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# Physical and chemical characterization of the polysaccharide capsule of the marine bacterium, *Hyphomonas* strain MHS-3

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***Hyphomonas* MHS-3 is a biphasic, marine bacterium that synthesizes an exopolysaccharide (EPS) capsule, which has a role in attaching the adherent, prosthecate developmental stages to solid substrata. To correlate structure with function, we characterized this integral EPS. It has a relatively homogeneous molecular weight of approximately 60 000 daltons, is acidic, and putatively contains large concentrations of *N*-acetylgalactosamine (GalNAc). The theoretical identity of the anionic component of the polymer, and the similarities between *Hyphomonas* MHS-3 EPS and other adhesive marine/aquatic bacterial EPS are discussed.**

**Keywords:** polysaccharides; bacterial capsule; adhesion; biofilm

## Introduction

The mechanism of bacterial adhesion to marine surfaces has not yet been fully elucidated. Previously, we reported evidence that a marine bacterium, *Hyphomonas* MHS-3 (MHS-3), adheres via an exopolysaccharide (EPS) capsule, which is produced in copious amounts. This paper reports on its structure and discusses how it may contribute to adhesiveness.

Hyphomonads have a biphasic life cycle, one phase being sessile, and the other flagellated and free-swimming (swarmers) [22]. *Hyphomonas* was chosen for this study, in part, because it is a primary colonizer of marine surfaces [3]. Only the sessile phase synthesizes the adhesive EPS capsule which also forms a large part of the biofilm matrix (Quintero and Weiner, submitted for publication). Multiple layers of cells become imbedded in this hydrated matrix [8]. In fact, more than 80% of the marine bacteria associated with deep sea aggregates possess EPS capsules, which are thought to be responsible for attachment to and formation of particular aggregates in the water column [11].

In addition to their putative role in adhesion, EPS biofilm matrices serve several different functions which enhance survival and influence the surrounding environment. They retain extracellular enzymes near the cell [32], protect against desiccation [5,7], and enhance virulence of pathogenic bacteria [10,23,25]. Some biofilms also cue marine invertebrate larval settlement and metamorphosis, which helps to establish a thriving community [16,31,37,38]. EPS are instrumental in metal binding [6], and generally protect cells from toxic compounds [9]. Finally, it has been demonstrated that EPS is critical in the second step of bacterial adhesion, that is, the irreversible attachment or cementation of the cells to surfaces [9,18].

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## Materials and methods

### *Bacterial strains, media and chemicals*

Wild-type *Hyphomonas* strain MHS-3 (MHS-3) was isolated from shallow water sediments in Puget Sound, WA, USA by J Smit, and kindly given to R Weiner. Reduced adhesion (MHS-3 rad) phase variants were isolated by their different colony morphology on agar plates (named for their low adhesion to surfaces and less biofilm formation in broth cultures). These strains were cultured in Marine Broth 2216 (MB; [41]) (37.4 g $L^{-1}$; Difco Laboratories, Detroit, MI, USA). Except where noted, *Hyphomonas* MHS-3 was always grown at 25° C. Marine agar (MA) was prepared by adding agar to Marine broth, to a final concentration of 2% w/v. Except when indicated otherwise, all chemicals were purchased from Sigma Chemical Co (St Louis, MO, USA) or Fisher Scientific (Pittsburgh, PA, USA).

### *EPS purification*

Teflon mesh was introduced into the culture vessels to provide more surface for biofilm formation (mesh opening 1.8 mm, thread diameter 0.5 mm; Tetko Inc, Briarcliff, NY, USA). Briefly, EPS was purified as follows: cultures were grown to early stationary phase, the spent medium was discarded, and the flocs and the biofilm (mechanically removed from culture vessel walls and from teflon mesh) were centrifuged at 16 000 × *g* for 20 min. The supernatant fluid was precipitated with four 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 to shear off the capsular EPS. The suspension was again centrifuged, the cell pellet discarded and the sheared EPS in the supernatant phase was precipitated with 2-propanol as described above. The precipitate was resuspended in minimum volume of $dH_2O$ and dialyzed exhaustively against $dH_2O$, and lyophilized. The following steps are a modification of the purification procedure of Read and Costerton [29]. The crude EPS 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 $ml^{-1}$ each, and incubated at 37° C for 4 h. Protease K was added to a final concentration of 0.1 mg $ml^{-1}$ and

the mixture was incubated at 37° C overnight. The residual protein was removed by hot phenol extraction, followed by a chloroform extraction. This was repeated as necessary until no protein was detected by the BCA assay (see colorimetric and enzymatic assays). The EPS solution was dialyzed exhaustively against $dH_2O$ and lyophilized. This partially purified EPS was redissolved in $dH_2O$, and further purified by gel permeation chromatography to separate the capsular EPS from contaminating lipopolysaccharide.

### Column chromatography

Solutions (1 mg $ml^{-1}$) of EPS were chromatographed on a column (45 × 1.5 cm) of Sephacryl S-400-HR gel permeation resin (Pharmacia, Piscataway, NJ, USA) using 50 mM ammonium acetate, pH 7.0 (containing 0.02% $NaN_3$) as the elution buffer, at a flow rate of 0.5 ml $min^{-1}$. Fractions of 3 ml were collected and analyzed for total carbohydrate by the colorimetric assay of Dubois *et al* [12]. Solutions (1 mg $ml^{-1}$) of dextran molecular weight standards of 5 000 000, 2 000 000, 500 000, 70 000, 40 000, and 10 000 daltons (Pharmacia; Sigma Chemical Co) were used to standardize the column. Ion-exchange chromatography was performed using a Mono Q anion exchange column (quaternary amine column) (Pharmacia) in a Pharmacia LKB (Piscataway, NJ, USA) FPLC system, comprised of a LCC-500 plus controller module, two P-500 pumps and a FRAC-200 fraction collector. EPS solutions (500 $\mu$l of 2 mg $ml^{-1}$ solutions) in 20 mM Tris buffer, pH 8.0, were loaded into the column. Samples were first eluted with 5 ml of Tris buffer, then with a linear gradient of NaCl from 0.05 to 1.0 M (in buffer) at a flow rate of 1 ml $min^{-1}$. Fractions of 1 ml were collected and analyzed for total carbohydrate.

### Colorimetric and enzymatic assays

All chemicals utilized to prepare reagents for these tests were high purity or reagent grade, and all glassware was acid-washed prior to use. The carbohydrate (CHO) assay of Dubois *et al* [12] was used to measure the neutral hexose content of samples. Glucose standards (10–100 $\mu$g $ml^{-1}$) were used to prepare the standard curve. Total protein was measured using the Pierce BCA (bicinchoninic acid) protein reagent (Pierce, Rockford, IL, USA), using reagents and protocols supplied by the manufacturer. Standards (10–100 $\mu$g $ml^{-1}$) were prepared with bovine serum albumin (BSA). Uronic acids were quantified following the procedure of Blumenkrantz and Asboe-Hansen [4]. The metaphenylphenol reagent was purchased from Eastman Kodak Co (Rochester, NY, USA).

The presence of acetyl groups on the EPS was assayed using a modified colorimetric procedure [14,20]. Just prior to the test, a working reagent was prepared by mixing equal volumes of 8.0 M hydroxylamine hydrochloride and glycine reagent (1.0 M glycine in 8.5 M NaOH); 200 $\mu$l of test sample were mixed with 400 $\mu$l of this reagent in a large glass test tube and incubated at room temperature for 3 h. Then, 2.5 ml of 1.0 M HCl and 6 ml of ferric chloride reagent (0.1 M $FeCl_3$ in 0.01 M HCl) were added, and the absorbance was measured immediately at 540 nm ($A_{540}$) before precipitate formed.

Pyruvylation of the EPS was tested using an enzymatic assay developed by Duckworth and Yaphe [13]. Briefly, 1.5 ml of 0.08 N oxalic acid was added to 5 mg of EPS (in 1.5 ml $dH_2O$) and refluxed for 5 h at 100° C to hydrolyse pyruvate from EPS backbone. After cooling, 230 mg of calcium carbonate were added to each tube to neutralize the solution, and the amount of free pyruvate present was assayed using lactate dehydrogenase (Sigma Chemical Co), following the manufacturer's specifications. The presence of lipopolysaccharide (LPS) was determined using the *Limulus* Amebocyte Lysate (LAL) assay (Associates of Cape Cod, Inc, Woods Hole, MA, USA) according to manufacturer's instructions. LPS was also identified using polyacrylamide gel electrophoresis with sodium dodecyl sulfate (SDS-PAGE), and the silver staining procedure of Tsai and Frasch [33], as modified by Hitchcock and Brown [15] to visualize it.

### Monosaccharide analysis of EPS

High performance anion exchange chromatography (HPAE) was used to identify the major monosaccharide components of the EPS, following a protocol described by Reddy *et al* [30]. Briefly, 200 $\mu$g of purified EPS were hydrolysed in 200 $\mu$l of 2 N HCl at 100° C for 2 h. Samples were dried under a stream of nitrogen and resuspended in 200 $\mu$l of $dH_20$; the injection volume was 20 $\mu$l. The system used for HPAE consisted of a Dionex BioLC gradient pump (Dionex Corp, Sunnyvale, CA, USA) with a pulsed amperometric detector (PAD). A Carbopac PA1 (4 × 250 mm) pellicular anion exchange column (Dionex Corp) with a Carbopac guard column was used at a flow rate of 1 ml $min^{-1}$ at room temperature. Two different eluants (degassed with helium) were used: eluant 1, 15 mM NaOH, useful for the analysis of neutral and amino sugars, and eluant 2, 100 mM NaOH, 150 mM sodium acetate, effective in the analysis of acidic monosaccharides. A monosaccharide standard solution (85 $\mu$g $ml^{-1}$ of each sugar, 20 $\mu$l injected) was run after each hydrolysed EPS sample to identify the monomers.

### Infrared spectroscopy (IR)

IR analysis was carried out to test for the presence of sulfate groups in the EPS [19]. One milligram of EPS was mixed with 100 mg of potassium bromide (IR grade), and ground with mortar and pestle. About half the fine powder was placed into a die and compressed into a translucent pellet. The pellet was placed into a Perkin Elmer 1600 Series FTIR (Perkin-Elmer Co, Norwalk, CT, USA), and a spectrum was obtained. Chondroitin sulfate was used as a standard.

## Results

### Chemical characterization of MHS-3 EPS

The partially purified capsular EPS (treated with nucleases, protease, hot phenol and chloroform extraction) was contaminated with small amounts (<2%) of LPS; however, as noted in the materials and methods section, no protein was detected (lower limit, 5 $\mu$g $mg^{-1}$ EPS). Anion exchange chromatography revealed that both the LPS and the EPS were negatively charged (Figure 1). The capsular EPS sharply eluted at 0.25 M NaCl; the LPS broadly eluted from

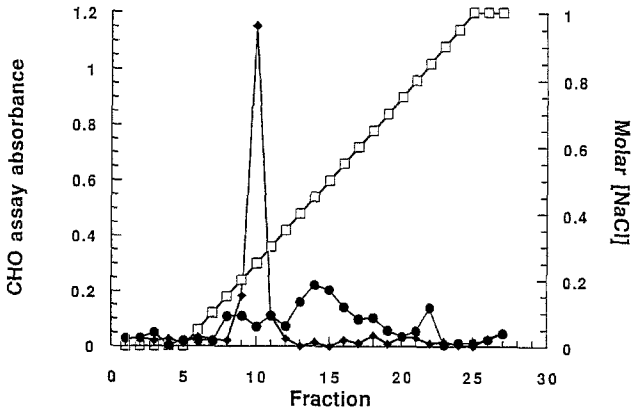


**Figure 1** Anion exchange chromatography of partially purified *Hyphomonas* MHS-3 LPS and capsular EPS. The samples were chromatographed in a Mono Q quaternary amine column. The capsular EPS eluted with 0.25 M NaCl. LPS eluted broadly, between 0.15 M and 0.8 M NaCl, with a major fraction which eluted at 0.4 M NaCl. —◆— Capsular EPS; —●— LPS; —□— molar (NaCl)

0.15 M to about 0.8 M NaCl. However, this procedure was not useful for purification, since both species coeluted.

In contrast, gel permeation chromatography did separate EPS and LPS (Figure 2). The separation was facilitated because the LPS aggregated into micelles and bilayer vesicles [42]. LPS aggregates eluted with an average molecular weight of approximately 2 000 000 daltons, and the EPS was calculated to have an average molecular weight of approximately 60 000 daltons (Figure 2). LPS was identified in silver-stained SDS-PAGE gels and by the results of the LAL assay (data not shown).

The purified capsular EPS was characterized using colorimetric and enzymatic assays. No LPS was detected (LAL assay, lower limit 6 pg LPS per mg EPS) after final gel permeation chromatography; nor was it detected in LPS-silver-stained gels (negative data not shown). The polymer

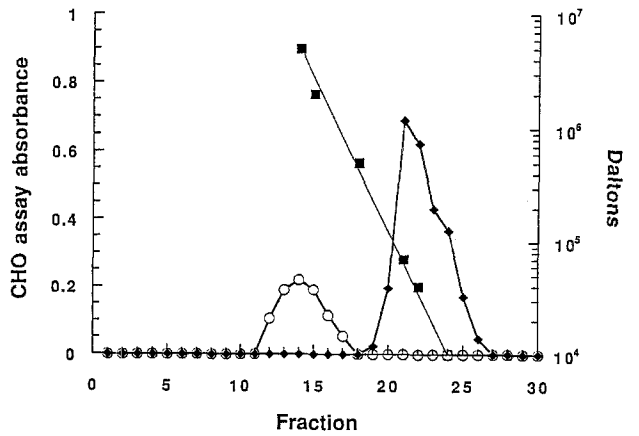


**Figure 2** Gel permeation chromatography of partially purified *Hyphomonas* MHS-3 LPS and capsular EPS. The medium was Sephacryl S-400-HR resin. The elution profile of the dextran molecular weight standards is indicated, and the average molecular weights can be read on the *y*-axis. The peak of capsular EPS elution centered around fraction #21, indicating that the average molecular weight of the EPS is 60 000 daltons. LPS usually aggregates into micelles in aqueous solution. This is reflected in its elution pattern at 2 000 000 daltons MW. Its true molecular weight is much lower, as indicated by SDS-PAGE. —○— LPS; —◆— capsular EPS; —■— molecular weight

**Table 1** Chemical characterization of *Hyphomonas* MHS-3 capsular EPS

| Group | Quantity ($\mu$g mg$^{-1}$ EPS)[a] | Assay reference |
|---|---|---|
| Neutral hexose | 710 | [12] |
| Uronic acid | ND[b] | [4] |
| Acetyl groups | 75 | [14,22] |
| Pyruvate | ND | [13] |
| Sulfate | ND | [21] |

[a]Amount detected per mg dry weight of purified EPS
[b]Not detected (<5 $\mu$g mg$^{-1}$)

was acetylated, but neither uronic acids nor pyruvate were detected (Table 1). However, the absence of uronic acid and pyruvate groups could not be confirmed by IR due to 'water noise' between the 1400–1900 cm$^{-1}$ frequencies. The absence of an absorbance peak at 1250 cm$^{-1}$ (Figure 3) suggested that the EPS does not have sulfate groups. On the other hand, an absorbance peak at 1654 cm$^{-1}$ confirmed the presence of acetyl groups. This absorbance frequency is in the area where the carbonyl stretching band of amides is found, characteristic of acetamido groups in *N*-acetylated sugars. In fact, most amino sugars found in microbial EPS are usually *N*-acetylated [8].

The High Performance Anion Exchange Chromatography analysis of the hydrolysed EPS revealed that the major monosaccharide component of the polymer is galactosamine (Figure 4a). Again, no uronic acids were detected (Figure 4b). Several minor unknown peaks (considered degradation products of acid-labile sugars) and a glucose peak were found in the neutral sugar chromatogram (Figure 4a), but the areas under these peaks were so small as to suggest they were contaminants.

## Discussion

Chemical characterization of the capsular EPS, synthesized by MHS-3, indicates that it contains *N*-acetyl-galactosamine. HPAE data confirms galactosamine as a major component, and the colorimetric assay for acetyl groups is posi-

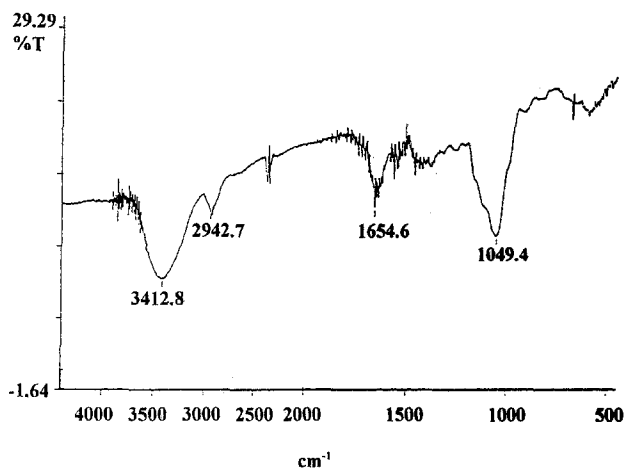


**Figure 3** IR spectrum of *Hyphomonas* MHS-3 capsular EPS. The absorbance peak at a frequency of 3413 cm$^{-1}$ is attributed to OH groups, at 1049 cm$^{-1}$ to C—O, and at 1654 cm$^{-1}$ to C=O in acetamido groups

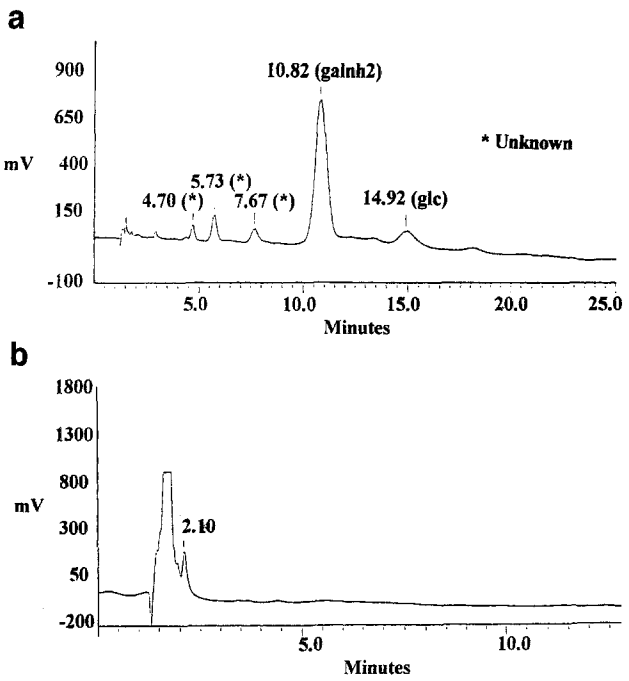


**Figure 4** HPAE chromatograms of hydrolysed purified *Hyphomonas* MHS-3 capsular EPS. Two eluants were used: eluant 1 (a), 15 mM NaOH, was used for the analysis of neutral and amino sugars. Galactosamine (galnh2) was the only major monosaccharide component detected. Unknown peaks and glucose (glc) were found in trace amounts and deemed to be contaminants. Eluant 2 (b) (100 mM NaOH, 150 mM sodium acetate) was used in the analysis of acidic monosaccharides; none was detected

tive. Finally, IR analysis reveals the presence of amide groups in the MHS-3 EPS, which implies the presence of acetamido groups (*N*-acetyl linkage).

*Hyphomonas* MHS-3 EPS is acidic, since it was retained in anion exchange columns, and because it binds polycationic ferritin (data not shown). Nonetheless, the identity of the negative charge still remains to be elucidated. Sulfate and pyruvate groups were not detected. Less commonly appearing acidic groups such as succinate (found in succinoglucans, [17]) and phosphate [24] were not screened. However, though uronic acids were not detected by colorimetric assay nor HPAE analysis, their presence cannot be ruled out. This is because aminuronic acids (amino sugars that are also uronic acids) are not detected by the existent colorimetric assays for uronic acids ([27]; E Rosenberg, personal communication). Also, since these sugars are acid-labile [27], they would have been destroyed during the acid hydrolysis of the EPS, and thus, they would not have been detected by HPAE either.

Therefore, the *Hyphomonas* MHS-3 EPS could conceivably contain *N*-acetylgalactosaminuronic acid. So far, only two bacterial polysaccharides have been reported to contain this sugar, the *O*-antigen polysaccharide of *Pseudomonas aeruginosa* LPS [40], and the major acidic EPS of *Pseudomonas solanacearum* [27]. *P. solanacearum* EPS and *Hyphomonas* MHS-3 EPS may share some features. *P. solanacearum* EPS is a heteropolymer composed of equimolar amounts of *N*-acetylgalactosamine (GalNAc), 2-*N*-acetyl-2-deoxy-L-galacturonic acid (*N*-acetyl-galactosaminuronic acid) (GalANAc), and the bacillosamine derivative 2-*N*-acetyl-4-*N*-(3-hydroxybutanoyl)-2,4,6-tri-deoxy-D-glucose (Bac2NAc4N(30HBut)) [27]. Interestingly, early characterization of *P. solanacearum* EPS revealed *N*-acetyl-galactosamine as the only component [1]. An acid-resistant glycosidic linkage between two acid-labile sugars could explain the fact that neither GalANAc nor Bac2NAc4N(30HBut) had initially been detected [27]. *Hyphomonas* MHS-3 capsular EPS could well have a similar structure, which would pose the same analytical problems.

The average molecular weight of *Hyphomonas* MHS-3 EPS is approximately 60 000 daltons. *P. aeruginosa* has been reported to produce a low molecular weight EPS (chemically distinct from the high molecular weight alginate also synthesized) only when growing in a biofilm [2]. The low molecular weight EPS is around 10 000 daltons, and contains mannose, galactose and an unidentified amino sugar. It has been proposed to be involved in the attachment of *P. aeruginosa* to surfaces [2]. *Staphylococcus aureus* also synthesizes a low molecular weight EPS (30 000 daltons) [26].

In the prosthecate genera *Caulobacter* and *Asticcacaulis*, attachment to surfaces appears to be mediated by a polar, acidic EPS organelle termed holdfast [28,36]. It is interesting that the *Hyphomonas* MHS-3 capsular EPS also shares certain commonalities with these adhesive EPS. The *Hyphomonas* MHS-3 EPS and those of *Caulobacter* spp [28] and *Asticcacaulis biprosthecum* [36] are all acidic exopolysaccharides. Furthermore, as we suggest above, *Hyphomonas* MHS-3 EPS contains the amino sugar, *N*-acetylgalactosamine. During lectin analysis of the adhesive holdfast of 26 different species of *Caulobacter*, 15 out of 16 marine species and 6 out of 10 freshwater species contained the amino sugar *N*-acetylglucosamine [21]. Additionally, the acidic, polar slime EPS synthesized by *Rhizobium japonicum* is bound by *Glycine max* lectin (SBA) [34,35]. This lectin binds polymers containing *N*-acetylgalactosamine. It is conceivable that the presence of acidic groups and amino sugars may be characteristic of a class of adhesive EPS.

The *Hyphomonas* MHS-3 capsular EPS appears to be an integral EPS, since it is not released into the culture medium. This is consistent with several general characteristics of adhesive EPS [8], since enhanced solubility should work against adhesion. Furthermore, adhesive properties are probably dependent on the conformational state of the polymer, since the arrangement of functional groups may change at different conformations. *Pseudomonas* sp strain S9 synthesizes two forms of EPS, one integral and the other peripheral [39]. The integral EPS was associated with cell adhesion to surfaces, and the peripheral EPS with cell desorption from the surface during starvation.

Thus, it is possible that in *Hyphomonas* MHS-3, structure follows function, and that the EPS capsule may be a primary adhesive, in addition to its role in the formation of biofilm.

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