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ADHESIVE EXTRACELLULAR POLYMERS OF *HYPHOMONAS* MHS-3: INTERACTION OF POLYSACCHARIDES AND PROTEINS

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The adsorption behavior of extracellular polymeric substances (EPS) from the marine bacterium *Hyphomonas* MHS-3 was investigated using attenuated total reflection Fourier transform infrared (ATR/FT-IR) spectrometry. The protein fraction of the crude EPS (EPS_c) (propanol precipitated/extracted with EDTA) dominated the adsorption onto the germanium substratum. Removal of the Protease K accessible portion of the EPS_c protein, and treatment with RNase and DNase, yielded a hygroscopic substance (EPS_p) which contained at least one adhesive polysaccharide component. Conditioning the substratum with EPS_c diminished adsorption of the polysaccharide fractions in EPS_p; pre-adsorbed EPS_c protein was not displaced. The rate of EPS_c adsorption on substrata conditioned with EPS_p was slower than to clean germanium; however, the projected surface coverage of protein after long times, based on an empirical datafit, was the same as that for a clean substratum; the EPS_c proteins did not displace the pre-adsorbed adhesive polysaccharide fraction. SDS-PAGE (Coomassie blue stain) revealed an extensive homology between proteins from cell lysates and EPS_c proteins. However, distinct differences in the banding pattern suggested that proteins did not originate primarily from cell lysis during the extraction procedure. The results indicate that adhesive components of EPS, with respect to a hydrophilic surface (germanium), can be either protein or polysaccharide and that they may compete for interfacial binding sites.

KEYWORDS: extracellular polymeric substances, adhesion, marine bacterium, ATR/FT-IR, adsorption

INTRODUCTION

Bacteria which have colonized a surface are typically enveloped in a matrix of extracellular polymeric substances (EPS) (Costerton *et al.*, 1987; Cooksey, 1992). Many of the functions of the EPS matrix are probably related to maintainance of a favorable local environment for cell subsistence (Geesey, 1992). The intercellular polymer network may serve to store nutrients (Lange, 1976), shield cells from antagonists (Hoyle *et al.*, 1990) or optimize biofilm architecture (de Beer *et al.*, 1993; Tetz *et al.*, 1993; Korber *et al.*, 1994). In addition, the composition of the EPS matrix can influence subsequent fouling by invertebrate larvae, presumably by providing chemical cues for settlement (Holmström & Kjelleberg, 1994). Scanning electron micrographs of biofilms typically reveal strands of EPS anchoring the cells to each other and to the substratum (Fletcher & Floodgate, 1973; Allison & Sutherland, 1987). These results suggest that the EPS plays an adhesive role.

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The cell adhesion process has been described in terms of a reversible adsorption step followed by processes which secure the cells to the substratum irreversibly (Allison & Sutherland, 1987; Croes *et al.*, 1993). Formation of this more tenacious anchor has been associated with extracellular polysaccharide production (Croes *et al.*, 1993). Presence of a polysaccharide rich capsule can either enhance (Shea *et al.*, 1994) or reduce (Pringle *et al.*, 1983; Rosenberg & Kjelleberg, 1986; Wrangstadh *et al.*, 1986) initial attachment to surfaces. In general, the results suggest that particular extracellular polysaccharides have an inherently adhesive character, especially with respect to hydrophilic surfaces. Although the primary research focus has been on the polysaccharide portion of the EPS, the EPS typically contains a protein component (Humphrey *et al.*, 1979; Abu *et al.*, 1991; Vincent *et al.*, 1994). Most proteins which have been examined adsorb strongly to a variety of substrata (Brash & Horbett, 1987). As a class, this implicates them as potential adhesive elements of the EPS matrix.

Much progress has been made in characterizing proteinaceous adhesins employed by pathogenic bacteria for initial attachment to specific host tissues (Jann & Jann, 1990). However, the adhesins and molecular interactions involved in securing other biofilm forming bacteria to substrata, including marine bacteria, have not been clearly identified. Many studies have attempted to delineate general attributes of substrata or cells which enhance or inhibit cell attachment *e.g.* wettability or hydrophobicity (Dexter *et al.*, 1975; Fletcher & Loeb, 1979; Rosenberg & Kjelleberg, 1986; Busscher *et al.*, 1990). There is a growing consensus that hydrophobic interactions play a dominant role (Doyle & Rosenberg, 1990). In one case EPS strands connecting cells to the substratum were identified as an acidic polysaccharide (Fletcher & Floodgate, 1973). However, in general, the primary adhesive biomolecules which lie immediately proximal to the surface have not been characterized. Identification of these primary adhesive biomolecules and the molecular interactions involved in binding to various substrata may be a prerequisite for further advances in control of microfouling.

Adsorption behavior has been used to determine whether EPS components mediate initial attachment by correlation with whole cell behavior (Pringle & Fletcher, 1986). Adsorption studies can also be used to identify EPS components which have inherently sticky properties and thus are candidates for primary adhesive EPS components. The present study investigates adsorption behavior of components of *Hyphomonas* MHS-3 (MHS-3) EPS on germanium using attenuated total reflection Fourier transform (ATR/FT-IR) spectrometry. MHS-3 is a prosthecate, biofilm-forming marine bacterium which can thrive under both oligotrophic and copiotrophic conditions and has been implicated as a primary colonizer in the microfouling process (Shen *et al.*, 1989; Quintero & Weiner, 1995a,b). Recent studies indicate that the MHS-3 polysaccharide-rich extracellular capsule serves as an adhesin (Quintero & Weiner, 1995b).

MATERIALS AND METHODS

Materials

Synthetic seawater consisted of the following (w/v): 2.3% NaCl, 0.24% Na_2CO_3, 0.033% KCl, 0.4% $MgCl.6H_2O$, 0.066% $CaCl_2.2H_2O$, pH adjusted to 8.0 with HCl. Electrophoresis reagents were from Bio-Rad Laboratories (Richmond, CA); cleaning reagents were from Aldrich Chemical Company; chemicals for colorometric assays were analytical grade. Trypsin (Bovine Pancreas Type I), RNase, DNase and Protease K were from the Sigma Chemical Company (St. Louis, MO).

Culturing of bacteria

Hyphomonas strain MHS-3 (MHS-3) was isolated from shallow water sediments in Puget Sound, WA and cultured in Marine Broth 2216 (37.4 $g \cdot l^{-1}$) (Difco Laboratories, Detroit, MI) at 25°C on a rotating shaker at 100 $rev \cdot min^{-1}$. Teflon™ mesh was introduced into culture vessels to provide greater surface for attached growth (mesh opening, 1.8 mm, thread diameter, 0.5 mm, Tetko, Incorporated, Briarcliff, NY).

Isolation and Purification of EPS

Cells were harvested from an early stationary phase culture (defined conventionally in terms of planktonic growth). The spent medium was discarded and the flocs and the biofilm were mechanically removed from the culture vessel walls and the teflon mesh and resuspended in synthetic seawater. The cell suspension was centrifuged at 16,000 xg for 20 min. The EPS in the supernatant was precipitated with 4 volumes of ice-cold 2-propanol and the cell pellet blended in a Waring blender with 10 mM EDTA, 3% NaCl for 1 min at 4°C. The cell suspension was again centrifuged for 15 min at 16,000 xg. The cell pellet was discarded and the supernatant was precipitated with 2-propanol as above. The precipitated EPS fractions were pooled and resuspended in a minimum volume of dH_2O and dialyzed exhaustively against dH_2O. This crude EPS (EPS_c) was lyophilized.

Polysaccharide-enriched EPS (EPS_p) was prepared by a modified protocol (Read & Costerton, 1987). EPS_c was dissolved in a minimum volume of 0.1 M $MgCl_2$. DNase and RNase were added to a final concentration of 0.1 $mg \cdot ml^{-1}$, and incubated at 37°C for 4 h. Protease K was added to 0.1 $mg \cdot ml^{-1}$ and incubated at 37°C overnight. The residual protein was removed with a hot phenol extraction, followed by a chloroform extraction. This preparation was dialyzed exhaustively against dH_2O and lyophilized. All EPS fractions were stored desiccated, at room temperature.

Electrophoresis (SDS/PAGE)

Tris/glycine-based gels (Shapiro *et al.*, 1967) were cast and run in a Model 220 Vertical Slab Electrophoresis Cell (Bio-Rad Laboratories, Richmond, CA). The composition (w/v) of the stacking gel was 3% acrylamide/0.08% bisacrylamide; separating gels were 9% acrylamide/0.24% bisacrylamide. The running buffer was (w/v) 0.1% SDS, 2.88% glycine, 0.6% Tris base. The sample buffer was 15% glycerol, 0.2% dithiothreitol. Molecular masses were estimated using Sigma SDS protein standards (M-2789).

MHS-3 lysate was prepared from log phase planktonic cultures. 1 ml aliquots were centrifuged (10,000 xg) for 10 min and the supernatant discarded.

Appropriate amounts of EPS_c and pelleted bacteria were suspended in 50 μl sample buffer, sonicated for 10 min and boiled for 10 min before being introduced to the sample wells.

Colorometric Assays

Neutral hexoses were determined using the phenol sulfuric assay with glucose as the standard (Dubois *et al.*, 1956). Proteins were estimated using the Lowry procedure (bovine serum albumin standard) (Lowry *et al.*, 1951). Uronic acid content was determined using the meta-hydroxy diphenyl method of Blumenkrantz and Asboe-Hansen (1973) modified by Kintner and Van Burren (1982) with glucuronic acid as the standard.

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 followed by a series of rinses which all consisted of ultrasonication in various liquids 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: ultrapure water (2×), ethyl alcohol, chloroform and dichloromethane. Auger electron spectroscopy (Phi 595 scanning Auger microprobe) indicated that the elemental percent composition of the first few monolayers of Ge coupons (Exotic Materials Incorporated, Costa Mesa, CA) cleaned by this protocol, was 9.11 ± 1.38% carbon, 6.51 ± 2.01% oxygen, and 83.92 ± 2.24% Ge. This is approximately the same level of hydrocarbon contamination obtained after exposing a carbon-free (Argon etched) Ge coupon to air for 1 min.

Adsorption Protocol

For adsorption experiments the cylindrical IRE was positioned within a stainless steel flow chamber (Circle Cell™, Spectra Tech). The interior cavity of the flow chamber is cylindrical with a diameter of 0.476 cm and a length of 2.7 cm. The volume contained in the annular region between the surface of the IRE and chamber wall is 0.289 cm^3. Fluid was introduced and displaced through entrance and exit ports at each end of the cavity. The IRE was held in place in the chamber by two teflon O-rings.

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 (0.08 cm ID). Tubing was cleaned after each experiment by sonicating in base bath. The section of tubing connecting the reservoir containing protein to the flow chamber was made as short as possible (~20 cm). Effluent tubing was teflon and viton™ (Cole-Parmer, Niles, IL). Fluid was pumped by threading the viton through a peristaltic pump (Sage Instruments, Cambridge, MA) at 0.5 $ml \cdot min^{-1}$. All glassware was acid cleaned (No Chromix™ in concentrated H_2SO_4).

Before each adsorption experiment the surface was exposed to flowing seawater for 20 min. A vial containing approximately 1 ml of the desired concentration of EPS 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 for 100 min. Adsorption was performed under these static conditions to conserve EPS. Flow was then resumed and the surface was rinsed with synthetic seawater for 120 min.

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 either EPS_c or EPS_p the spectrum acquired immediately before the second adsorption reaction was initiated was used as the background.

Transmission spectra were measured in a 15 μm pathlength demountable cell with calciumfluoride windows (Spectra-Tech).

Estimation of Surface Coverage

Surface coverage of the protein portion of the EPS_c was estimated using a published correlation based on the area of the amide II band (Pitt & Cooper, 1988). This correlation can be converted for application to the present experimental ATR configuration by utilizing the water absorbance at 1640 cm^{-1} (Pitt, 1987) as a normalization factor. The normalized conversion factor is 0.746 ± 0.104 $\mu g \cdot cm^{-2}$ area amide II band. Surface coverage of putative polysaccharide fractions was estimated by comparing absorbances obtained in transmission mode with those obtained in the ATR mode. The estimates are approximate and are predicated on the assumption that the extinction coefficient for IR absorbance is the same for solution phase and adsorbed molecules. This assumption is supported by data on ATR/FT-IR of adsorbed protein (Pitt & Cooper, 1988), but has not been tested for polysaccharides. With this assumption the following relation can be derived:

$$\Gamma = \ln 10[A_{w,t}/A_{a,t}][1/(1-T_{w,e})][c_{a,t}][d_p/2]A_{a,e} \qquad \text{Eqn 1}$$

where Γ is the surface coverage of adsorbed substance (a) in $mg \cdot cm^{-2}$, ln10 is the natural log of 10, $A_{w,t}$ is the absorbance of water at 1640 cm^{-1} in the transmission cell (baseline subtracted: 1740 to 1540 cm^{-1}), $A_{a,t}$ is the absorbance of the substance in the transmission cell, $T_{w,e}$ is the transmittance of water in the ATR mode at 1640 cm^{-1}, $c_{a,t}$ is the concentration of the substance in the transmission cell ($mg \cdot ml^{-1}$), d_p is the penetration depth of the evanescent field, and $A_{a,e}$ is the absorbance of the adsorbed substance in the ATR mode. The penetration depth, d_p, can be calculated from the incident angle of the internal reflections and the relative refractive index at the interface for a particular wavenumber (Knutzen & Lyman, 1985). Equation 1 employs the absorbance of water in the transmission and ATR modes as a normalization factor. The expression yields a conversion factor of 0.97 $\mu g \cdot cm^{-2} \cdot$area amide II band for a bovine serum albumin standard which is close to that of Pitt and Cooper (1988) for human serum albumin. In order to use Equation 1 to determine the surface coverage of putative polysaccharides in EPS_p, $c_{a,t}$ was assumed to be equivalent to the carbohydrate content of EPS_p.

Langmuir and Exponential Fits

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

$$A = A_s[((1/Kc_b)+1)^{-1}] \qquad \text{Eqn 2}$$

where K is the binding (association) constant, c_b is the concentration of the substance in bulk solution, A is the absorbance (or area in $abs \cdot cm^{-1}$) 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 can be converted to projected surface coverage (Γ) using Equation 1.

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.

In order to quantify the hindered adsorbance of protein on pre-adsorbed putative polysaccharide another empirical fit was employed:

$$A = p_1(1-\exp[(t-p_2)/\tau]) \qquad \text{Eqn 3}$$

where A is the absorbance at time t and a best fit for parameters p_1, p_2 and τ (the time constant) is found using nonlinear regression as described above. The static adsorption conditions preclude discrimination of adsorption kinetics from diffusion limited mass transfer to the interface. However, the above empirical (essentially first order) fit allows a semi-quantitative comparison of data sets to be made.

RESULTS

Biochemical Assays

Composition of the major components of *Hyphomonas* crude EPS (EPS_c) and partially purified EPS (EPS_p) are shown in Table 1. Extraction of protein and nucleic acids from EPS_c had a profound effect on the solubility characteristics. Whereas EPS_c must be sonicated for an extended period (> 1 h) in order to solubilize 1 $mg \cdot ml^{-1}$ in seawater, EPS_p is completely dispersed instantly in seawater at > 30 $mg \cdot ml^{-1}$. This difference in solubility is probably a reflection of a difference in the colloidal structure and also suggests that EPS_p is relatively hydrophilic compared to EPS_c.

Coomassie blue stained SDS-PAGE (Fig. 1) of cell lysates from planktonic cultures and EPS_c indicated an extensive homology between the two sets of proteins. At least one distinct high molecular weight band ((approximately 154kD) and two faint lower molecular weight bands (approximately 44 and 49kD) were present in the cell lysate which were absent, or at least much less prominent, in the EPS_c proteins implying that EPS_c proteins did not originate primarily from bacteria lysed during the extraction procedure. However, many of the EPS_c proteins could well have originated from cells lysed during biofilm development. Slight differences in the banding pattern between 11kD and 13kD suggest that some proteins are present in EPS_c which are absent in the cell lysate. These are candidates for specialized EPS proteins.

IR Spectra and Time Course of Binding

Three IR spectra of EPS_c are shown in Figure 2. The spectrum of the bulk EPS_c taken in transmission mode (Fig. 2a) contains bands at 1645 cm^{-1} and 1550 cm^{-1} which are typical

Table 1 Major components of *Hyphomonas* MHS-3 EPS ($\mu g \cdot mg^{-1}$)

Component	*EPS_c*	*EPS_p*
Neutral hexose	277	635
Protein	409	135
Uronic acids	5	30

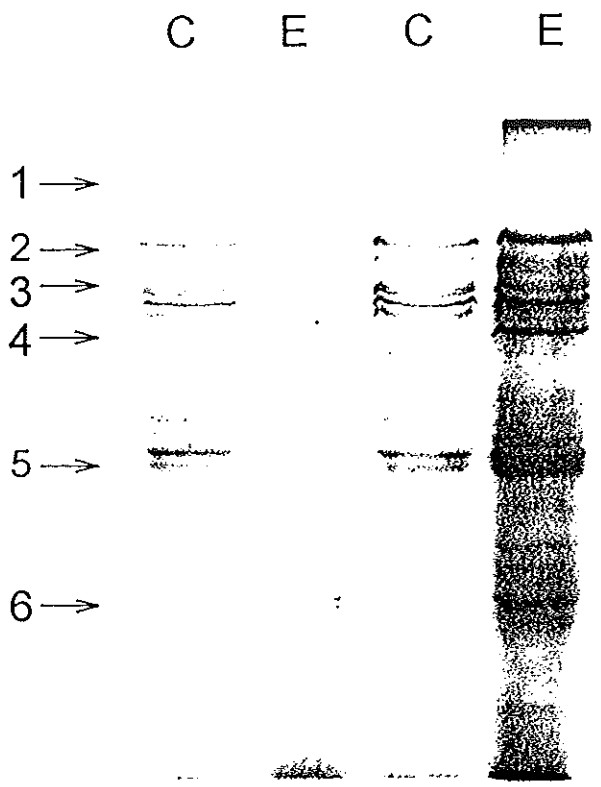

Fig. 1 Coomassie blue stained SDS-PAGE. C = cell lysate; E = EPS_c. Molecular weight calibration is indicated as follows (kD): 1 = 205, 2 = 116, 3 = 97.4, 4 = 66, 5 = 29, 6 = 14.2.

of the protein amide I and II bands, respectively (Miyazawa & Blout, 1961; Bandekar & Krimm, 1980). The broad band centered at approximately 1086 cm^{-1} is in a region where carbohydrates (including polysaccharides) have characteristic vibrations (Cael *et al.*, 1975). RNA and DNA also contain overlapping bands in this region which have a prominent maximum between 1085 cm^{-1} and 1090 cm^{-1} (Taillandeir *et al.*, 1985). Figures 2b and c are spectra of EPS_c, taken in ATR mode, during adsorption and after a 120 min rinse period, respectively. The amide I and II bands contribute a larger proportion of the spectral absorbance for these two latter spectra than the broad spectral feature contained within the carbohydrate/RNA/DNA region (1120 cm^{-1} to 900 cm^{-1}). Although a small amount of absorbance is present in this region of the spectrum after the rinse period (Fig. 2c), the amide bands dominate the spectrum.

Figure 3 shows the time course of adsorption and desorption of the EPS_c based on the area of the amide II band. Although there is a small amount of material lost from the interface when the rinse#1 is initiated, the adsorption is essentially irreversible. After an hour exposure to trypsin (1 $mg \cdot ml^{-1}$, under static conditions) the adsorbed material can be easily removed by flowing seawater (rinse#2).

Three spectra of EPS_p are shown in Figure 4. The spectra are displayed in the same order as in Figure 2, *i.e.* Figure 4a is a transmission spectrum of bulk EPS_p and Figures 4b and c are spectra taken in ATR mode during adsorption (Fig. 4b) and at the end of the rinse period (Fig. 4c). In contrast to EPS_c the broad spectral feature in the region of the spectrum associated with carbohydrate resonance frequencies (1120 cm^{-1} to 900 cm^{-1}) is prominent for both the bulk (Fig. 4a) and adsorbed (Fig. 4b,c) material. This broad feature contains no apparent band or shoulder at 1085 cm^{-1} to 1090 cm^{-1} which would suggest the presence of nucleic acids. Although the bands centered at 1646 cm^{-1} and

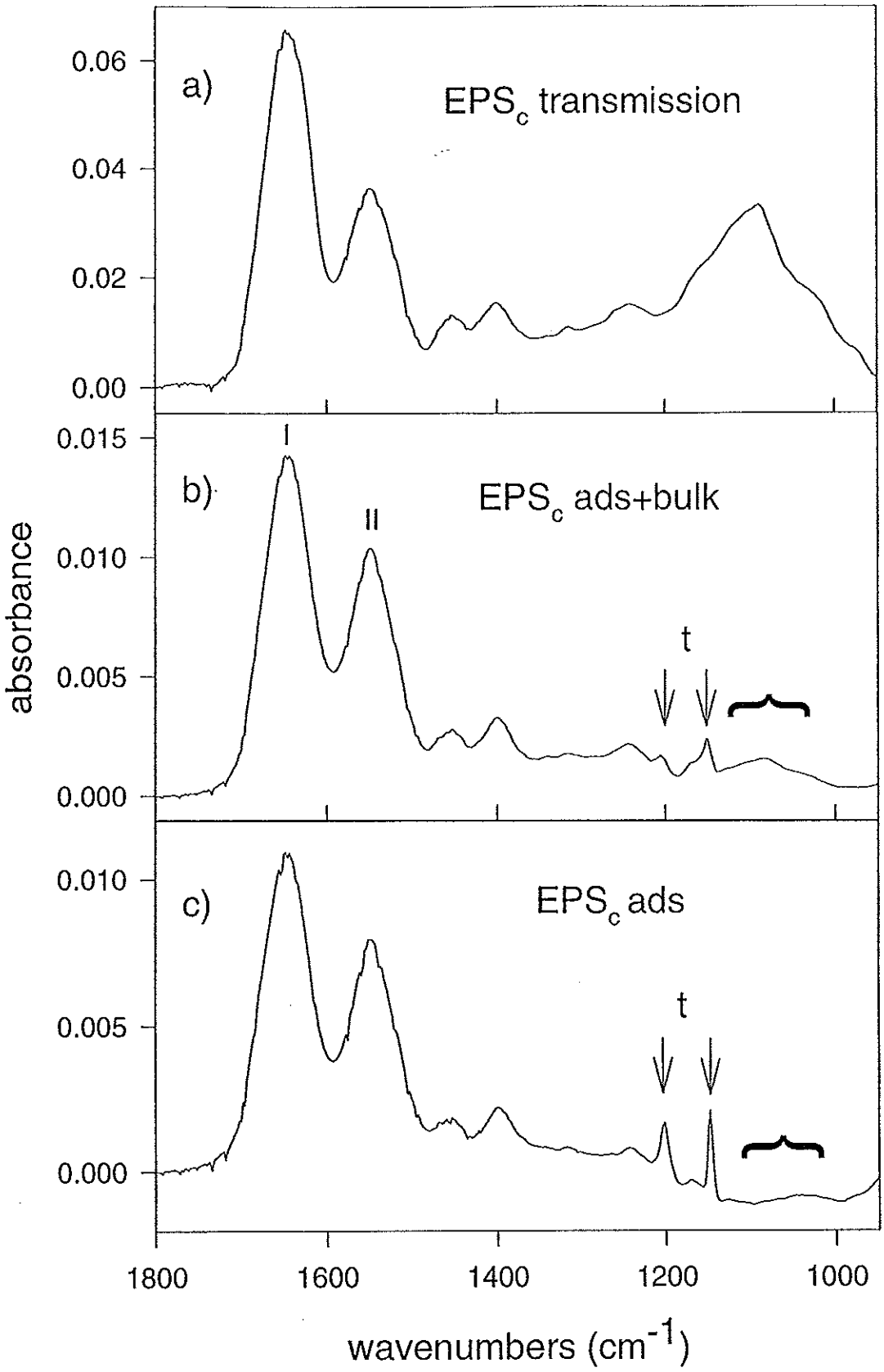

Fig. 2 a = IR transmission spectrum of EPS_c (approximately 20 mg·ml^{-1}); b = ATR spectrum of 1 mg·ml^{-1} EPS_c in the flow chamber; amide I and II bands are indicated; bracketed portion contains bands emanating potentially from carbohyrates, RNA and DNA; c = ATR spectrum of adsorbed EPS_c at the end of the rinse period. Arrows in b and c indicate positions of bands emanating from Teflon O-rings.

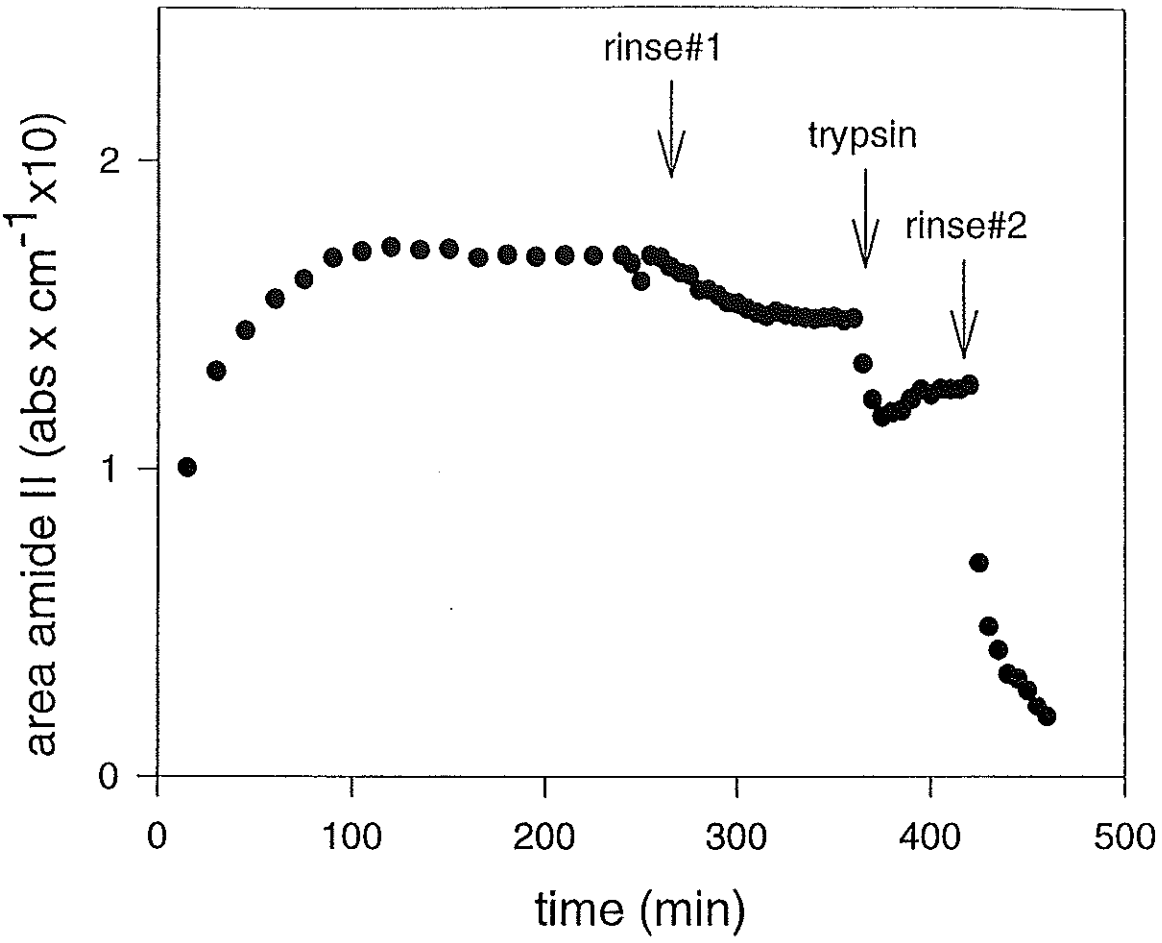

Fig. 3 Time course of adsorption and desorption of EPS_c protein based on areas of the amide II band. Times of rinses with seawater (rinse#1 and #2) are indicated, as well as time of introduction of trypsin into the flow chamber.

1561 cm^{-1} in the spectrum of the bulk EPS_p (Fig. 4a) may be protein amide bands, they are somewhat atypical in this respect. It is rare to find a protein amide II absorbance above 1560 cm^{-1} (Krimm, 1962; Nevskaya & Chirgadze, 1976; Moore & Krimm, 1976). Furthermore, the amide I and II bands of proteins are typically of more equal half-widths (Fink *et. al.*, 1987; Ishida & Griffiths, 1993a). The wide band at 1561 cm^{-1} may be composed of overlapping bands from protein peptide linkages and N-acetyl groups of a polysaccharide (Quintero & Weiner, 1995a). Amide vibrations of N-acetyl groups of hyaluronic acid are close to 1565 cm^{-1} (Parker, 1983) and that of N-methylacetamide is at 1567 cm^{-1} (Miyazawa *et al.*, 1958). In contrast to the EPS_c, the putative amide I and II bands contribute proportionately less to the spectra of the adsorbed material (both during adsorption and after the rinse) than to the spectrum of the bulk material. This is especially evident for the band centered at 1561 cm^{-1} which is undetectable for adsorption experiments with bulk EPS_p less than 0.2 $mg \cdot ml^{-1}$ (spectra not shown). In summary, the carbohydrate fraction of EPS_p (rather than any residual protein) appears to dominate the adsorption process.

In Figure 4c bands used to follow the adsorption reaction involving components of EPS_p are indicated (EPS_p I and II). These bands were chosen for the technical reason that they are less obscured by the water background absorbance centered at 1640 cm^{-1} and the

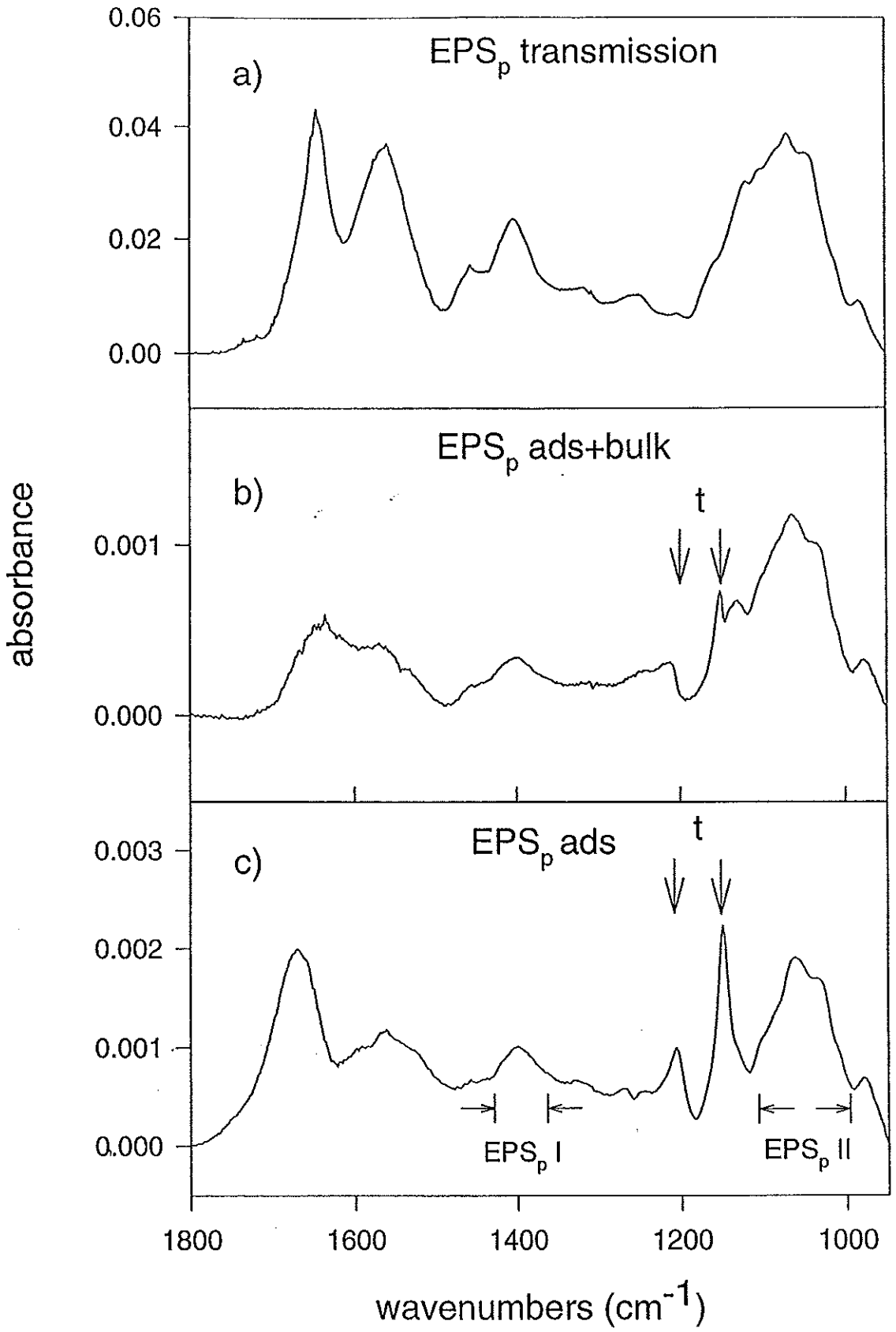

Fig. 4 a = IR transmission spectrum of EPS_p (30 mg·ml^{-1}); b = ATR spectrum of 0.2 mg·ml^{-1} EPS_p in the flow chamber; positions of bands emanating from the Teflon O-rings are indicated; c = ATR spectrum of adsorbed EPS_p at the end of the rinse period. Spectral features (EPS_p I and II) used to follow the adsorption reaction are indicated.

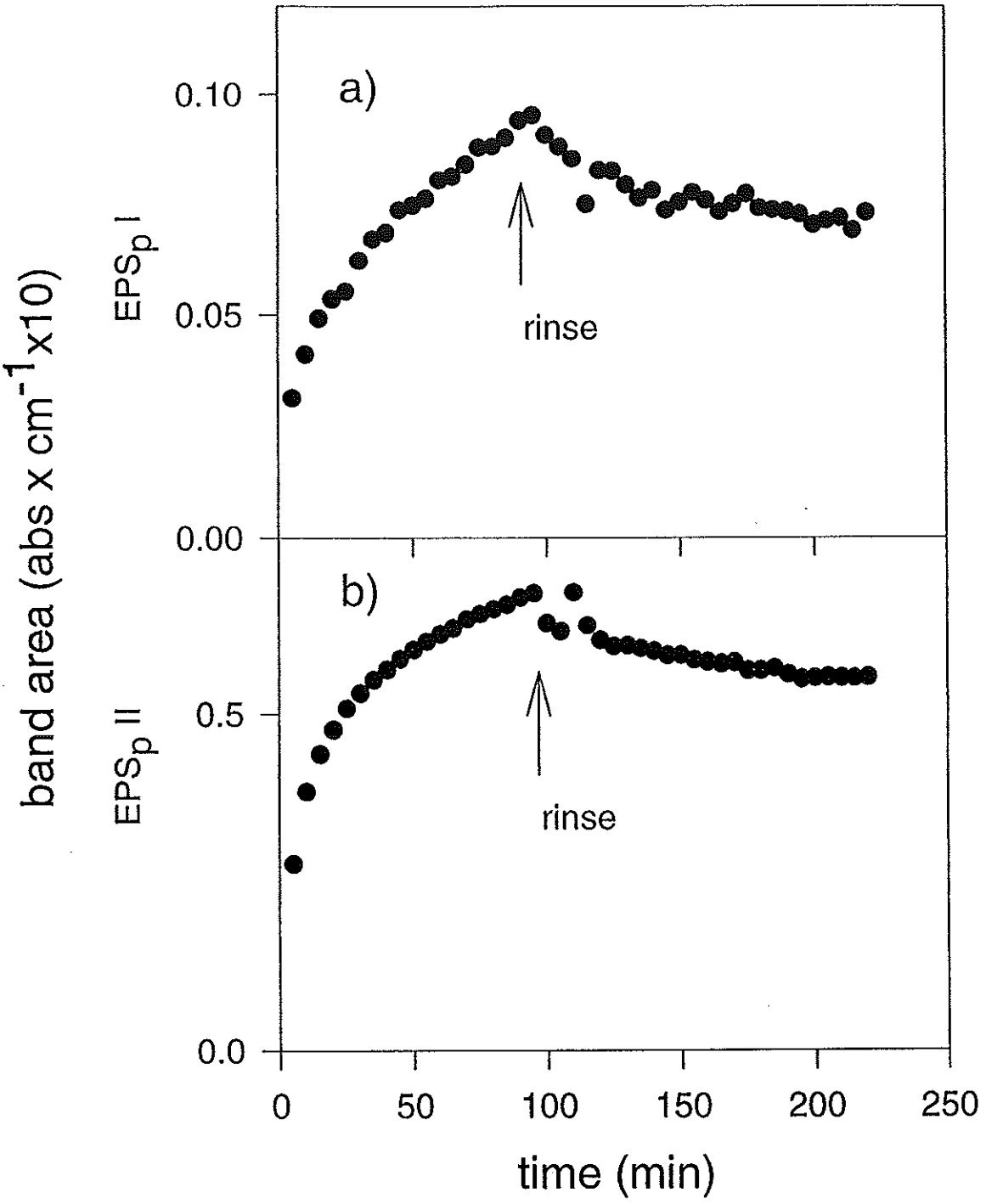

Fig. 5 Time course of adsorption and desorption of EPS_p based on areas of spectral features. a = EPS_p I; b = EPS_p II.

teflon bands at 1200 cm^{-1} and 1150 cm^{-1} (which emanate from the O-rings) than other spectral features. In addition, the wide spectral feature named EPS_p II is located within the prospective carbohydrate region. For the adsorbed material (Fig. 4c) this spectral feature appears to be composed primarily of bands centered at 1060 cm^{-1} and 1033 cm^{-1}. Vibrations near 1030 cm^{-1} have been assigned to C-O-H deformations or C-O stretch of the pyranose ring (Cael *et al.*, 1975; Parker, 1983; Ishida & Griffiths, 1993b).

Polysaccharides containing N-acetyl groups such as hyaluronic acid have bands near 1070 cm^{-1} (Parker, 1983). The spectral feature named EPS_p I, centered at 1395 cm^{-1}, cannot be given an unambiguous assignment. Carboxylate salts commonly have a symmetric stretch in this region (L-Vien *et al.*, 1991) with an accompanying asymmetric stretch in the region from 1650–1540 cm^{-1}. However, for uronic acids this latter band is typically located near 1600 cm^{-1} and is prominent (Parker, 1975). Proteins normally display a band in this region. However, as mentioned above, for low surface coverages of EPS_p, the putative amide II band centered at 1561 cm^{-1} becomes undetectable, while EPS_p I remains conspicuous. At these low surface coverages, it becomes impossible to distinguish the band centered at 1646 cm^{-1}, (another candidate for the asymmetric carboxylate stretch), from the strong background water absorbance at 1640 cm^{-1}.

Figure 5 shows the time course of EPS_p adsorption/desorption from the substratum based on the areas of EPS_p I and II (Fig. 4c). Although there is some desorption after the rinse is initiated, a major fraction of the material is retained on the surface at the end of the rinse period.

EPS Binding Curves

In order to construct binding curves for the various EPS preparations, the areas of pertinent spectral features at the end of the rinse period have been plotted against the bulk concentration to which the substratum was exposed. The binding curves have been fit with a Langmuir model (Eqn 2). Parameters (K^{-1} and Γ) obtained from the Langmuir fits to binding curves are listed in Table 2. Binding curves presented below (Figs 6, 7a, 7b) have been plotted in terms of area of the spectral feature *vs* total concentration of bulk EPS. To obtain the parameters K^{-1} and Γ, ordinate and abscissa values must be converted to surface coverage and concentration of the specific EPS component, respectively (see Methods). Using the total carbohydrate content of EPS_p for these estimates yields maximum estimates of K^{-1} and Γ.

The binding curve for the protein fraction of EPS_c (amide II band *vs* bulk concentration) is shown in Figure 6. The fit to the Langmuir model is shown by the dotted line. The adsorbed quantity at a 1 $mg \cdot ml^{-1}$ bulk concentration exhibits an extent of variability not observed at lower concentrations. The mean of these five adsorption experiments is indicated by the open square symbol.

Table 2 Parameter fits to the Langmuir model (Eqn 2). Data are presented in Figures 6 and 7. See text for conversion of spectral features to surface coverage (Γ). Lower values of K^{-1} imply greater binding affinity.

	Hyphomonas EPS[a]			*Literature (proteins)*		
parameter[b]	Amide II *(EPS_c)*	EPS_p I	EPS_p II	i	ii	iii
Γ ($\mu g.cm^{-2}$)	0.179 ± 0.019	0.143 ± 0.015	0.143 ± 0.017	0.281 ± 0.010	0.5	0.25
K^{-1}($mg.ml^{-1}$)	0.049 ± 0.015	0.270 ± 0.047	0.033 ± 0.010	0.017 ± 0.002	0.103	0.006

i = mussel adhesive protein on germanium; binding curve previously published (Suci & Geesey, 1995)
ii = bovine serum albumin on methylated surface (estimated from Fig. 5), Koltisko & Walton, 1985
iii = gamma-globulin on silicone rubber (Watkins & Robertson, 1977);
[a]Identified by IR spectral feature
[b]Fits for EPS_p I and II are to data points for bulk concentrations < 5$mg \cdot ml^{-1}$

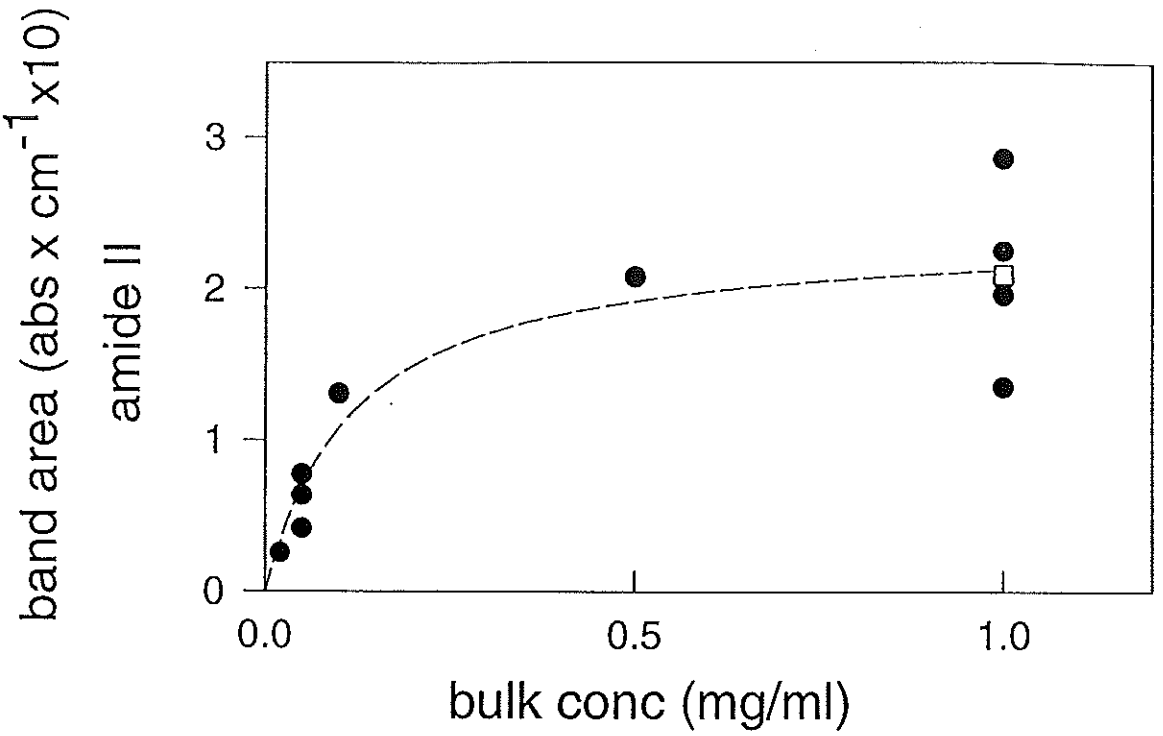

Fig. 6 Binding curve of the EPS_c protein fraction. □ = the position of the average of the five data points at 1 mg·ml^{-1} bulk concentration; --- = the Langmuir fit.

The binding curves for EPS_p are shown in Figure 7. Areas of spectral features named EPS_p I and II (Fig. 4c), determined at the end of the rinse period, have been plotted against the total bulk concentration to which the substratum was exposed. For each plot two fits of the Langmuir model are indicated. The broken line is the fit to all data points, while the dotted line is the fit to data points for bulk concentration below 5 mg·ml^{-1}. The mean value for data points at 5 mg·ml^{-1} bulk concentration is designated by an open square. The variation in the areas of EPS_p II at this concentration is extreme. An obvious explanation is that the extent of adsorption varies sensitively with the degree of surface contamination. However, the extent of adsorption at this relatively high bulk concentration is uncorrelated to general hydrocarbon contamination estimated by absorbances in the CH_2 and CH_3 stretch region between 2980 and 2820 cm^{-1} immediately before the adsorption was initiated (data not shown). It is possible that a small fraction of the adhesive protein, which resides in the EPS_p after the purification, begins to compete with the polysaccharide components for adsorption sites above a certain threshold concentration which is just barely achieved at 5 mg·ml^{-1} bulk concentration of total EPS_p. However, this does not explain the enhancement of adsorption above the mean for two experiments.

Interaction Between Proteins and Polysaccharides

Conditioning the germanium substratum with EPS_c impedes adsorption of the putative polysaccharides in EPS_p. This is indicated by results presented in Figure 8. Germanium IREs were first exposed to EPS_c, and then rinsed as described above. Substrata which had different degrees of surface coverage of EPS_c were then exposed to a 0.1 mg·ml^{-1} bulk concentration of EPS_p. This second adsorption was performed with the same protocol as

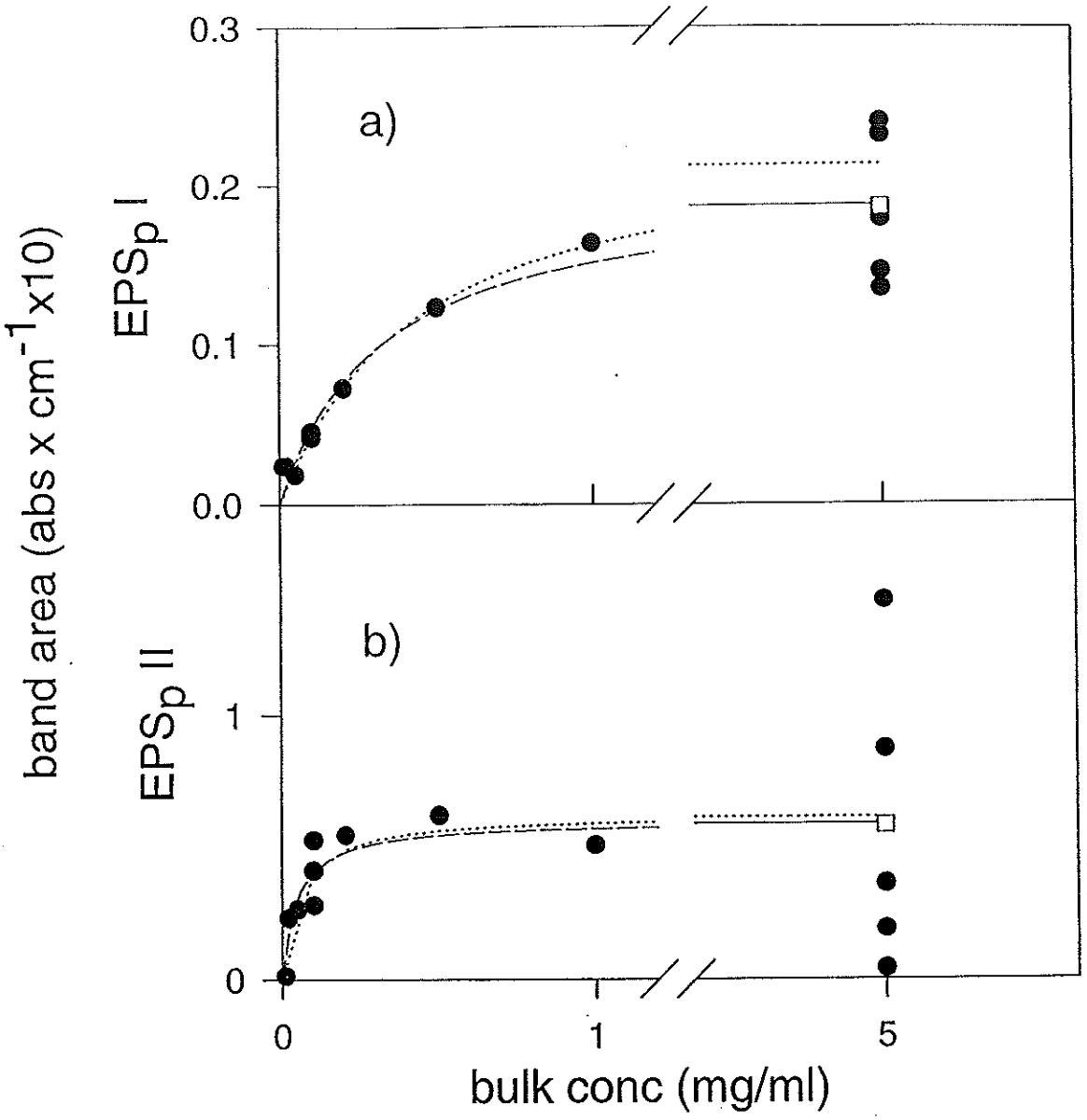

Fig. 7 a, b = binding curves for putative polysaccharide fractions of EPS_p □ = the position of the average of the five data points at 5 mg·ml^{-1} bulk concentration; --- = the Langmuir fit to all data points; ... = the fit to data points for bulk concentrations < 5 mg·ml^{-1}.

for adsorption experiments to the clean substratum (100 min adsorption/120 min rinse). (The adsorption attained a plateau during the 100 min time period). The results are summarized in Figure 8 in which the areas of the spectral feature EPS_p II at the end of the second rinse period are plotted against the amount of pre-adsorbed protein from EPS_c (amide II area). (Results for EPS_p I are similar). The negative correlation between surface coverage of pre-adsorbed EPS_c and area of the spectral feature EPS_p II is apparent.

In order to see whether the protein from the EPS_c would displace the putative polysaccharides (from which spectral features EPS_p I and II emanate), EPS_p was adsorbed at a concentration of 1 mg.ml^{-1} using the standard protocol. The surface conditioned with EPS_p was then exposed to a 1 mg·ml^{-1} bulk concentration of the EPS_c. Figure 9 shows the time course of the change in the amide II band (Fig. 9a) and in EPS_p II (Fig. 9b) during

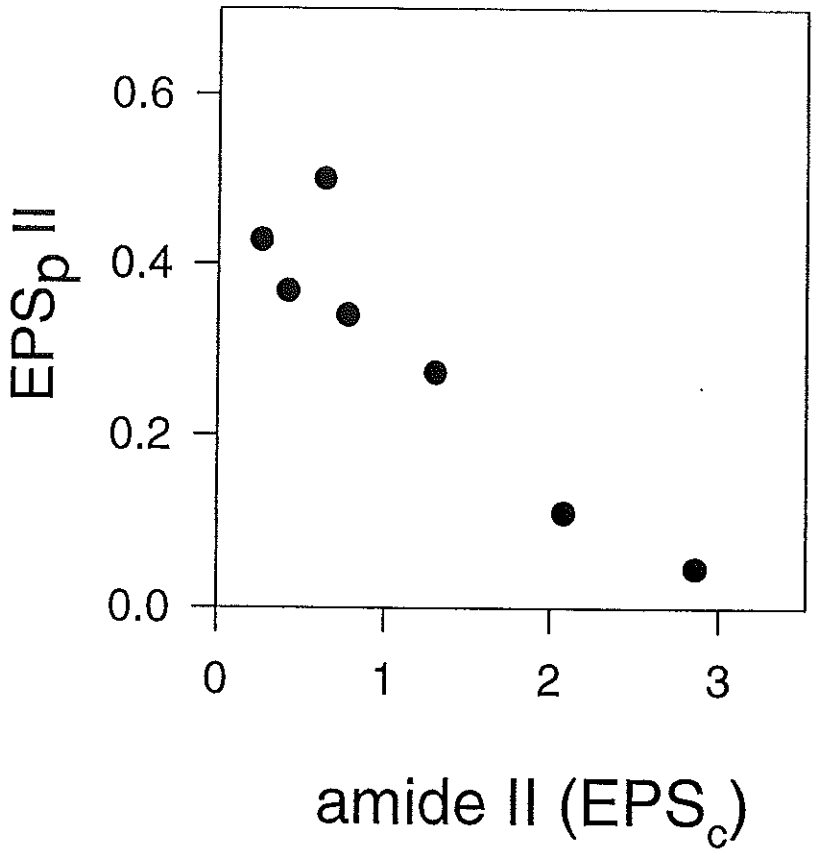

Fig. 8 Relation between surface coverage of pre-adsorbed protein from EPS_c and putative polysaccharide fraction from EPS_p (EPS_p II) adsorbed onto the conditioned substratum.

this second adsorption and the rinse. The solid line through the initial data points in Figure 9a is an exponential fit as described in Methods. This empirical fit allows assignment of a "time constant" to the adsorption kinetics (τ in Eqn 3) of 100 min. The average τ value for adsorption of protein from EPS_c onto a clean substratum is 40 ± 10 min (5 experiments).

Pre-adsorbed material from EPS_p is not displaced by the protein fraction from EPS_c. There is little change in the area of EPS_p II (Fig. 9b) and the insert reveals that the material remaining on the surface resembles the material adsorbed on a clean germanium substratum from EPS_p: the peak at 1060 cm^{-1}, the shoulder at 1034 cm^{-1} and the small peak at 976 cm^{-1} are evident. Although the rate of adsorption is slower, the empirical fit to Eqn 2 indicates that, if the same general trend in the time course of adsorption is followed, the protein will eventually reach a plateau area of 0.208 abs·cm^{-1} or 4 mA peak height above baseline (A_p in Eqn 2), which is in the range for adsorption to a clean substratum after the rinse period (mean for 4 experiments: 0.257 ± 0.505 abs.cm^{-1}) (see also Fig. 6).

DISCUSSION

Characterization of adsorption behavior is one approach which has been used to investigate the potential adhesive role of EPS matrix components (Pringle & Fletcher,

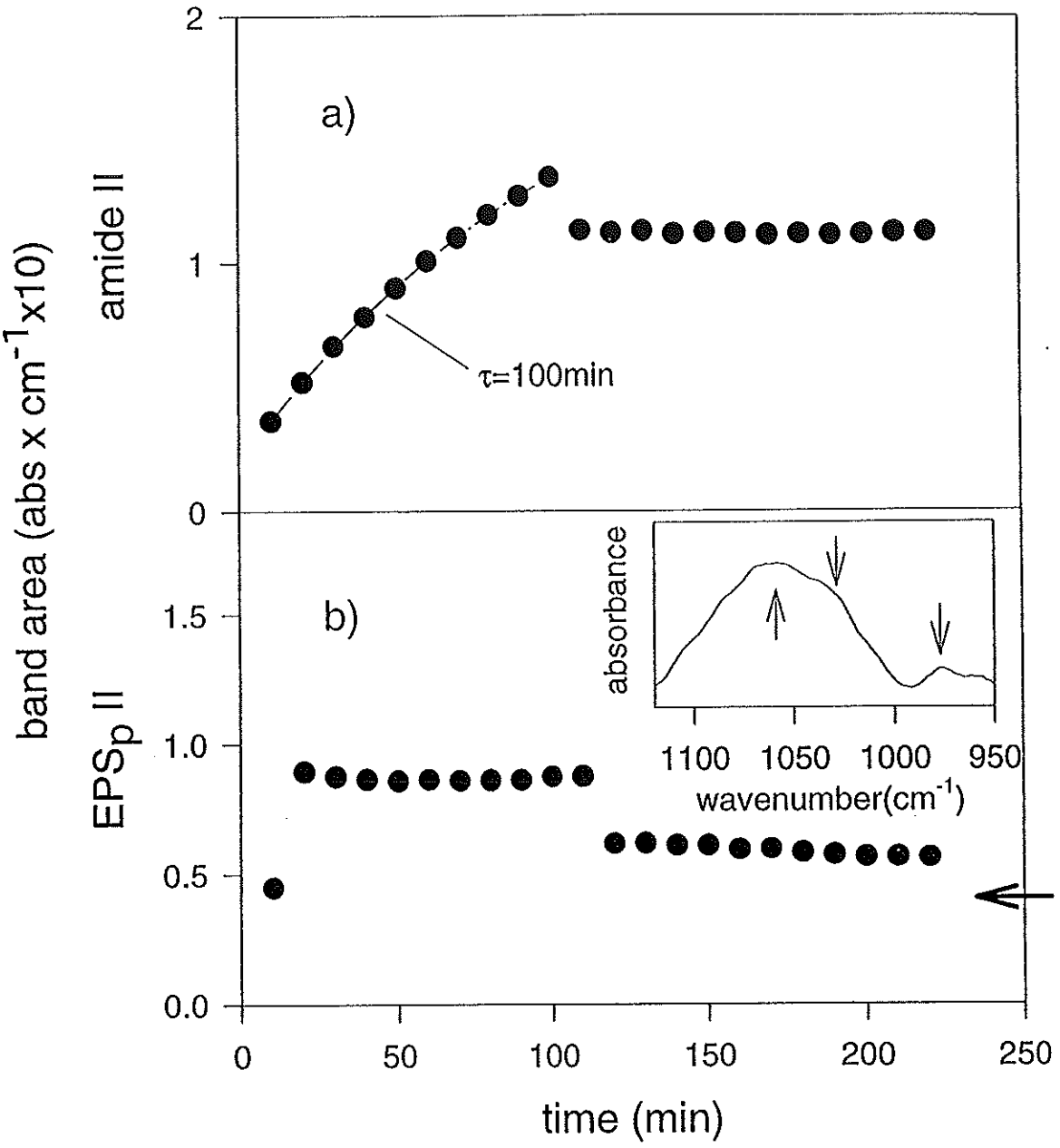

Fig. 9 a = time course of binding of protein for EPS_c onto substratum conditioned with EPS_p at 1 mg·ml^{-1} with time constant indicated (see text); b = change in area of EPS_p during protein binding; arrow indicates area of this region before exposure to protein; insert is difference spectrum of this region at the end of the rinse period with characteristic features indicated.

1986). In order to make definite structure/function assignments these studies must be supplemented with data acquired using other methods *e.g.* direct observation (Fletcher & Floodgate, 1973) or footprint analysis (Neu, 1992). In this context, adsorption studies can indicate interactions between biomolecules and surfaces which merit scrutiny.

In this report, adsorption behavior of crude EPS (EPS_c) from biofilms of *Hyphomonas* MHS-3 has been compared to EPS from which a large portion of the protein, DNA and

RNA has been removed (EPS_p). The protein dominates the adsorption process for EPS_c. Removal of the Protease K accessible protein allows adsorption of two putative polysaccharide components to be detected. In terms of adsorption behavior, the interaction between protein and polysaccharide in the EPS is apparently antagonistic; they appear to compete for the same sites on the substratum. It is apparent from the data that even EPS_p consists of a mixture of biomolecules. It is likely that the chaotic adsorption behavior at 5 $mg \cdot ml^{-1}$ bulk concentration (Fig. 7b) can be attributed to interactions between minor components of this mixture which begin to exert an influence at some threshold concentration. However, the nature of the critical governing interactions remains illusive.

Characterization of adsorption behavior yields an estimate of affinity for the surface of the various molecular species. The Langmuir model has been applied here to allow a quantitative appraisal of binding affinity, with the understanding that its application is not rigorous. Intuitively, it might be expected that an adsorbent with a strong affinity for a surface would adsorb out of relatively dilute bulk solutions and would have a high density of coverage at saturation. The parameters K^{-1} and Γ listed in Table 2 reflect these adsorption tendencies. In addition, this approach allows comparison of adsorption behavior with results of other investigators, some of whom have applied the Langmuir model to quantify irreversible adsorption behavior (Pringle & Fletcher, 1986; Watkins & Robertson, 1977).

Using the parameters obtained from the Langmuir fit as an index of binding affinity, both the protein fraction from the EPS_c and the putative polysaccharide fraction of EPS_p corresponding to the spectral feature EPS_p II are quite sticky. The EPS component responsible for the spectral feature EPS_p I (which cannot be unambiguously assigned) is somewhat less sticky. This assessment has been made based on a comparison with typical values for globular blood proteins (Brash & Horbett, 1987), which are conventionally considered to be strong adsorbents, from comparison to adsorption data for an EPS from another marine biofilm forming organism (Pringle & Fletcher, 1986), and from data collected in the authors' laboratory on the adsorption of adhesive protein from *Mytilus edulis* (Suci & Geesey, 1995).

It is notable that the *Hyphomonas* EPS contains at least one adhesive protein and at least one adhesive polysaccharide. The literature indicates that, in general, proteins adsorb strongly to most surfaces. However, it is important to mitigate this generalization with the qualification that the majority of research in this respect has been done on globular blood proteins, which may have inherently adhesive properties.

Polysaccharides are not considered to be universally strong adsorbents. Results indicate that presence of a preformed polysaccharide rich capsule may impede initial cell attachment to hydrophobic surfaces (Wrangstadh *et al.*, 1986; Rosenberg & Kjelleberg, 1986) or enhance binding preferentially to hydrophilic surfaces (Shea & Smith-Somerville, 1994). The uronic acid-containing polysaccharide, alginate, adsorbs only weakly to both germanium and polystyrene (Ishida & Griffiths, 1993b, Suci & Geesey, 1995). This suggests that affinity for hydrophilic surfaces is not a universal characteristic of functionalized polysaccharides, and must be conferred by appropriate molecular architecture. It has been proposed that polysaccharides functionalized with methyl or acetyl groups may exhibit protein-like adsorption behavior as a result of the consequent hydrophobic domains (Christensen *et al.*, 1985). Extracellular polysaccharides produced after cell attachment could presumably attain sufficient concentration to confer an extent of adhesion originating purely from their viscosity (Humphrey *et al.*, 1979). The *Hyphomonas* polysaccharide responsible for the spectral feature EPS_p II adsorbs from dilute solutions which suggests that it may have been specially synthesized as a primary adhesive EPS component.

Although chemical characterization of bacterial EPS has revealed that it is typically composed of significant amounts of both protein and carbohydrate (Humphrey *et al.*, 1979; Abu *et al.*, 1991), there are few studies which have investigated the interaction between the protein and polysaccharide EPS components in mediating adhesion of bacteria to surfaces. The SDS-PAGE banding pattern suggests that many EPS_c proteins originate from processes related to cell lysis which occur during biofilm development. There is some slight evidence for the presence of specialized EPS matrix proteins. Collectively these proteins do not appear to perform the clear function of serving to mediate adhesion between the substratum and (structural) polysaccharides. However, the possibility remains that there may be particular proteins contained within this set which do serve this function.

The proteins contained within EPS_c do not readily displace the adhesive EPS_p polysaccharide fraction. In contrast, they adsorb either to unoccupied sites on the substratum or to the bound polysaccharides. This leads to the possibility that they may serve to pin these polysaccharides more tenaciously to the surface. This type of interaction has been observed between macromolecules adsorbing to substrata (Johnson & Granick, 1992).

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