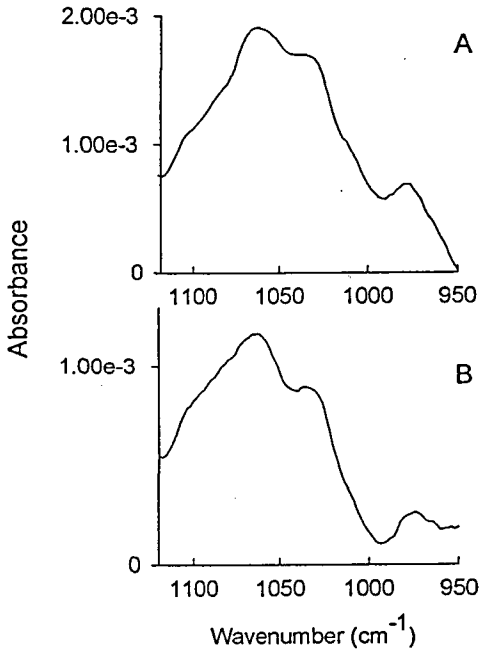

Fig. 2 ATR/FT-IR spectra of the carbohydrate C-O stretch region of EPS_p (A), and fr2PS (B), adsorbed to Ge.

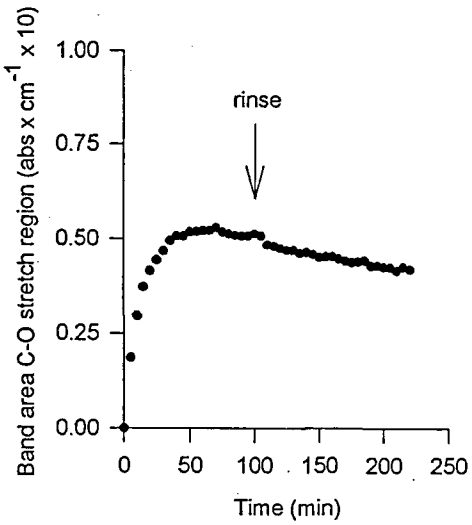

Fig. 3 Kinetics of adsorption of fr2PS onto Ge. Rinse was initiated at 100 min.

the first 10 min, but adsorption is essentially irreversible. Langmuir fits to binding curves were used to measure the "stickiness" of fr2PS with respect Ge. The Langmuir fit yields two parameters, *viz.* surface coverage (Γ) and bulk concentration to reach half saturation (K^{-1}), where a high Γ and low K^{-1} indicate a relatively sticky substance. In order to place this quantitation in context, Γ and K^{-1} have been obtained for an adhesive protein mixture from *M. edulis* (MAP) and an acidic polysaccharide (kelp alginate) using an identical protocol as for fr2PS (Table 2). According to this ranking the fr2PS has an adhesive quality (or "stickiness") comparable to MAP. The acidic polysaccharide, alginate, is relatively non-adhesive with respect to Ge.

The effect of two different protein conditioning films on the adsorption behavior of fr2PS was examined. Binding curves were obtained using a protocol identical to that used to obtain binding curves on clean Ge. Binding curves obtained by ATR/FT-IR for conditioned and unconditioned substrata are presented in Figure 4. It is evident from the binding curves that the affinity of fr2PS for both conditioned substrata is severely depressed. The Langmuir model did not converge when attempting to fit data for Ge conditioned with BSA or MAP, precluding a quantitative comparison of the Γ and K^{-1} values. There was no indication of any loss of the protein conditioning film during the

Table 2 Binding parameters for Langmuir fit to binding curves of fraction 2 polysaccharide, MAP, and alginate to Ge. A lower value of K^{-1} and a higher value of Γ implies greater stickiness.

	Fraction 2	MAP	Alginate
Γ (μg cm^{-2})	0.158 ± 0.009^a 0.029 ± 0.017^b	0.305 ± 0.016	0.039 ± 0.003
K^{-1} (mg l^{-1})	0.018 ± 0.002	0.024 ± 0.003	0.738 ± 0.179

[a] = estimated minimum value; [b] = estimated maximum value.

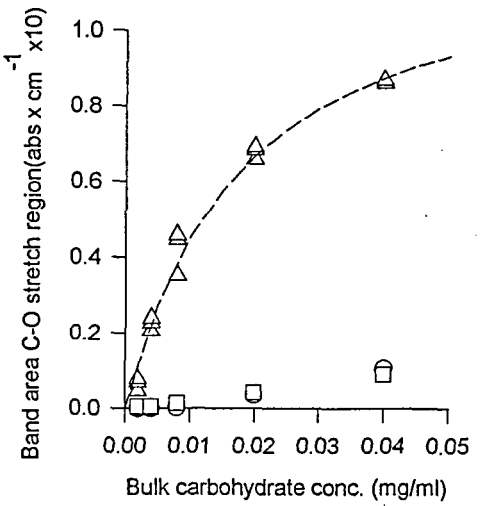

Fig. 4 Binding curves of fr2PS adsorbed on Ge, (△), Ge conditioned with MAP (□) and Ge conditioned with BSA (○). Broken line = Langmuir model fit to three data sets for unconditioned Ge.

acquisition of the step isotherm. Any such loss would be evident from a change in prominent amide bands located at approximately 1650 and 1550cm^{-1} (Suci & Geesey, 1995).

The ATR/FT-IR spectra of the absorbed fr2PS on Ge and Ge conditioned with protein are shown in Figure 5. The absorbances are low and very close to background noise. However, differences in the adsorbed material from fr2PS between the two conditioned substrata are revealed. The spectrum of the absorbed EPS_p on BSA resembles the spectrum obtained on Ge, suggesting that the same components are adsorbed. The spectrum of adsorbed components on MAP bares less resemblance to the spectrum on clean Ge, suggesting adsorption of a trace contaminant.

The large differences in the affinity of fr2PS for clean Ge and Ge conditioned with proteins offered an opportunity to see whether the adsorption behavior of fr2PS could be extrapolated to whole cell adhesion behavior (Fig. 6). The results showed that MHS-3 attaches to a much greater extent to clean Ge than to Ge conditioned with MAP or BSA. Thus, the cells show the same preference for unconditioned Ge that fr2PS displays in its adsorption behavior. The number of cells attached to Ge after the 30 min exposure period and the rinse was significantly larger than cells attached to Ge conditioned with BSA (2 sample t-test, $P < 0.95$). The number of cells attached to Ge conditioned with BSA was significantly larger than cells attached to Ge conditioned with MAP (2 sample t-test, $p < 0.95$).

DISCUSSION

It has previously been demonstrated that MHS-3 rad lacks diffuse capsular material which is present on MHS-3 (Quintero & Weiner, 1995). The capsular material binds gold labelled *Bauhinia purpurea* lectin (BPA) and calcofluor, both of which reduce MHS-3 attachment to the level observed in MHS-3 rad. Thus, the capsular material has been suggested to be both a polysaccharide and an adhesin. MHS-3 EPS, isolated from

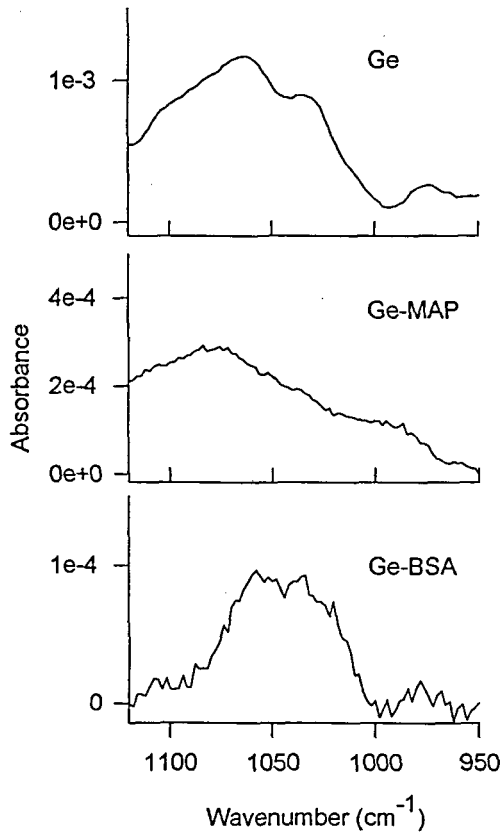

Fig. 5 Spectra of fr2PS adsorbed to Ge, and Ge conditioned with MAP (Ge-MAP), and BSA (Ge-BSA).

biofilms and flocs, has been reported to contain an adhesive polysaccharide which adsorbs preferentially out of a mixture from which the protease K accessible proteins have been removed (EPS_p) (Suci *et al.*, 1995). Using HPSEC, an adhesive polysaccharide fraction has been isolated and identified as the same substance which adsorbed preferentially out of the EPS_p mixture. This isolated adhesin is referred to as fraction 2 polysaccharide (fr2PS).

One significant implication is that a subclass of EPS molecules mediate MHS-3 adhesion to hydrophilic substrata. fr2PS is apparently dispersed throughout the EPS matrix of the biofilm since HPSEC of the more loosely associated and EDTA extracted EPS yielded similar chromatograms (data not shown). This was also indicated by labelling MHS-3 microcolonies with BPA lectin and calcofluor (Quintero & Weiner, 1995). On individual planktonic cells fr2PS is located integrally, extending from the cell envelope to a distance of approximately 0.2 μm (Quintero & Weiner, 1995). It may be that this polysaccharide functions both to mediate initial adhesion and as an EPS matrix polymer to anchor the biofilm to the substratum. Previously, it was found that calcofluor inhibited long term (5 d) biofilm formation (Quintero & Weiner, 1995).

Isolation of the adhesin in research quantities has allowed characterization of its adsorption behavior. It has been found that its "stickiness" (assessed by surface

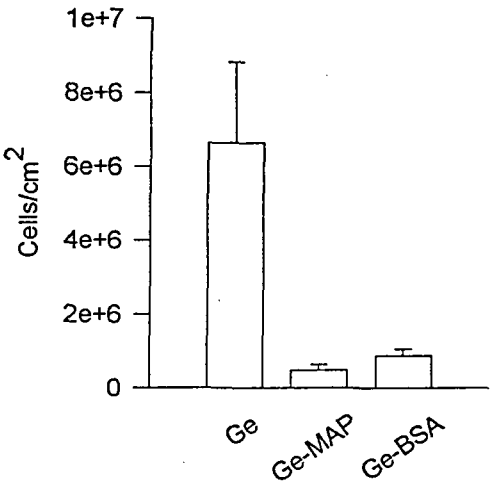

Fig. 6 Adhesion assays for MHS-3 on Ge, Ge conditioned with MAP, and Ge conditioned with BSA. Error bars designate standard deviation for 4 independent assays.

coverage and affinity) with respect to a hydrophilic surface is comparable with a protein mixture which constitutes the resin of a natural thermoset composite (MAP) (Waite, 1990). The driving forces behind non-specific adsorption of globular blood proteins have been well studied (Andrade, 1985; Brash & Horbett, 1987; Haynes *et al.*, 1994). Interactions responsible for adsorption of marine proteins are just beginning to be investigated (Baty *et al.*, 1996a). There is only speculation concerning structural features, molecular bonds, *etc.* which promote binding of adhesive polysaccharides (Allison, 1994) and fr2PS should provide a good model system in this respect.

It was found previously that proteins in a crude MHS-3 EPS preparation competed with the adhesive polysaccharide(s) for interfacial binding sites (Suci *et al.*, 1995). It appears from the results presented here that this polysaccharide binds poorly to substrata conditioned with two proteins which have completely different properties. BSA is a globular blood protein having a rich secondary structure (Peters, 1985) and a net negative charge at pH 8.0. It has been hypothesized that presentation of one of three, hydrated, negatively charged domains to the aqueous phase creates an interface which is unfavorable for bacterial attachment (Al-Makhlafi *et al.*, 1994). Mefp-1, the primary component of MAP, is densely populated with lysine residues which are protonated at this pH and is reported to have an open conformation in solution with little secondary structure (Waite, 1990). It is sold commercially as a coating to enhance cell adhesion (Cell-Tak, Collaborative Biomedical Products, Bedford, MA). Previously, it was found that MAP enhanced adsorption of kelp alginate above that found on clean Ge (Suci & Geesey, 1995). It was concluded that the driving force for adsorption was primarily electrostatic, even at the relatively high ionic strength of seawater. Conditioning with BSA was found to have little effect on the adsorption of alginate when compared to clean Ge.

The cumulative results presented here and in two other publications (Quintero & Weiner, 1995; Suci *et al.*, 1995) suggest the following interpretation. MHS-3 has evolved a capsular extracellular polysaccharide (fr2PS) that binds *via* non-electrostatic interactions. It has been suggested on the basis of inhibition assays that hydrogen bonding is involved (Quintero & Weiner, 1995). Hydrogen bonds involving both organic acids

and bases can form with mineral surfaces (oxides) in marine sediments (Thurman, 1985). Therefore, this may be a common adhesive molecular strategy of marine organisms which occupy these environments.

It seems likely that the EPS matrix of most marine biofilms is composed of a large portion of proteins, some which reach the substratum and adsorb tenaciously; this is the case for MHS-3 (Suci *et al.*, 1995). Thus, in general, the first microorganisms which colonize a surface condition it with a film which consists partially of adsorbed protein. The molecular bonds which are established between fr2PS and the Ge surface (an oxide) form poorly, or not at all, with adsorbed proteins. Therefore, the adhesive polysaccharide (fr2PS) may be the implement for a binding strategy which is selective for relatively pristine mineral surfaces that have not been previously colonized. This would be consistent with the proposed niche it fills in the succession of microfouling as a primary colonizer (Quintero & Weiner, 1995). An interesting research direction could be initiated by posing the question: to what extent do the different adhesive strategies of a variety of microorganisms occupying a similar niche determine the ecology of the local microfouling process?

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