ARTICLE

BIOTECHNOLOGY and BIOENGINEERING

Yield and Physicochemical Properties of EPS From *Halomonas* sp. Strain TG39 Identifies a Role for Protein and Anionic Residues (Sulfate and Phosphate) in Emulsification of *n*-Hexadecane

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ABSTRACT: In this study, we investigated the yield and physicochemical properties of the high molecular weight extracellular polymeric substance (HMW–EPS) produced by *Halomonas* sp. strain TG39 when grown on different types and ratios of substrates. Glucose (1% w/v) and a peptone/yeast extract ratio of 5.1 (0.6% w/v final concentration) yielded an EPS fraction (HMW-glucose) exhibiting the highest anionic activity (20.5) and specific emulsifying activity ($EI_{24} = 100\%$) compared to EPS produced by cells grown on mannitol, sucrose, malt extract or no carbon source. The HMW–EPS fractions were capable of binding ≈255–464 mg of methylene blue (MB) per gram of EPS, which represents the highest reported binding of MB by a bacterial EPS. A comparative evaluation of these properties to those of commercial hydrocolloids indicated that the combined effect of protein and anionic residues of the HMW–EPS contributed to its ability to emulsify *n*-hexadecane. Liquid chromatography revealed the HMW-glucose EPS to be a heterogeneous polymer with a polydispersity index of 1.8. This work presents evidence of a correlation between the anionic nature and protein content of bacterial EPS with its emulsifying qualities, and identifies EPS produced by strain TG39 as a high MB-binding bacterial sorbant with potential biotechnological application.

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KEYWORDS: exopolysaccharides (EPS); *Halomonas*; anionic residues; methylene blue; emulsification; hexadecane

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Introduction

Microbial exopolysaccharides (EPS) are high molecular-weight polysaccharides excreted by many bacteria. Their diverse physicochemical properties has seen their use in many commercial applications, such as to stabilize, thicken, gel, coagulate, and promote adhesion and water retention (Sutherland, 1998). Those with amphipathic properties are of particular interest because they can interface between hydrophobic and hydrophilic surfaces, as in the mixing of oil and water (Rosenberg, 1993; Shepherd et al., 1995). Products derived from microbial EPS are considered advantageous compared to their synthetically produced counterparts because of generally lower associated levels of toxicity, higher degradability, and greater structural and functional diversity (Banat et al., 2000).

In recent times, the marine environment has been recognized as a rich and largely untapped source of microbial EPS that can be harnessed and developed for potential biotechnological applications (Mancuso Nichols et al., 2005; Rosenberg, 1993; Weiner, 1997). Unlike EPS from terrestrial microorganisms or aquatic microalgae, marine bacterial EPS are often highly polyanionic (Kennedy and Sutherland, 1987), a property attributed to their relatively high uronic acid content (Corpe, 1970; Kaplan et al., 1987b). Hence, these compounds are often highly active at surfaces and represent a potential source of commercially valuable surface-active agents. Some polyanionic macromolecules exhibit amphipathic properties that allow them to interact with oily substrates, in some cases enhancing their dissolution by the process of emulsification or lowering their surface tension.

Their amphipathic nature is often attributed to the presence of a hydrophobic component(s) attached to the polysaccharide backbone, such as the fatty acids of RAG-1 emulsan (Belsky et al., 1979), or protein of some glycoprotein emulsifiers (Garti and Leser, 1999; Kaplan et al., 1987a). Little, however, is known on whether anionic moieties, such as carboxyl and sulfate residues, contribute amphipathic activity to polysaccharides.

The genus *Halomonas* has received increasing interest because of a number of reports describing species that produce significant quantities of EPS with high surface activity and/or rheological properties (Calvo et al., 1998, 2002; Martinez-Checa et al., 2002; Pepi et al., 2005). With the aim to exploit their potential biotechnological uses, optimization of the culture conditions used to grow these organisms is an important initial step towards maximizing the production yield of these types of polymers whilst also aiming to improve on their functionality (Giavasis et al., 2000; Laws et al., 2001). The influence of abiotic parameters (e.g., pH, temperature, agitation, salinity) and different nutritional conditions have been shown to influence the production and physico-chemical characteristics of EPS produced by *Halomonas* species (Arias et al., 2003).

In this study we investigated the influence of different growth substrates on the production and physicochemical qualities of EPS produced by the halomonad, TG39, previously reported to produce a high molecular weight glycoprotein emulsifier (Gutierrez et al., 2007). This study represents, for the first time, a comparative evaluation between the anionic activity and emulsifying qualities of an EPS from a bacterial species.

Materials and Methods

Microorganism, Media, and Culture Conditions

Halomonas sp. TG39 (Gutierrez et al., 2007) was grown in a Zobell's 2216 (ZM/1) medium composed of 3/4-strength filtered seawater, 0.5% peptone, 0.1% yeast extract, and various vitamins and mineral nutrients, as described previously (Green et al., 2004). To test the effects of different sugar substrates on EPS production, 250-mL Erlenmeyer flasks were used containing 100 mL of ZM/1 and one of the following sugar substrates (1% w/v final concentration): glucose, sucrose, mannitol, malt extract, and no sugar. Similarly, to test the effects of varying the organic nitrogen source on EPS production from glucose, the amounts of peptone (P) and yeast extract (YE) were adjusted to achieve the following final ratios of P/YE (w/w): 5:1, 2:1, 1:1, 1:2, and 1:5, with each flask amended with 1% (w/v) glucose. The final combined concentration of P and YE in each flask was 0.6% (w/v). All the flasks were inoculated using ZM/1-grown cells, and the cultures incubated with shaking (150 rpm) at 28°C. After 3 days incubation, the biomass was collected for dry weight determinations, and the EPS from whole spent media was extracted as described below. All growth experiments were performed in triplicate using independent flasks, and the yields of EPS extracted were averaged and their standard deviations calculated. Statistical data are reported for EPS yields in which the standard deviation exceeded 10%.

Production and Isolation of EPS

Emulsifying activity was only found associated with cell-free extracts, as washed whole-cell suspensions did not emulsify *n*-hexadecane (Gutierrez et al., 2007). Consequently, EPS from cultures grown on the different sugar substrates and P/YE ratios were isolated by removing the cell biomass (10,000*g*; 20 min) and any residual cells by filtration (0.22 μm). The cell-free filtrate was then adjusted to 7% (w/v) KCl and two volumes of cold 99% ethanol added to precipitate the EPS. After standing overnight at 4°C, the precipitate was recovered by centrifugation (4,500*g*; 10 min) then dialyzed against 20 L of distilled water and lyophilized. This was labeled total EPS extract.

Isolation of the high molecular weight (≥100 kDa) fraction from each of the total EPS extracts was performed by ultrafiltration of the EPS extracts dissolved in distilled water through YM-100 Centricon filter units (Millipore, Dundee, UK). The retentate (≥100 kDa) and filtrate (<100 kDa) fractions were then dialysed against 20 L of distilled water and then lyophilized. Respectively, these fractions were labeled HMW and LMW.

Methylene Blue Binding Assay

The anionic nature of the EPS fractions was determined using a colorimetric assay which is based on the formation of metachromatic complexes between negatively charged polymers with methylene blue (MB) (Gurr, Searle Diagnostic, UK), as developed by Soedjak (1994). For this, a stock solution of each polymer was prepared in distilled water to a final concentration of 0.2 mg mL^{-1}. Increasing volumes (0, 25, 50, 75, 100, 150, and 200 μL) of each EPS stock solution were added to a series of 1-mL disposable plastic cuvettes and the final volume adjusted to 0.9 mL with distilled water. Then, 100 μl of a 0.41 mM MB solution was added to each cuvette, and the solutions were mixed thoroughly prior to recording the change in absorbance from 610 and 664 nm, to 567 nm (i.e., the absorbance maximum for the MB used in this study) due to complex formation. This was performed against water as the blank and using a Nicolet Evolution 300 spectrophotometer. Absorbance readings (A_{567}) versus polymer concentrations (10^{-3}%) were plotted and the slope from each of these curves was calculated ($\Delta A_{567}/10^{-3}$%) to represent the anionic activity of each respective EPS extract for MB. The concentration of EPS at which the absorbance at 567 nm reached a plateau is defined as the saturation point—that is, when all 0.041 μmol of MB were complexed. This is the binding capacity expressed as the milligrams of MB bound per gram of polymer. All determinations were performed in

triplicate for each EPS concentration and their standard deviations calculated. Statistical data are reported for EPS yields in which the standard deviation exceeded 5%.

As previously described (Soedjak, 1994), the effect of NaCl on the complexation of each high molecular-weight (HMW) EPS extract was investigated by comparing the absorbance measured at 567 nm before and after additions of NaCl. For this, incremental additions of 2 M NaCl were dispensed into already formed complexed mixtures until a final concentration of 120 mM NaCl was reached. A decrease at A_{567} was indicative of salt-induced dissociation of the EPS–MB complex.

To quantify the number of sulfate and carboxyl residues that are available in each of the EPS fractions for binding cations, the MB assay was used as described above, though this time it was conducted in both water and in phosphate buffer (25 mM, pH 6). The specificity of this method in distinguishing between these residues relies on the ability of phosphate buffers (pH range 1.75–12.0) to inhibit MB-polymer complex formation as a result of competition between phosphate and carboxyl groups for binding to the dye. Binding of the dye to sulfate groups, on the other hand, is not affected because phosphate does not compete with strongly acidic sulfate groups (Soedjak, 1994). Thus, sulfated polymers, such as *k*-carrageenan, produce approximately identical A_{567} values when the assay is conducted in either water or in phosphate buffer. The A_{567} of 41 μM MB in phosphate buffer was slightly higher (0.020) than that measured in water, possibly due to binding of phosphate to the dye. This value was subtracted from all A_{567} values measured in phosphate buffer. These recalculated A_{567} values represent a measure of the carboxyl groups that are available for binding to the dye. The difference between these A_{567} values obtained in phosphate buffer to those measured in water are a measure of the sulfate groups available for binding by the dye. For comparison, the following hydrocolloid controls were used: alginate and gum arabic (both carboxylated polymers); κ-carrageenan (a sulfated polymer); and guar gum (a neutral polysaccharide). All four commercial hydrocolloids were obtained from Sigma–Aldrich (Poole, Dorset, UK).

Emulsification Assays

The emulsifying potential of each EPS fraction was determined using a modified version of the method described by Cooper and Goldenberg (1987). For this, EPS fractions were dissolved in water at various specified concentrations, and then 0.5 mL aliquots of these aqueous solutions were mixed with an equal volume of *n*-hexadecane in acid-washed (0.1 N HCl) screw-cap glass tubes (100 mm × 13 mm). Mixing was performed by manually shaking (15 s) and vortexing at 250 rpm (15 s) to homogeneity, allowing the emulsions to stand for 10 min, and then shaken as before. The emulsions were allowed to stand for 24 h at 21°C, and then the height of the emulsion layer was measured. The emulsifying activity (EI_{24}) was expressed as a percentage of the original height occupied by the oil. All determinations were performed in triplicate. For comparison, the following hydrocolloid controls (as above) were used: alginate, κ-carrageenan, guar gum (largely polysaccharide in composition), and gum arabic (a protein-containing polysaccharide).

Quaternary Ammonium Precipitation

Quaternary ammonium precipitation, using cetyltrimethylammonium bromide (CTAB), was used to isolate the anionic component of the HMW-glucose EPS fraction. This method was adapted from that described by Goldman et al. (1982). Briefly, 50 mg of EPS was dissolved in 10 mL of distilled water and precipitated with CTAB, initially using a CTAB-to-polymer ratio of 1:1 (w/w). Additional amounts of CTAB were added until no further material was observed to precipitate out of solution. The mixture was centrifuged and the supernatant removed and stored aside as fraction F1. The pellet was re-suspended in 50 mM Na_2SO_4. Any insoluble material was then removed by centrifugation and stored separately as fraction F2. Potassium iodide was then added to the supernatant fluid to a final concentration of 2 mg mL^{-1} to precipitate the remainder of the CTAB, which was subsequently collected by centrifugation. The supernatant solution, labeled F3, contained the purified anionic component of the HMW-glucose extract. Fractions F1, F2, and F3 were extensively dialyzed against distilled water and lyophilized prior to emulsification and chromatographic analysis.

Size-Exclusion Chromatography

Purity and molecular composition of the EPS extracts were evaluated by high-performance size-exclusion chromatography (HPSEC) using a Waters Ultrahydrogel 2000 column operated at 30°C which was fitted to an Agilent 1100 chromatograph equipped with a refractometer and diode-array UV detector. The eluent used was either 0.1 M $NaNO_3$ (pH 7), distilled water (18 MΩ cm^{-1} quality) or filtered seawater (0.2 μm). The flow rate used was 0.6 mL min^{-1}. Dextran standards of molecular weight range 1,270–1,400,000 Da (Sigma) were used to calibrate the column for molecular weight estimation. The absolute molecular weight and polydispersity were determined using size exclusion chromatography coupled to multi-angle laser light scattering (SEC-MALLS) (Wyatt, 1992). A DAWN-EOS multi-angle laser light scattering detector and an Optilab rEX refractometer (Wyatt Technologies, Santa Barbara, CA) were used for light scattering intensity and concentration detection respectively. The eluent was acetate buffer (0.1 M, pH 7) at a flow rate of 0.8 mL min^{-1} and 100 μL samples of the polymer were injected onto the columns (TSK Gel G6000 PW, TSK Gel G4000 PW with a TSK Gel guard column) (Tosoh Biosciences, Tokyo,

Japan) after filtering through 0.45 μm filters (Whatman, Maidstone, UK). A standard polysaccharide refractive index increment (dn/dc) of 0.150 mL g^{-1} (Theisen et al., 2000) was used. Polydispersity index (I_p) is a measure of the distribution of different molecular weights within a polymer sample and is the ratio of the weight average molecular weight (M_w) and the number average molecular weight (M_n) where

$$M_n = \frac{\sum M_i N_i}{\sum N_i} = \frac{\sum c_i}{\sum \frac{c_i}{M_i}} \quad (1)$$

and

$$M_w = \frac{\sum M_i^2 N_i}{\sum M_i N_i} = \frac{\sum c_i M_i}{\sum c_i} \quad (2)$$

where N_i and c_i are the numbers of and concentrations respectively of molecules of molecular weight M_i and therefore is always $I_p \geq 1$ (Harding et al., 1991).

Statistical Analysis

The Pearson rank correlation coefficient was calculated for each pair of data within Microsoft Excel, and the significance determined where the coefficient (r) exceeded the Pearson critical value for two-tailed tests at $P < 0.05$.

Analytical Techniques

Total uronic acids content of the EPS extracts were determined by the method of Cesaretti et al. (2003), but using an incubation temperature of 80°C instead of 100°C as originally specified. Total protein concentrations were determined using the BCA protein assay kit (Sigma, St. Louis, MO) with bovine serum albumin as the standard. Total sulfur content was determined by Inductively Coupled Plasma-Atomic Emission Spectrometry (ICP-AES), using a Perkin Elmer DV4300 spectrometer equipped with an AS93 autosampler (Perkin Elmer Life and Analytical Sciences, Inc., Boston, MA). Standard sulfur solutions were prepared in-house from 10,000 μg. mL^{-1} single element sulfur solution (CPI International, Amsterdam, The Netherlands). The samples were diluted in 5% nitric acid solution, and determined in axial mode by standard additions at 181.975 nm. All analytical determinations were performed with triplicate samples of EPS derived from independent cultures.

Results and Discussion

Influence of Sugars and P/YE Ratios on EPS Yield and Emulsifying Activity

Initial experiments were performed to design a growth medium, based on the modified Zobell's 2216 marine broth (ZM/1), which would yield the highest quantity of EPS with no loss in emulsifying activity against *n*-hexadecane. As shown in Table I, glucose- and mannitol-grown cells produced the highest dry cell yield of 2.69 and 2.19 g L^{-1}, respectively, compared to those grown on malt extract (2.16 g L^{-1}) or sucrose (2.00 g L^{-1}). As expected, the cell yield was lowest (0.43 g L^{-1}) for cells grown without any added substrate (i.e., in ZM/1 alone). Cells grown with malt extract produced the highest yield of crude EPS (1.75 g L^{-1}), but its specific emulsifying activity (40%) was comparatively lower to EPS produced by cells grown on glucose (60%) or mannitol (70%). Control experiments using uninoculated media showed that unidentified components within malt extract, possibly of high molecular weight, were co-precipitated with the EPS and remained associated with the precipitated material even after extensive dialysis (results not shown). This could explain the poor specific emulsifying activity of the EPS from malt extract, and for this reason it was not included in the analysis of any correlations between the emulsifying activities of the various EPS fractions and their respective protein content and anionic activities (see below). Although the crude EPS extract from mannitol-grown cells exhibited the highest specific emulsifying activity, subsequent fractionation of the various whole EPS extracts through a 100 kDa membrane revealed that EPS from glucose-grown cells contained the highest fraction of high molecular weight (HMW) EPS (i.e., ≥100 kDa) and exhibited the highest specific emulsifying activity (Table I). Hence, glucose was selected as the optimal substrate for producing EPS of high emulsifying activity and relatively high yield.

Table I. Yield and properties of total, HMW, and LMW EPS fractions extracted from strain TG39 after growth in ZM/1 broth amended with different sugar substrates[a].

	Substrates				
Property	None	Glucose	Mannitol	Sucrose	Malt
Extract					
Cell biomass (g L^{-1} dry)	0.43	2.69	2.19	2.00	2.16
Total EPS extract					
Yield (g L^{-1})[b]	0.40	0.66	0.59	0.61	1.75
Specific EI_{24} (%)[c]	5	60	70	45	40
HMW (≥100 kDa) EPS fraction					
Yield (%)[d]	12.8	85.0	71.6	79.3	29.5
Specific EI_{24} (%)[c]	20	100	70	60	50
LMW (≤100 kDa) EPS fraction					
Yield (%)[d]	55.0	15.0	26.3	24.1	70.2
Secific EI_{24} (%)[c]	0	0	0	0	0

[a]Values represent the average of three independent replicates; the standard deviations were below 10%.
[b]Values are the total yield of polymer extracted from cell-free spent medium.
[c]Values are the specific emulsifying activity expressed as the percentage height of *n*-hexadecane oil emulsified by 0.02% of EPS. The standard deviations of triplicate measurements were below 5%.
[d]Values are the percentage amount of polymer recovered from 50 mg of total EPS extract. Standard deviations were below 10%.

The influence of different P/YE ratios on the yield and emulsifying quality of EPS produced by cells grown on glucose was investigated. A decrease in the P/YE ratio from 5.0 to 0.5 produced a proportional increase to cell biomass and EPS production (Fig. 1). The EPS exhibiting the highest specific emulsifying activity (80%), however, was derived from cells grown on a P/YE ratio of 5.0, indicating that the higher organic nitrogen concentrations favored the production of EPS with better emulsifying qualities. Preliminary results have shown that the use of synthetic seawater-based media, or a nutrient poor version of modified Zobell's medium (ZM/10) containing the same P/YE ratio of 5.0, resulted in lower yields of EPS with low specific emulsifying activities (results not shown). A high organic nitrogen loading may be expected to influence protein synthesis, possibly of certain types of proteins. The proteinaceous component of TG39s EPS molecules may confer these glycoproteins an ability to adsorb onto and stabilize oil droplets, as has been reported for other proteinaceous polymeric emulsifiers (Garti and Leser, 1999; Kaplan et al., 1987a; Williams et al., 1990). Further discussion on the role of the protein component and total anionic activity in the emulsification of *n*-hexadecane by EPS from strain TG39 is presented below.

Effect of Ionic Strength on EPS Recovery

Various parameters can significantly influence the recovery and quality of EPS extracts during downstream processing. In this study, the ionic strength of the cell-free permeate was found to influence the yield of HMW EPS recovered by ultrafiltration (100 kDa cutoff). As shown in Table I, up to 85% of the whole EPS extract produced by cells grown on glucose was found to consist of macromolecules $\geq$100 kDa.

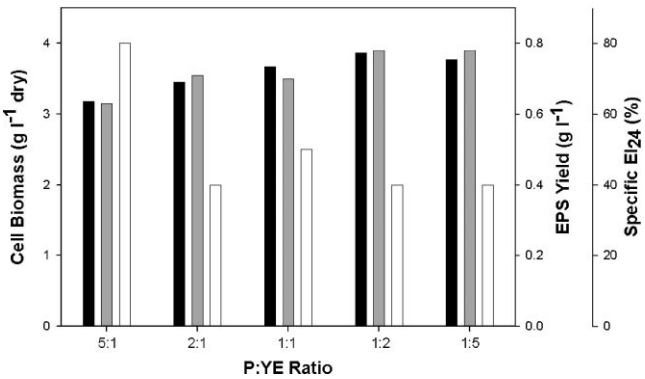

Figure 1. Yield and properties of EPS derived from strain TG39 after growth on glucose in ZM/1 broth containing different ratios of peptone and yeast extract. Values for cell biomass (■) and EPS yield (▩) represent the average of three independent replicates, the standard deviations of which were below 10%. Values for emulsifying activity (□) are expressed as the percentage height of *n*-hexadecane oil emulsified by 0.02% of EPS, with standard deviations from triplicate measurements being below 5%.

This equated to a yield of 0.53 g L^{-1} for the HMW-glucose EPS, which is $\approx$4-fold greater than that previously reported (0.131 g L^{-1}) under identical culture conditions (Gutierrez et al., 2007). In this previous study, the cell-free permeate was at 75% salinity and subjected to ultrafiltration directly following cell removal. In the present study, however, we added an equal volume of distilled water (18 MΩ cm^{-1} quality) to the cell-free permeate prior to ultrafiltration, thereby reducing the ionic strength of the cell-free spent liquid by half. The HPLC chromatograms shown in Figure 2 illustrate the influence of ionic strength on the heterogeneity of the EPS in solution. Under low ionic strength conditions (i.e., 18 MΩ cm^{-1} quality water), macromolecular species complexed forming high molecular-weight aggregates that eluted as three poorly resolved peaks within the void volume (>2,000 kDa) of the HPLC column (Fig. 2a). These peaks, with retention times of 7.8, 8.7, and 12.8 min, would easily be recovered within the retentate volume during the ultrafiltration step. High ionic strength conditions (i.e., filtered seawater), however, resulted in the dissociation of these aggregates to form lower-molecular-weight macromolecules that resolved into six distinct peaks with retention

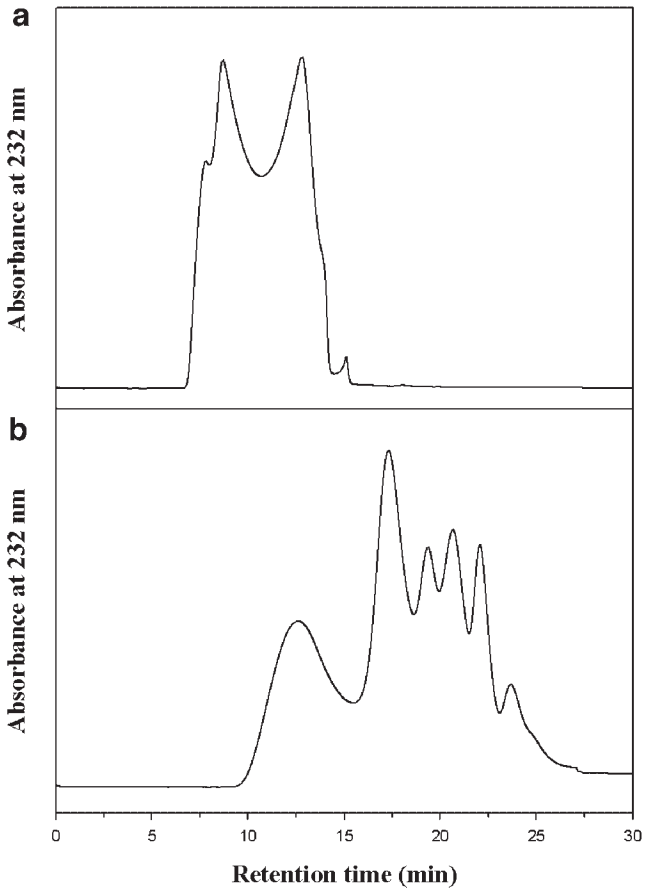

Figure 2. Chromatographic analysis of the HMW–EPS produced by TG39 grown on glucose on an Ultrahydrogel 2000 size-exclusion column using 18 MΩ cm^{-1} quality water (**a**) or filtered seawater (**b**) as eluent.

times of 12.6, 17.3, 19.4, 20.7, 22.1, and 23.7 min (Fig. 2b). Excluding the first two peaks (>2,000 and 247 kDa, respectively), the remaining four peaks represented macromolecules of molecular weights less than 100 kDa and would have been lost in the filtrate volume during ultrafiltration.

Furthermore, our analysis of the HMW-glucose EPS dissolved in deionized water by SEC-MALLS showed only 2 partially resolved components eluting near the void volume (Fig. 3): one minor peak at retention time 15.6 min was composed of a very high molecular weight component ($2{,}300 \pm 200$ kDa) constituting ≈4% of the total material, and a second major peak at retention time 18.1 min with a molecular weight of 620 ± 60 kDa. The polydispersity index (M_w/M_n) of this major component was 1.8. As polydispersity provides an indication of the molecular size distribution of a polymer in solution, this value for the HMW-glucose EPS denotes that this polymer fraction is heterogenous compared to, for example, commercially available pullulan ($I_p \leq 1.1$). Generally, a polydispersity index of ≥1.6 is indicative of a polydisperse polymer (Harding et al., 1991). This data corroborates the HPLC traces presented in Figure 2a and b showing the heterogeneity of this EPS fraction. It should be noted that the weight-average molecular weight of a mixture of components will be biased toward higher molecular weights due to the way weight-average molecular weights are calculated. This salt-induced heterogeneity is likely to be attributed to the high content of uronic acids in the EPS because these acids tend to ionize at seawater pH and salinity, and cause the low molecular-weight species to dissociate and exist freely in solution (Decho, 1990).

Our data shows that by reducing the ionic strength of the cell-free permeate prior to ultrafiltration, the recovery of charged macromolecules could be maximized. These observations emphasize the importance of performing liquid chromatography analysis using a mobile phase that closely resembles the ionic strength of the growth medium when evaluating marine-derived EPS extracts, as it would reveal a more accurate measure of their purity and composition. Furthermore, it would also shed greater insight into ways that could optimize their recovery and increase yields.

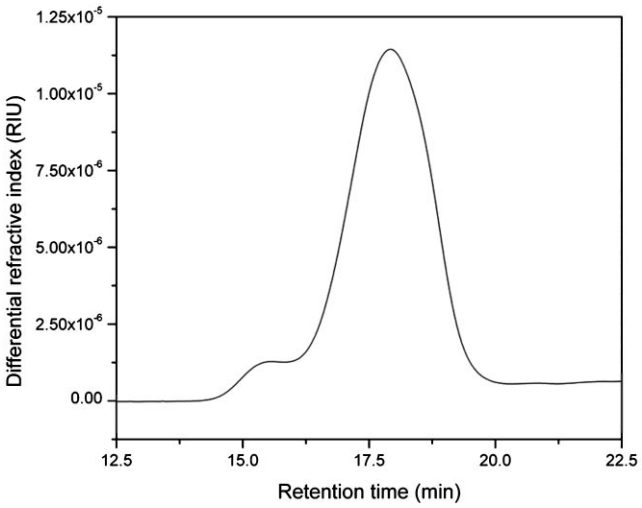

Figure 3. SEC-MALLS analysis of the HMW–EPS produced by TG39 grown on glucose.

Anionic and Emulsifying Activities of EPS

Most reports in the literature describing amphipathic microbial polymers have generally used total uronic acid analysis as a measure of the anionic nature of their polymers. Whether performed colorimetrically using sulfuric acid and carbazole (Filisetti-Cozzi and Carpita, 1991), or by gas chromatography after methanolysis and subsequent acetylation (Bertaud et al., 2002; Huang et al., 1992), such methods can suffer from poor sensitivity and reproducibility. The MB assay employed in this study, however, has been shown to be highly sensitive, quantitative and reproducible for determining the anionic nature of polymers (Soedjak, 1994). It measures total anionic activity, as contributed by different anionic residues (e.g., carboxyls and sulfates) and can be applied to a wide variety of different biopolymers. The interaction of MB with polyanions is a stoichiometric process (1:1 ratio between the dye and anionic sites) which can be quantified based on the absorbance of the metachromatic complex formed when using a known amount of the dye. Using this assay, we were able to quantify the specific binding of the different EPS fractions for the dye and present this as a measure of their anionic activity.

As shown in Table II, different growth substrates yielded EPS exhibiting different anionic activities based on their reactivity with MB. A linear least-squares analysis for plots of anionic activity versus increasing concentrations of each EPS resulted in correlation coefficients that were ≥0.9982 (not shown). The linear correlation between A_{567} and EPS concentrations is in accordance with data from various sulfated and carboxylated hydrocolloid polymers (Soedjak, 1994). The HMW-glucose EPS exhibited the highest anionic activity (21.0), MB binding capacity (464.4 mg g^{-1} of EPS) and specific emulsifying activity (100%; Table I). Conversely, the HMW-control and HMW–ME EPS fractions were found to have the lowest anionic activities (12.4 and 6.7, respectively), MB-binding capacities (326.2 and 255.5 mg g^{-1} EPS, respectively) and specific emulsifying activities (20% and 50%, respectively; Table I). Pearson rank correlation coefficient analysis showed a significant ($P < 0.05$) correlation between anionic activity and emulsifying activity of the HMW–EPS fractions. The HMW–EPS derived from cells grown on malt extract (i.e., HMW–ME) was excluded from this statistical analysis for reasons described above. To our knowledge, this is the first observation of a correlation between anionic activity and

Table II. Yield and physicochemical properties of the high molecular weight (≥100 kDa) EPS fractions from strain TG39[a].

EPS (>100 kDa)	Anionic activity ($\times 10^2$)[b]	Binding capacity[c]	Carboxyl (%)[d]	Sulfate (%)[d]	Uronic acids (%)	Protein (%)
HMW-control	12.4	326.2	ND	ND	25.2	11.1
HMW-glucose	21.0	464.4	71.6	28.4	23.6	31.8
HMW-mannitol	19.2	414.3	75.6	24.4	25.3	25.2
HMW-sucrose	17.4	340.7	64.2	35.8	22.0	23.8
HMW-malt extract	6.7	255.5	ND	ND	23.4	27.4
Alginate	31.8	1,277.5	100.0	0.0		
κ-carrageenan	28.0	1,226.4	24.0	76.0		
Gum Arabic	14.0	319.4	97.0	3.0		
Guar gum	0.0	0.0	ND	ND		

ND, not determined.
[a]TG39 was grown in ZM/1 medium amended with different sugar substrates. Values represent the average of independent replicates; the standard deviations were below 5%.
[b]Values are the calculated slopes ($\times 10^2$) from plots of A_{567} against polymer concentrations (10^{-3}%).
[c]Binding capacity represents the amount of methylene blue (mg) complexed per gram of EPS.
[d]Values are the ratio of the A_{567} values calculated for the carboxyl and sulfate residues in each polymer, as described in the Materials and Methods Section.

emulsification for EPS derived from a bacterium. Only one report has shown this correlation in polysaccharide fractions of pectins from sugar beet (Dea and Madden, 1986). Interestingly, as the uronic acid content of the different HMW–EPS fractions (22.0–25.3%) did not correlate with emulsifying activity (Table II) ($P < 0.1$), the structural orientation of these acids on the EPS, not their overall content, may be a more important parameter in conferring their ability to emulsify *n*-hexadecane. Alternatively, other chemical components may be involved in this process.

To investigate this further, the anionic activities of the commercial hydrocolloids alginate, *k*-carrageenan and gum arabic were also measured. Compared to the various TG39 EPS fractions, alginate and *k*-carrageenan exhibited higher anionic activities—31.8 and 28.0, respectively. Neither of these gums, however, emulsified *n*-hexadecane (results not shown), thus indicating that a high negative charge density does not necessarily confer polysaccharides with an ability to emulsify this oil. In other experiments, we found that other anionic polysaccharides, including carboxymethylcellulose, poly-L-glutamate and heparin also did not emulsify *n*-hexadecane (data not shown). Although this appears to contradict the correlation we observed between the anionic and emulsifying activities of the various HMW–EPS fractions, we believe that in addition to their high negative charge density, the presence of protein, which both alginate and *k*-carrageenan lack, is involved in emulsification activity of TG39 EPS fractions. Pearson rank correlation coefficient analysis showed a highly significant correlation ($P < 0.01$) between emulsification and total protein content of the HMW–EPS fractions. This suggests that protein may play an important role in emulsification of *n*-hexadecane. Collectively, the correlations found between anionic and emulsifying activities, as well as protein content and emulsifying activity, appear to indicate that both the protein and the anionic residues of the polysaccharide component contribute to conferring the various HMW–EPS fractions with their capacity to emulsify *n*-hexadecane. Similar protein-polysaccharide interactions have been described in applications of the food industry where they play a key role in determining the final qualities of many foods (Samant et al., 1993). For example, the emulsifying activities and emulsion stability of certain types of proteins have been shown to be improved in the presence of anionic polysaccharides (Gurov et al., 1983; Larichev et al., 1983).

To evaluate the influence of protein to emulsification, we found that the addition of bovine serum albumin (2% w/v) to solutions of alginate and *k*-carrageenan, which alone do not emulsify *n*-hexadecane, resulted in a partial increase in emulsification ($EI_{24} = 25\%$) of this oil (results not shown). We also found that gum arabic, which by its nature has a high protein content of ≈10% of total gum (Williams et al., 1990), could emulsify *n*-hexadecane ($EI_{24} = 50\%$; result not shown). As with gum arabic (Dickinson et al., 1988; Nakamura, 1986; Randall et al., 1989; Williams et al., 1990) and some microbial-derived polysaccharides (Baird et al., 1983; Boyle and Reade, 1983; Kaplan et al., 1987a), the protein component has been shown to play an important role in emulsion formation. Therefore, the protein component of the HMW–EPS fractions from TG39 may play a contributing, and possibly major, role in their ability to emulsify *n*-hexadecane. The mechanism by which this occurs may be via the formation of electrostatic interactions between anionic groups on the polysaccharide with cationic amino acid residues on the protein, thereby coordinating steric adsorption onto droplets of *n*-hexadecane (Snoeren, 1976).

Collectively, these results indicate that polysaccharides alone, even those endowed with a high negative charge density (e.g., alginate and *k*-carregeenan), are less likely to exhibit emulsifying activity for *n*-hexadecane. However, the combination of protein and a high negative charge density, as is inherent to gum arabic (Williams et al., 1990), may contribute a synergistic role to conferring the various HMW–EPS fractions from TG39 with their ability to emulsify *n*-hexadecane.

Quantitative Sequestration of MB

The HMW-glucose EPS produced by TG39 was found to bind up to 464.4 mg MB per gram of EPS under non-optimized conditions. Lower quantities of MB were sequestered by EPS derived from cells grown on mannitol (414.3 $mg\,g^{-1}$), sucrose (340.7 $mg\,g^{-1}$), malt extract (255.5 $mg\,g^{-1}$) or no added sugar substrate (326.2 $mg\,g^{-1}$). The addition of NaCl to the reaction complex resulted in an increase to the absorbance of the free dye at 610 nm. This indicated that MB-EPS complex formation was reversible. Previously, the highest reported binding capacity of a microbial biosorbent for MB was 339.2 mg per gram of *Corynebacterium glutamicum* EPS (Vijayaraghavan et al., 2008). Clearly, TG39 produces a more potent MB-binding microbial sorbent. Overall, the high binding of MB by TG39 EPS, coupled to the potential ease with which this EPS can be regenerated by the addition of electrolytes (e.g., NaCl), suggests that it may find use as a biosorbant material, for example, to treat wastewaters contaminated with cationic dyes and toxic metal species (Veglio and Beolchini, 1997; Zemaitaitiene et al., 2003).

An interesting aspect of these results is that the high cationic-binding capacity of this EPS suggests that in the marine environment, where it would be produced naturally, it could influence biogeochemical processes such as the cycling of heavy metal species and trace metal nutrients. To investigate this, we are presently evaluating the metal ion binding specificity and affinity of EPS from strain TG39 under experimental conditions simulating seawater ionic strength, as previously performed by our group using other marine bacterial EPS (Gutierrez et al., 2008).

Anionic Residues of TG39 EPS

To determine the contribution of carboxyl and sulfate residues to the total anionic activity of each EPS fraction, MB assays were conducted in phosphate buffer and the measurements for anionic activity compared to those measured in deionized water. As previously described (Soedjak, 1994), any decrease in the MB binding activity in the presence of phosphate buffer, is attributed to competition between carboxyl residues and phosphate for binding the dye. This decrease represents the anionic activity conferred by carboxyl groups, whereas the remaining activity may be attributed to the binding of MB by strongly anionic sulfate residues. Table II shows the percent contribution that each of these residues confers to the total anionic activity of each EPS fraction. The anionic activities of the HMW-glucose, HMW-mannitol and HMW-sucrose EPS fractions were primarily contributed by carboxyl residues—71.6%, 75.6%, and 64.2%, respectively. That contributed by sulfates was 28.4%, 24.4%, and 35.8%, respectively. Corroborating the presence of sulfate in the EPS, ICP-AES analysis showed that elemental sulfur comprised 4.6% of the HMW-glucose EPS. The total anionic activity of the highly carboxylated polysaccharide, alginate, was found to be solely conferred by carboxyl residues since phosphate was found to completely inhibit MB-alginate complex formation. The proteinaceous hydrocolloid, gum arabic, was also found to be highly carboxylated (97% of its total anionic activity), whereas the total anionic activity of *k*-carrageenan was primarily contributed by sulfates (76% of total anionic activity), which is in agreement with this polymer's high sulfate content (Stanley, 1987).

Overall, we infer from these results that the different HMW–EPS fractions produced by TG39, contain anionic groups (i.e., primarily carboxyl residues of uronic acids, and to a lesser extent sulfates, and possibly the carboxyl moieties of some amino acids) that participate together with protein in emulsification of *n*-hexadecane. Furthermore, since sulfate groups are recognized to significantly contribute to the anionic quality of marine EPS (Leppard et al., 1996), this may confer them with some pharmaceutical potential, such as antiviral (Okutani, 1992), antitumoral (Inoue et al., 1988), or anticoagulant (Nishino et al., 1989) agents. Thus, the relatively high sulfate content in TG39 EPS warrants further investigation in this respect.

Purification of the Anionic Component With CTAB

By using CTAB quaternary ammonium precipitation, a total of 2.6 mg of anionic material (i.e., fraction F3) was isolated from 50 mg of HMW-glucose EPS. This constituted 5.2% of the total dry weight of polymer. Chromatographic analysis of this fraction on a calibrated Ultrahydrogel 2000 column using 0.1 M $NaNO_3$ (pH 7) as the mobile phase, revealed a well resolved peak at retention time 17.3 min (Fig. 4a). Using a plot of $\log M_r$ of standards versus retention time, its relative molecular mass was estimated to be 150 kDa. This peak was found to exhibit high emulsifying activity against *n*-hexadecane and corresponded to the major peak of the original HMW-glucose extract (Fig. 4b). This provides further evidence that the active emulsifying component of the HMW-glucose EPS is anionic in nature. Precipitation with CTAB also resulted in the removal of 38.6 mg of insoluble material (F2) that could not be reconstituted back into solution. This was likely to be CTAB that was irreversibly complexed to the EPS via hydrophobic-hydrophobic interaction. A total of 5.6 mg of the EPS (F1) did not complex and precipitate out during precipitation with CTAB, suggesting that 11.2% of this EPS was not anionic.

Conclusion

In this study, we have examined the influence of different growth substrates on the anionic activity of EPS produced by TG39, and demonstrated a correlation between the protein content and anionic activity of the different EPS with their emulsifying activities for *n*-hexadecane. The mechanism

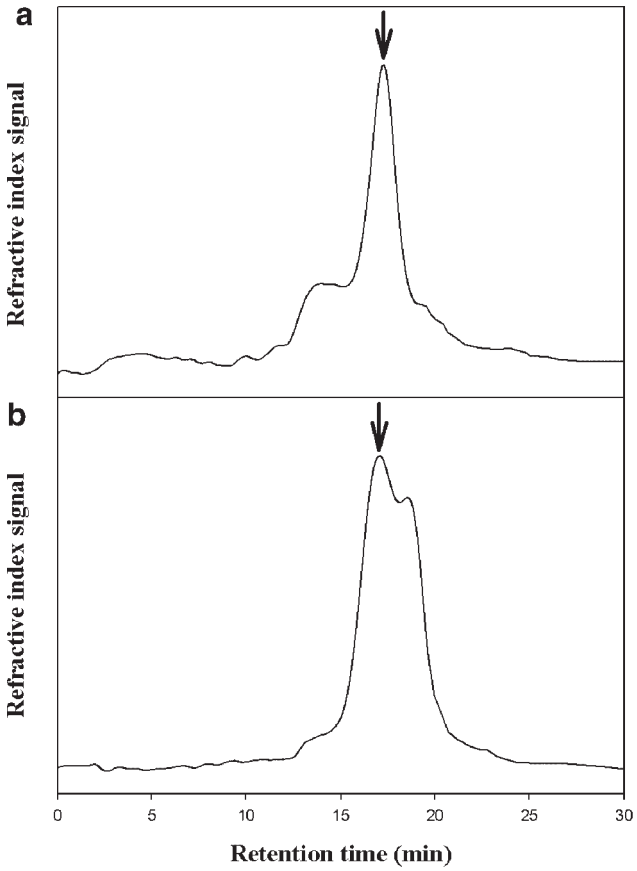

Figure 4. Chromatographic analysis of Fraction 3 from CTAB precipitation (**a**) and the HMW-glucose EPS (**b**) on an Ultrahydrogel 2000 size-exclusion column. Arrows denote corresponding peaks.

involved in this process may be via a synergistic interaction between the protein and anionic residues on the polysaccharide in coordinating the steric adsorption of the EPS macromolecules to the oil-water interface. We anticipate that this mechanism may be a common feature of amphipathic bacterial EPS, especially those from marine bacteria because their EPS is often highly polyanionic (Kennedy and Sutherland, 1987). Furthermore, EPS from this strain was found to complex high concentrations of MB compared to that by other reported microbial-derived biosorbant materials, suggesting the potential application of this EPS for the removal of cationic dyes from wastewater effluents. Future studies investigating the amphipathic qualities of bacterial polymers would, therefore, benefit by applying the MB assay because it provides a standardized means to measure the anionic activity of polymers. The optimal conditions for EPS production and emulsification required a P/YE ratio of 5:1 amended with glucose. Its purification required a reduction in the ionic strength of the cell-free spent medium coupled with 100 kDa ultrafiltration to give a yield of 0.5 g/L. Based on the polymer's amphipathic qualities and polyanionic nature, we anticipate that it may find uses in various commercial sectors, from healthcare products to the bioremediation of hydrocarbons and cationic pollutants.

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