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Polysaccharide-specific probes inhibit adhesion of *Hyphomonas rosenbergii* strain VP-6 to hydrophilic surfaces

SE Langille[1], GG Geesey[2] and RM Weiner[1]

[1]*Department of Cell Biology and Molecular Genetics, University of Maryland, College Park, MD 20742, USA;* [2]*Department of Microbiology and Center for Biofilm Engineering, Montana State University, Boseman, MT 59717-3980, USA*

Biofilm formation commences with the adhesion of microorganisms to surfaces. Information regarding the initial bond between a bacterium and a solid surface is essential for devising methods to inhibit the onset of biofilm formation. Three different types of polysaccharide-specific probes, cationic metals, dyes, and lectins, were used to bind the exopolysaccharide of *Hyphomonas rosenbergii*, a budding, prosthecate marine bacterium. Probes, which specifically bind complex carbohydrates, inhibit the adhesion of *H. rosenbergii* to hydrophilic surfaces. These results suggest that the polysaccharide portion of *H. rosenbergii* capsular, extracellular polymeric-substance is involved in the primary adhesion process. *Journal of Industrial Microbiology & Biotechnology* (2000) **25,** 81–85.

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Introduction

Efforts to deter biofilm formation at its onset have focused on inhibition of the initial stages of bacterial adhesion to surfaces. However, the exact mechanism by which microorganisms adhere to solid surfaces in aqueous environments has yet to be elucidated [4]. It is important to determine whether an extracellular protein, polysaccharide or other polymer mediates primary adhesion in order to develop colonization-resistant surfaces and methods for removing attached microorganisms.

The role of proteinaceous adhesions such as fimbrae and flagella in the initial adhesion of bacteria to surfaces has been well documented [8,10,18,19,24]. It is generally believed that extracellular polymeric substances (EPS), consisting of both protein and polysaccharide components, are responsible for permanent adhesion of bacteria to surfaces as biofilm cement. Far less is known about the role, if any, of the exopolysaccharide component of the EPS in mediating primary adhesion to surfaces.

Specific probes that bind polysaccharide and disrupt its function can be utilized to investigate the role of exopolysaccharide in adhesion to solid surfaces. Metallic cations can bind to anionic exopolysaccharide and become concentrated there [1,5,6,11,26]. Carbohydrate binding dyes such as Calcofluor and Congo Red have been used to screen for exopolysaccharide mutants and characterize the glycosidic linkages within the polymer [2,9,12,16,25]. Finally, lectins that bind specific glycosidic linkages have also been used to bind exopolysaccharides and block bacterial adhesion to surfaces [15,21]. Merker and Smit [15] demonstrated that wheat germ agglutinin inhibited adhesion of *Caulobacter crescentus* by binding the holdfast exopolysaccharide.

Quintero and Weiner [21] reported similar results using a lectin specific for the holdfast exopolysaccharide of *Hyphomonas adhaerens*.

Correspondence: Dr RM Weiner, MD, Department of Cell Biology and Molecular Genetics, University of Maryland, College Park, MD 20742, USA
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Hyphomonas is a genus of Gram-negative prosthecate, budding marine bacteria. It is a primary and ubiquitous colonizer of marine biofilms and may represent up to one third of the total biomass of some ecosystems [3,16,27]. *Hyphomonas* strain VP-6 was recently classified and speciated as *H. rosenbergii* on the basis of 16S rDNA analysis and other criteria [28]. The adherent stage has neither fimbriae nor flagella. *H. rosenbergii* synthesizes large quantities of EPS making it an excellent model organism for the study of exopolysaccharide-mediated adhesion.

Immunofluorescence and electron microscopy showed that *H. rosenbergii* produces two spatially and structurally distinct EPS: a polar holdfast EPS that is bound by coral tree lectin (CTL) and a second acidic, capsular EPS bound by sweet pea lectin (SPL) [14]. Unlike CTL, which labeled the cell at one pole, indicative of a holdfast, SPL labeled the entire biofilm matrix, which completely surrounded the cell. Adhesion assays verified that both EPS were adhesive, with cells initially tethering themselves to surfaces with the holdfast, and the capsular EPS later forming more permanent bonds [14].

This report details the use of polysaccharide-specific probes to investigate the role of EPS in the adhesion of *H. rosenbergii* to surfaces. Our results suggest that the exopolysaccharide component of *H. rosenbergii* EPS functions in the primary adhesion of cells to surfaces.

Materials and methods

Strains, culture conditions, and EPS purification

H. rosenbergii strain VP-6 was isolated from deep-sea water, sampled with a Niskin bottle, just above the hydrothermal vents at the Guaymas Basin, Gulf of Mexico (approx. 2000 m deep) by H. Jannasch and generously donated to our laboratory. All cultures were grown in marine broth 2216 (MB; Difco Laboratories, Detroit, MI, USA) at 25°C with aeration. Unless indicated, all chemicals were purchased from Fisher Scientific (Pittsburgh, PA, USA) or Sigma Chemical Company (St. Louis, MO, USA). EPS was purified from 1-week-old MB cultures grown on Teflon mesh

Table 1 Percentage of elements found in *H. rosenbergii* biofilms grown in the presence of platinic or cupric chlorides

Element	Control	Copper	Platinum
Aluminum	33	32	15
Phosphorus	17	21	9
Sulfur	4	7	5
Chlorine	1	9	29
Calcium	6	17	6
Iron	39	10	9
Copper	0	4	0
Platinum	0	0	27

The percentage of elemental metals found in control biofilms and those treated with cupric or platinic chlorides was determined by the area of each elemental peak.

inserted into the beakers. Biofilms were scrubbed from the mesh and blended in a solution of 10 mM EDTA and 3% NaCl. The EPS was precipitated with four volumes of ice-cold isopropanol, dialyzed against three changes of distilled water, and lyophilized. EPS was further purified by dissolving it in a minimum volume of 10 mM $MgCl_2$ and treating it with 100 μg/ml of DNAse and RNAse for 4.0 h at 37°C followed by 100 μg/ml of protease K overnight at 37°C. The enzyme-treated EPS was then phenol- and chloroform-extracted to remove protein and lipopolysaccharide, dialyzed against distilled water, and lyophilized.

Energy dispersive X-ray analysis (EDAX)

H. rosenbergii biofilms were grown on carbon planchets (EY Labs, San Mateo, CA, USA) at 25°C for 1 week. The biofilms were treated with 2.5 mM solutions of either platinic or cupric chloride dissolved in 10 mM $CaCl_2$ buffer (pH 4.0). Control biofilms were incubated in buffer alone. After 30 min, the biofilms were fixed with 1% glutaraldehyde and washed three times in $CaCl_2$ buffer. Planchets were dehydrated by subsequent 10-min incubations in a graded series of ethanol solutions (75%, 90%, 100%, 100% and 100%). The planchets were then critical-point dried in a Denton DCP-1 critical point drying apparatus (Denton Vacuum, Moorestown, NJ, USA) and subjected to energy dispersive spectrometry (EDS, EDAX Int. Spectrophotometer, KLA-Tenor/Amray, Bedford, MA, USA., 1820D 20 kV). Biofilms were counted for 300 s to determine elemental metal concentrations.

Coverslip adhesion assays

Mid-logarithmic phase cultures of *H. rosenbergii* were incubated for 30 min with each dye, metal, or lectin. Cupric and platinic chlorides were added to the medium at a final concentration of 0.25, 2.5 or 25 mM. Calcofluor, Congo Red and Toluidine Blue O were added at concentrations of 75 μg/ml, 100 μg/ml and 80 μg/ml respectively. CTL, SPL, and peanut lectin (PNL) were added at a concentration of 100 μg/ml. Monosaccharides were added at a concentration of 10.0 mg/ml of culture. Glass coverslips were precoated with sterile MB for 5 min to create a conditioning film. Following incubation with each probe, 100 μl of *H. rosenbergii* cultures were layered on the coverslips and incubated in a humid chamber at 25°C for 60 min. The coverslips were washed with 10 ml of artificial seawater (23.0 g NaCl, 0.24 g Na_2CO_3, 0.33 g KCl, 4.0 g $MgCl_2 \cdot 6H_2O$, and 0.66 g $CaCl_2 \cdot H_2O$/l distilled water) eluted in a continuous stream from a 10-ml pipette to remove unattached cells. Coverslips were inverted on to glass slides and viewed with a Zeiss Axiophot microscope at 1250× magnification. The number of cells attached to 6400-μm^2 areas was counted. Ten separate areas were counted on three different coverslips per experiment for a sample size of 30. The number of cells attached to each group of coverslips was divided by the average number of untreated (control) cells attached to coverslips in order to calculate the adhesion percentage and standard error.

Electrophoretic separation and staining of EPS

One hundred micrograms dry weight of partially purified *H. rosenbergii* wild type EPS was loaded on to 7.5% polyacrylamide minigels and run at 30 mA/gel for 1.5 h. The gels were cut and stained with either Toluidine Blue O (0.08% in 7% acetic acid), Congo Red (0.01%), or Calcofluor (0.01%) for 1 h. Gels were destained with distilled water.

Labeling of EPS with sweet pea lectin

Mid-logarithmic phase *H. rosenbergii* cells were washed once with sterile PBS then incubated with 50 μg/ml of FITC conjugated SPL for 60 min at room temperature. The cells were then washed three times in sterile PBS, observed and photographed with a Zeiss Axiophot microscope using incident light fluorescence microscopy (60× objective, numerical aperture 1.32).

Results

To determine whether *H. rosenbergii* biofilms sequestered metallic cations, cells were grown in the presence of either platinic or cupric chloride and subjected to EDS analysis. Table 1 shows that *H. rosenbergii* biofilms sequestered both platinic and cupric chloride comprising 9% and 4%, respectively, of the elemental weight of *H.*

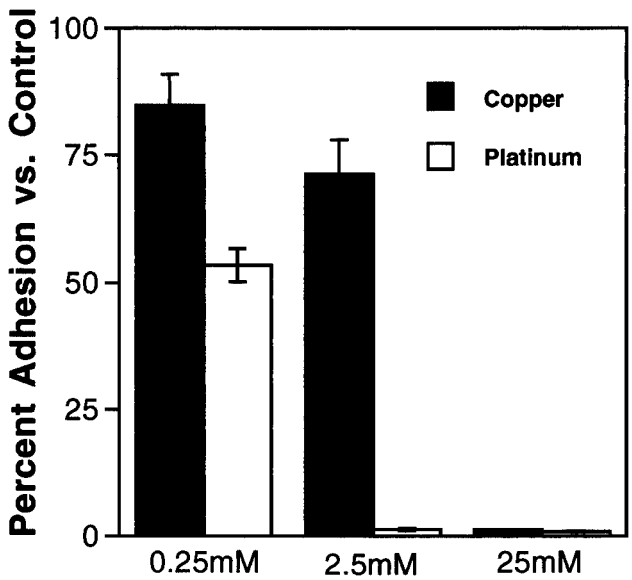

Figure 1 Adhesion inhibition of *H. rosenbergii* by cationic metals. *H. rosenbergii* was treated with 0.25, 2.5 or 25 mM concentrations of either cupric or platinic chlorides for 30 min then layered on glass coverslips. Control cells were treated with artificial seawater. The percent adhesion was determined by dividing numbers of attached bacteria by the number of untreated control attached bacteria. Bars represent the standard deviations.

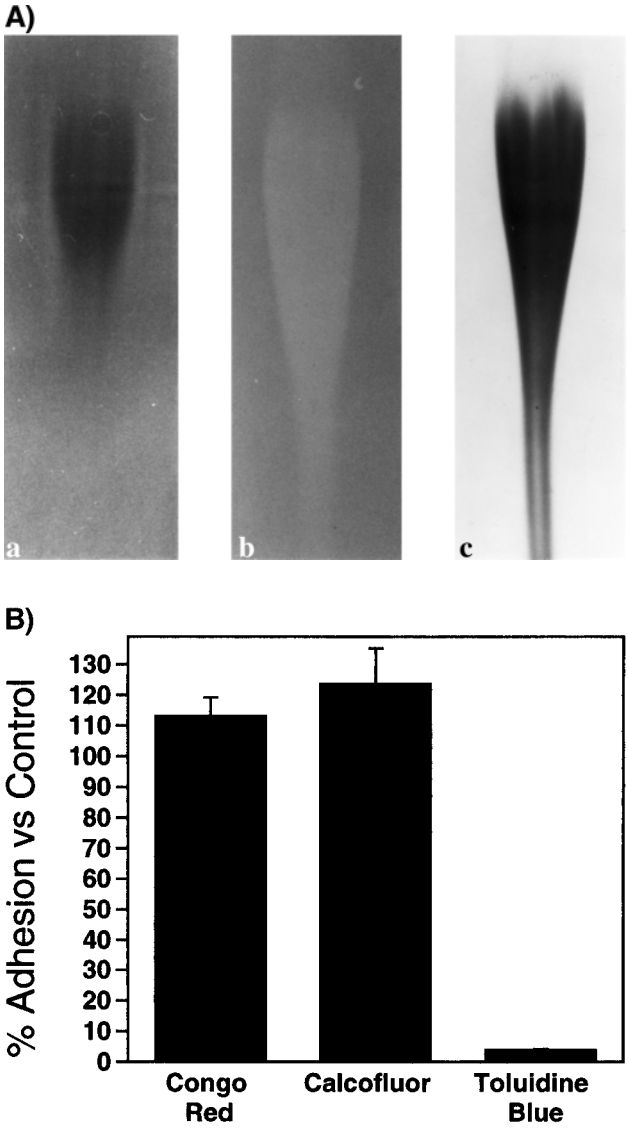

Figure 2 (A) Adhesion inhibition with carbohydrate-specific dyes. One hundred micrograms dry weight of partially purified VP-6 EPS was separated on 7.5% SDS-PAGE gels and stained with either (a) Calcofluor (0.01% wt/vol), (b) Congo Red (0.01%) or (c) Toluidine Blue O (0.08%). Toluidine Blue positively stained the partially purified EPS while Congo Red and Calcofluor were retained within the gel but did not visibly bind this EPS. (B) Cells were incubated with either Calcofluor (75 μg/ml), Congo Red (100 μg/ml) or Toluidine Blue O (80 μg/ml), layered on glass coverslips and subjected to the coverslip adhesion assay. Percent adhesion was determined by dividing the number of attached, dye-treated cells by the average number of attached, untreated control cells. Bars represent the standard deviations.

rosenbergii biofilms. To determine whether these metals interfere with the adhesion of the cells to surfaces, cells were pretreated with three different concentrations of platinic or cupric chloride and assayed for adhesion. As shown in Figure 1, the percentage of adhesion inhibition by these metals was dependent on their concentration. Interestingly, platinic chloride, which was complexed with a higher affinity by *H. rosenbergii* biofilms than was cupric chloride (as determined by EDS analysis) also inhibited adhesion at a far lower ambient concentration than cupric chloride.

Due to its negative charge, *H. rosenbergii* EPS can be run on SDS-PAGE gels and stained with carbohydrate-specific dyes. This is an effective method for ensuring the purity and homogeneity of the exopolysaccharide and also eliminates the risk of nonspecific binding between the dyes and putative exopolysaccharide contaminants (e.g., LPS and protein). We used this approach to correlate the affinity of three different dyes for *H. rosenbergii* exopolysaccharide with the ability of these dyes to inhibit adhesion. Congo Red and Calcofluor did not stain *H. rosenbergii* exopolysaccharide separated on SDS-PAGE gels (Figure 2A) and were ineffective at inhibiting cellular adhesion to surfaces (Figure 2B). Conversely, Toluidine Blue, positively stained *H. rosenbergii* exopolysaccharide (Figure 2A) and

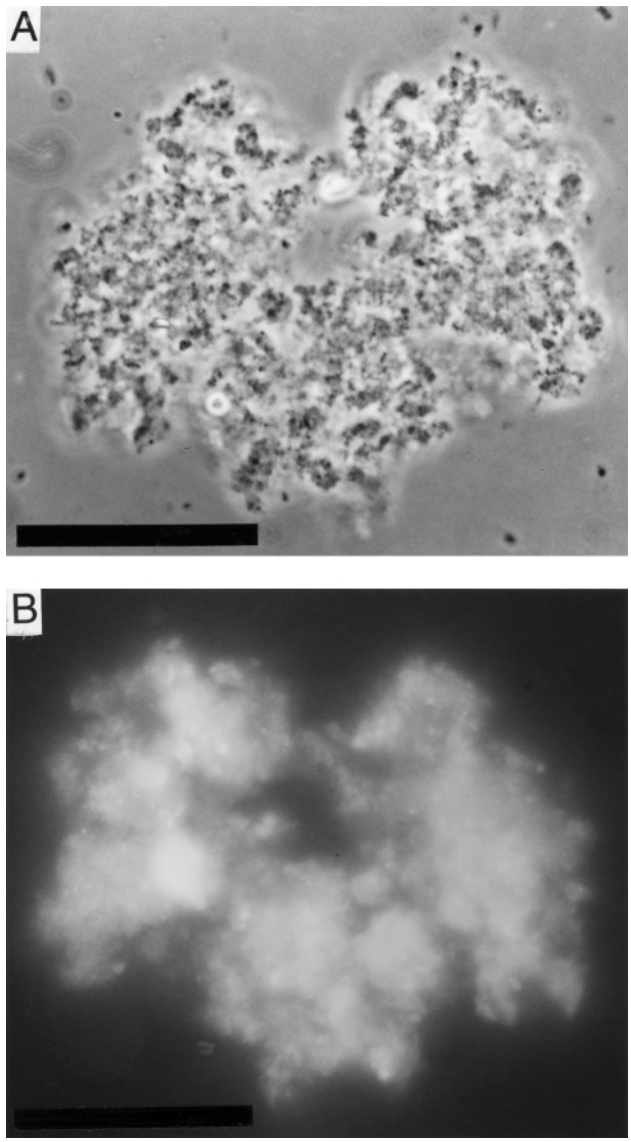

Figure 3 *H. rosenbergii* labeled with FITC conjugated SPL. Cells were harvested during middle logarithmic phase and washed once in sterile PBS. Cells were then resuspended in PBS along with 50 μg/ml of FITC-labeled SPL. (A) Phase contrast image of VP-6 labeled with FITC conjugated SPL and (B) the same cells viewed with incident light fluorescence. 1250× magnification. Bar = ~50 μm.

Table 2 Percent adhesion of *H. rosenbergii* cells after treatment with lectins

Lectin[a]	Treatment[b]	% Adhesion vs. control[c]	Significance[d]
Peanut	none	83.2	−
CTL	none	54.4	+
	mannose	64.1	+
	N-acetyl galactosamine	85.1	−
SPL	none	15.5	+
	N-acetyl galactosamine	13.1	+
	mannose	47.7	+

[a]Lectins were added at a concentration of 100 μg/ml for 30 min.
[b]Monosaccharides were added at a concentration of 10 mg/ml.
[c]The average number of cells attached to 6400 μm^2 areas for each experimental sample was divided by the average number of untreated control cells attached to the same area. The average number of control cells per 6400 μm^2 area ranged from 25 to 53. A sample size of 30 was used for each experimental and control sample.
[d]The paired Student's *t*-test was used to determine the significance of each experimental sample. (+) $p<0.05$; (−), $p>0.05$.

inhibited adhesion by >95% (Figure 2B). Thus, the ability of these dyes to bind purified EPS coincided with their ability to inhibit adhesion.

Two lectins, SPL and CTL, known to bind *H. rosenbergii* EPS at different cellular locations, were tested for their ability to block adhesion. SPL bound a large part of the surface of *H. rosenbergii* as evidenced in part by incident light fluorescence microscopy (Figure 3). In contrast, CTL bound only the holdfast. Adhesion assays of cells treated with both SPL and CTL demonstrated that both the holdfast and capsular exopolysaccharides are involved in adhesion. Treatment of cells with 50 μg/ml of SPL or CTL inhibited adhesion by approximately 85% and 46% respectively (Table 2). Cells treated with PNL, which binds neither *H. rosenbergii* exopolysaccharide as determined by immunofluorescence microscopy (data not shown), adhered as well as untreated cells. SPL-mediated adhesion inhibition was partially neutralized by the addition of mannose, postulated to compete with *H. rosenbergii* exopolysaccharide for the SPL carbohydrate-binding site. *N*-acetyl galactosamine had no effect on SPL mediated adhesion inhibition. Conversely, *N*-acetyl galactosamine, the monosaccharide target of CTL, neutralized CTL-mediated adhesion inhibition while mannose had little effect.

Discussion

This paper describes the use of polysaccharide-specific probes and a simple cell-adhesion assay to demonstrate the role of polysaccharide adhesins in the initial colonization of surfaces by marine bacteria. Cationic metals were used to investigate the role of *H. rosenbergii* exopolysaccharides in adhesion because previous studies showed that marine biofilms sequester metal ions, presumably through interactions of cationic metal ions with anionic exopolysaccharide [1,5,6,11,26]. Metallic cations bind to electron-donating groups such as carboxyl moieties in the form of succinate, formate, pyruvate, and acetate groups [11]. Oxygen atoms of hydroxyl groups are weaker electron donors but still capable of binding metallic cations [11]. Most binding occurs through an ion-exchange process in which water molecules are displaced on the metal or organic compound for the opposite charge [7]. One example of this cation–polysaccharide interaction is that of Ca^{2+} and the glucuronate residues of alginate, which promotes gelling of the polymer [5].

Railkin *et al.* [23] showed that Co^{2+} and Ni^{2+}, when bound to exopolysaccharide, inhibit cell adhesion to glass surfaces. It was proposed that both of these metals occupied sites used by Ca^{2+} and Mg^{2+} to promote cell adhesion. Our data show that platinum and copper cations, which are sequestered by *H. rosenbergii* biofilms, actively block adhesion of the cells to glass surfaces. The binding of these cations to electron-donating groups on the exopolysaccharide may displace cations such as Ca^{2+} and Mg^{2+}. The replacement of naturally occurring cations with platinum and copper may adversely affect salt bridges used to maintain the tertiary structure of the EPS or overcome electrostatic repulsion between the negatively charged bacterium and the surface.

The only dye that positively stained *H. rosenbergii* exopolysaccharide and blocked adhesion was Toluidine Blue. Calcofluor, which binds β-1 and 4β-1,3 glycosidic linkages, [13], and Congo Red, which binds $\alpha(1-4)$ glycosidic linkages, basic, and neutral polysaccharides [26], were ineffective at either staining *H. rosenbergii* exopolysaccharide or inhibiting adhesion. The specificity of Toluidine Blue is not known but it is a positively charged and planar molecule that may bind negatively charged or helical exopolysaccharides [17]. *H. rosenbergii* exopolysaccharide is negatively charged as determined by its electrophoretic mobility [22]. To our knowledge, there have been no other reports of carbohydrate specific dyes inhibiting the adhesion of bacteria to solid substrata.

Work in our laboratory has previously shown that plant lectins specifically and fastidiously bind *Hyphomonas* exopolysaccharide [14,21] and block adhesion of *H. adhaerens* to surfaces [21]. In this study, we used both the CTL, which binds holdfast exopolysaccharide, and the SPL, which binds capsular exopolysaccharide, to inhibit the adhesion of *H. rosenbergii* to surfaces. Interestingly, both of these lectins, as well as the *Glycine max, Malcura pomifera, Arachis hypogea* and *Bauhinia purpuria* lectins which bind *H. adhaerens* exopolysaccharide [20], are specific for N-acetylated amino sugars. N-acetylated amino sugars have been found in the exopolysaccharide of several other adhesive bacteria such as *Rhizobium japonicum* and 14 different species of *Caulobacter*. Our data support the notion that amino sugars may be general components of adhesive polysaccharides [22]. In any case, those agents that bound exopolysaccharide also inhibited cell adhesion to inanimate substrates. This correlation strongly implicates the role of exopolysaccharide not only as biofilm cement, but also as a nonspecific adhesin.

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