

Chemical Characterization of Exopolysaccharides from Antarctic Marine Bacteria

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Abstract

Exopolysaccharides (EPS) may have an important role in the Antarctic marine environment, possibly acting as ligands for trace metal nutrients such as iron or providing cryoprotection for growth at low temperature and high salinity. Ten bacterial strains, isolated from Southern Ocean particulate material or from sea ice, were characterized. Whole cell fatty acid profiles and 16S rRNA gene sequences showed that the isolates included representatives of the genera *Pseudoalteromonas*, *Shewanella*, *Polaribacter*, and *Flavobacterium* as well as one strain, which constituted a new bacterial genus in the family *Flavobacteriaceae*. The isolates are, therefore, members of the "Gammaproteobacteria" and *Cytophaga-Flexibacter-Bacteroides*, the taxonomic groups that have been shown to dominate polar sea ice and seawater microbial communities. Exopolysaccharides produced by Antarctic isolates were characterized. Chemical composition and molecular weight data revealed that these EPS were very diverse, even among six closely related *Pseudoalteromonas* isolates. Most of the EPS contained charged uronic acid residues; several also contained sulfate groups. Some strain produced unusually large polymers (molecular weight up to 5.7 MDa) including one strain in which EPS synthesis is stimulated by low temperature. This study represents a first step in the understanding of the role of bacterial EPS in the Antarctic marine environment.

Introduction

Particulate aggregates are ubiquitous and abundant in the world's oceans [31]. As complex assemblages of zooplankton fecal pellets, phytoplankton, and other material enriched in bacteria [53, 57], marine aggregates are centers of high bacterial activity and are believed to have a significant role in the downward transport of carbon [46]. Bacteria in marine aggregates are at an advantage compared to free-living cells [53]. Their proximity to other cells and surfaces provides opportunities for interaction and nutrient uptake. Exopolysaccharides (EPS) excreted by bacteria are among the polymeric substances that provide a network to hold these structures together [28]. Microscopic and laboratory studies have shown that bacterially produced EPS have a major role in aggregate formation [7, 38, 49].

Bacteria contribute significantly to secondary production in sea ice communities and to the overall carbon cycle in the Antarctic environment. In thick pack ice, bacterial secondary production even exceeds primary production as the light supply to the bottom layers of ice is reduced [35]. Bacteria are found in abundance in the bottom layers of the ice or in brine channels and are often attached to detrital particles or to living microalgal cells [2, 25, 67]. Delille and Rosier [25] also suggest that the high numbers of particle-associated bacteria found in sea ice may explain observations of underlying water enriched in bacterial biomass relative to the open ocean [35]. More recently, studies of Arctic sea ice in winter showed that at temperatures as low as -20°C , active bacteria were found in the brine channels and were particle associated [42]. Exopolysaccharides may be providing a cryoprotectant role in these environments of high salinities and low temperature [47]. The same authors showed that high

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Table 1. Source information for exopolysaccharide producing Antarctic marine bacterial isolates

Isolate	Position of sample site	Type of source material	Size fraction of source material (μm)	Treatment prior to culturing	Taxonomic grouping
CAM005	66°38'10"S, 141°42'76"E	Melted fast ice	>0.8	Filtering/enrichment	<i>Flavobacterium</i> sp.
CAM030	65°32'06"S, 143°10'16"E	Plankton tow	>20	None	<i>Flavobacteriaceae</i>
CAM006	65°32'06"S, 143°10'16"E	Plankton tow	>20	None	<i>Polaribacter</i> sp.
CAM003	63°52'41"S, 139°41'47"E	Melted pack ice	>0.8	Filtering/enrichment	<i>Pseudoaltermonas</i> sp.
CAM015	65°03"S, 142°31'61"E	Melted fast ice	<0.8	Filtering/enrichment	<i>Pseudoaltermonas</i> sp.
CAM023	65°32'06"S, 143°10'16"E	Plankton tow	>20	None	<i>Pseudoaltermonas</i> sp.
CAM025	63°52'04"S, 139°41'47"E	Melted pack ice	<0.8	Filtering/enrichment	<i>Pseudoaltermonas</i> sp.
CAM036	65°32'06"S, 143°10'16"E	Plankton tow	>20	None	<i>Pseudoaltermonas</i> sp.
CAM064	65°03"S, 142°31'61"E	Melted fast ice	>0.8	Filtering/enrichment	<i>Pseudoaltermonas</i> sp.
CAM090	66°38'10"S, 141°42'76"E	Melted fast ice	>0.8	Filtering/enrichment	<i>Shewanella</i> sp.

concentrations of EPS were found in brine channels and could have been produced by the abundant bacteria or diatom populations present.

Exopolysaccharides appear to be an integral component of particulate material and sea ice microbial communities; however, very little is known about the chemical structures of EPS from cold-adapted bacteria or bacteria from Antarctic waters. Basic knowledge of the chemical composition of EPS gives an insight into potential physicochemical interactions that may take place within or between these polymers or between these polymers and cells or other substances in aquatic environments [40, 64]. Within the polymer, the individual monosaccharides and functional groups covalently linked to them influence the weak cohesive forces that affect the behavior of these polymers [23]. Chemical analysis of EPS from specific bacterial members of natural assemblages is difficult because of the low abundance of any one polymer and the complexity of tracing a single polymer back to its source [21]. Therefore, Antarctic marine bacteria and the EPS they produced in pure liquid cultures were examined in this study as a first step in understanding the ecological significance of these abundant polymers in the Antarctic marine environment.

Materials and Methods

Isolation of Bacteria. Samples were obtained during the November/December 2001 voyage of RSV *Aurora Australis* and included sea ice and seawater. The geographical position, the type of sample material, and the size fraction from which bacterial isolates described in this study were obtained are shown in Table 1. Strains CAM006, CAM030, CAM023, and CAM036 were isolated from particulate material from the Southern Ocean, where the sea temperature was approximately 4°C and salinity was 3.5%. CAM005, CAM003, CAM015, CAM025, CAM064, and CAM090 were obtained from melted sea ice. Pieces of sea ice (250–500 mL volume) from the bottom layer of ice floes (containing a visible

basal algal band assemblage) were collected with a long-handled sieve. Sea ice samples including interstitial seawater were combined with 500 mL sterile artificial seawater (2.2% w/v; Sigma) at 2°C. Aliquots were plated directly or the samples were stored at 2°C for 2 weeks. This storage temperature was preferable to the alternative (–20°C) for shipboard storage, as the latter may have caused additional freezing of the core and associated seawater, resulting in dramatically reduced viability of isolates. Upon return to Hobart, aliquots of 200 mL of melted sea ice were passed through a Nucleopore 0.8- μm filter placed over a glass fiber filter (Schleicher & Schuell), which had been preheated to 450°C for 1 h. The 0.8- μm filter or aliquots of 200 μL of the filtrate were placed in separate glass McCartney bottles containing 10 mL seawater nutrient broth (SNB) [13]. These enrichment cultures containing medium and filters or medium and filtrate were incubated for 24 h at 2°C, then mixed; 200 μL aliquots were then removed and spread onto SNAgar (SNB with 12 g⁻¹ agar added prior to sterilization) and SNAgar + Glucose (2% w/v). Agar plates were incubated at 4°C for 4 weeks. Additional isolates were obtained from a plankton net (20 μm) trawled through the Southern Ocean. Aliquots of 20 μL of material from the cod end of the plankton net were spread onto SNAgar and SNAgar + Glucose (2% w/v) and the plates were incubated at 2°C.

After initial isolations, strains were subcultured onto marine agar (MA; 1 g yeast extract, Oxoid L21; 5 g bacteriological peptone, Oxoid L37; 32 g artificial sea salts, Sigma S9883; 15 g agar; 1000 mL distilled water) and MA supplemented with 3% (w/v) glucose (MA+Glu). A glucose solution was prepared and autoclaved separately before being combined with MA. Strains were selected if they displayed a mucoid morphology when grown on MA+Glu. Approximately 300 isolates demonstrating a mucoid morphology were obtained initially from sea ice and marine particulate samples. The 10 strains that showed the best growth on MA+Glu at 20°C with enhanced mucoid morphology were chosen for further analysis.

Characterization of Bacterial Isolates

16S rRNA Gene Sequence Analyses. The 16S rRNA genes of ten strains were amplified by polymerase chain reaction (PCR) according to procedures described by Bowman et al. [11] using DNA primers 10F and 1519R. The PCR products were purified by Prep-A-Gene purification (Bio-Rad, Hercules, CA), and the concentration of purified DNA in each sample was measured with a Smart Spec 3000 (Bio-Rad, Regent Park, NSW, Australia). Sequences were obtained with a Beckman Coulter CEQ 2000 automated sequencer according to the protocol specified by the manufacturer (Beckman Coulter, Inc, Fullerton, CA).

Sequences were manipulated and aligned using Bio-Edit v. 5.0.9 [37]. Sequences were compared to 16S rDNA genes available in the GenBank library by BLAST searching [4] through the National Center for Biotechnology Information (U.S. National Institutes of Health) internet site as described by Bowman et al. [12]. Sequences were aligned to their closest related sequences determined from the BLAST searches. PHYLIP (version 3.57c) [26] was used to analyze the sequence data and sequence similarities with the maximum likelihood algorithm option were determined using DNADIST. Phylogenetic trees were constructed by the neighborliness method with the program NEIGHBOR. The sequence for *Escherichia coli* (J01695) was included as an outgroup. Partial sequences for the 10 isolates were deposited into GenBank under accession numbers AY243365, AY243366, and AY586522 to AY586529.

Whole-Cell Fatty Acid Analyses. Growth conditions were the same for all strains so that whole cell fatty acid profiles could be compared. The 10 isolates were grown on MA at 12°C for 4 weeks. This incubation temperature was chosen as it approximates a median growth temperature for Antarctic sea ice bacteria [10]. Whole-cell fatty acids were extracted from cell material according to the MIDI protocol [61]. Fatty acid methyl esters (FAME) were treated with N,O-bis-(trimethylsilyl)-trifluoroacetamide to convert hydroxy acids to their corresponding trimethylsilyl (TMSi) ethers for analysis by gas chromatography (GC) and GC-mass spectrometry (GC-MS). Double bond position and geometry of monounsaturated FAME for selected strains were determined after the formation of dimethyl-disulfide (DMDS) adducts prepared according to methods described previously [58].

Gas chromatography analyses were performed on a Hewlett Packard 5890A GC fitted with an HP-5 cross-linked methyl silicone fused capillary column (50 m × 0.32 mm i.d.) and flame ionization detector (FID, 310°C) and an HP 7673A auto-sampler. Helium was the carrier gas. Samples were injected in splitless mode at an oven temperature of 50°C. After 1 min, the oven temperature

was raised to 150°C at 30°C min⁻¹ then to 250°C at 2°C min⁻¹, and finally to 300°C at 5°C min⁻¹. Gas chromatography–mass spectrometry analyses of the FAME were performed with a Finnigan GCQ Plus GC/MS system fitted with on-column injection set at 45°C. Samples were injected with an AS2000 auto sampler into a retention gap attached to a HP 5 Ultra 2 (50 m × 0.32 mm i.d., and 0.17 μm film thickness column, with helium as the carrier gas. The chromatograms and mass spectra were analyzed by Excalibur software. Peaks were identified by comparison to known and laboratory standards, the library included with the software, and by consideration of the mass spectra.

Fatty acids are designated by the total number of carbon atoms: number of double bonds, followed by the position of the double bond from the terminal (ω) end of the molecule. The suffixes *c* and *t* indicate *cis* and *trans* geometry, and the prefixes *i* and *a* indicate iso and anteiso branching. The position of hydroxyl group (OH) may occur on the first (α) or second (β) carbon from the carboxyl end of the molecule.

EPS Production and Characterization Analyses

Growth of Antarctic Isolates in Batch Cultures for EPS Production. A McCartney bottle containing 20 mL of marine broth supplemented with 3% (w/v) glucose (MB+Glu) was inoculated with approximately 10 colonies of each strain from an agar plate (MA+Glu) inoculated 10 days earlier and incubated at 20°C. The 20-mL cultures were shaken for 24 h (200 rpm) at 20°C. The cultures were used to inoculate 200 mL of the same media, and the resulting culture was shaken (200 rpm) and incubated at 20°C. After 48 h, this broth was checked for contamination by subculturing onto a MA+Glu plate and a 10 mL aliquot was removed to measure pH. The remaining broth was used to inoculate 500 mL MB+Glu (pH 7) in a 2 L Schott bottle.

This 500-mL broth culture was bubbled with compressed air (138 kPa). Inlet and outlet air was filtered through a 0.2-μm Midisart filter (Sartorius Australia Pty. Ltd, VIC). Broth cultures were shaken (150 rpm) at 20°C for 1 week. Subculturing onto MA+Glu was used to check for contamination.

Isolation and Purification of Bacterial EPS. EPS were harvested according to procedures described previously [55]. Briefly, culture broths were centrifuged at 30,000 g for 2 h at 4°C, and the supernatants were pressure filtered through cellulose nitrate filters (Sartorius Australia Pty. Ltd., East Oakleigh, VIC). Exopolysaccharides were precipitated from the final filtrate after the addition of cold ethanol, and the resulting precipitates were washed with 70% to 100% ethanol–water mixtures. The EPS were dried in a desiccator and stored at room temperature. To

remove excess salts, the EPS were redissolved in distilled water and dialyzed (molecular weight cut-off of 0.1 MDa; Spectra/Por, Spectrum Laboratories, Rancho Domingues, CA) against distilled water for two days at room temperature ($\sim 25^{\circ}\text{C}$). Excess water was removed under vacuum before lyophilization. The EPS were stored at room temperature until analysis.

EPS Characterization

Colorimetric Analyses. Uronic acid content of the EPS was determined by the meta-hydroxydiphenyl method [8, 27]. Protein content was determined by the Lowry protein assay [54] with bovine serum albumin as the standard. The total neutral carbohydrate content was determined by the orcinol-sulfuric acid method modified by Rimington [59].

FT-IR Spectroscopy. Pellets for infrared analysis were prepared by grinding a mixture of 2 mg polysaccharide with 200 mg dry KBr, followed by pressing the mixture into a 16-mm-diameter mold. The Fourier transform-infrared (FT-IR) spectra were recorded on a Bruker Vector 22 instrument with a resolution of 4 cm^{-1} in the $4000\text{--}400\text{ cm}^{-1}$ region. Approximate sulfate content was determined by FT-IR spectroscopy according to the method of Lijour et al. [51].

Monosaccharide Analyses. To a solution containing 250 μg total EPS, 50 μg erythritol was added as an internal standard. Samples for GC analyses were prepared in triplicate and freeze-dried. The polymer was hydrolyzed by the addition of methanolic HCl (3N, Supelco, Bellefonte, PA) and heating for 16 h at 80°C [43] (modified by Montreuil et al. [56]). The monosaccharides were converted to their TMSi derivatives by the addition of *Bis*-(trimethylsilyl)trifluoroacetamide:trimethylchlorosilane / 99:1 (Supelco) and pyridine. The samples were held at room temperature for 2 h, then dried under nitrogen and redissolved in dichloromethane prior to analyses.

Gas Chromatography. Analyses of the monosaccharides as TMSi derivatives were performed on a GC8000 (Fisons, Paris, France) GC fitted with an automatic injector, an FID (300°C), and a CP-Sil-5CB glass capillary column ($0.32\text{ mm} \times 60\text{ m}$; Chrompac, Varian, Les Ulis, France). Hydrogen was the carrier gas. The GC oven was temperature programmed as follows: 50°C for 1 min then an increase of $20^{\circ}\text{C}/\text{min}$ upto 120°C , followed by a gradient of $2^{\circ}\text{C}/\text{min}$ upto 240°C .

Size Exclusion Chromatography. Molecular weights of EPS were determined by size exclusion chromatography (SEC). The eluent was 0.1 M NaNO_3 in water with 0.02% (w/v) sodium azide added. The analyses were carried out

at room temperature. An isocratic pump (Spectra Physics P100, Thermo Separations, Bordeaux, France) with a flow rate of 1 mL min^{-1} and a TSK gel PW6000 column ($60\text{ cm} \times 0.7\text{ cm}$); Toyo Haas, Frankfurt, Germany) with pectin as a standard, were used. For detection, a differential refractometer (Shodex RI71, Shodex, Tokyo, Japan) and a multiangle light-scattering detector described elsewhere [19] were employed. The polydispersity (I_p) is the ratio of the molecular weights based on the weight of the molecules (M_w) to the molecular weight based on the number of molecules (M_n), relative to the pectin standard ($M_w/M_n = I_p$).

Results

Isolation and Characterization of Bacteria. Several hundred isolates from sea ice and particulate material displayed mucoid morphology at 2°C on media with added glucose. Owing to time constraints, the 10 isolates that showed the best growth and enhanced mucoid morphology on MA+GIu, relative to MA at 20°C were selected for further characterization. All 10 isolates were Gram-negative rods and showed growth in the temperature range of 4°C to 20°C . None of the strains was able to grow at 37°C . These isolates therefore appear to be psychrotolerant.

According to 16S rRNA sequencing results, the 10 isolates belonged to the order *Alteromonadaceae* (Fig. 1) and the family *Flavobacteriaceae* (Fig. 2). CAM003, CAM015, CAM023, CAM025, CAM036, and CAM064 were closely related and grouped in a sub-branch of the genus *Pseudoalteromonas* in which many Antarctic strains are found [9]. The fatty acid profiles for these six *Pseudoalteromonas* isolates were consistent with the 16S rRNA identifications and are supported by other studies [9, 16] (Table 2). Based on 16S rDNA data, CAM090 was most similar to *Shewanella livingstonensis* (Fig. 1) [15]. The whole-cell fatty acid profile of CAM090 was very similar to that presented by Bozal et al. [15] for *Shewanella livingstonensis* and included i13:0 and 16:1 ω 7c as the most abundant whole-cell fatty acids. The whole-cell fatty acid profiles for CAM003, CAM015, CAM023, CAM025, CAM036, and CAM064 showed many similarities to that of CAM090 (Table 2). As the genera *Pseudoalteromonas* and *Shewanella* belong to the order *Alteromonadaceae*, these seven isolates are therefore members of the class, “*Gammaproteobacteria*.”

CAM006 was identified by 16S rDNA sequencing as being closely related to *Polaribacter irgensii* [33], and CAM005 was related to *Flavobacterium frigidarium* [41]. Strain CAM030 was distant from validly described species within family *Flavobacteriaceae* and was most closely related to the species *Myroides odoratus* [32, 70] (Fig. 2). As a group, CAM005, CAM006, and CAM030 had whole-cell fatty acid profiles typical of the *Cytophaga-Flexib-*

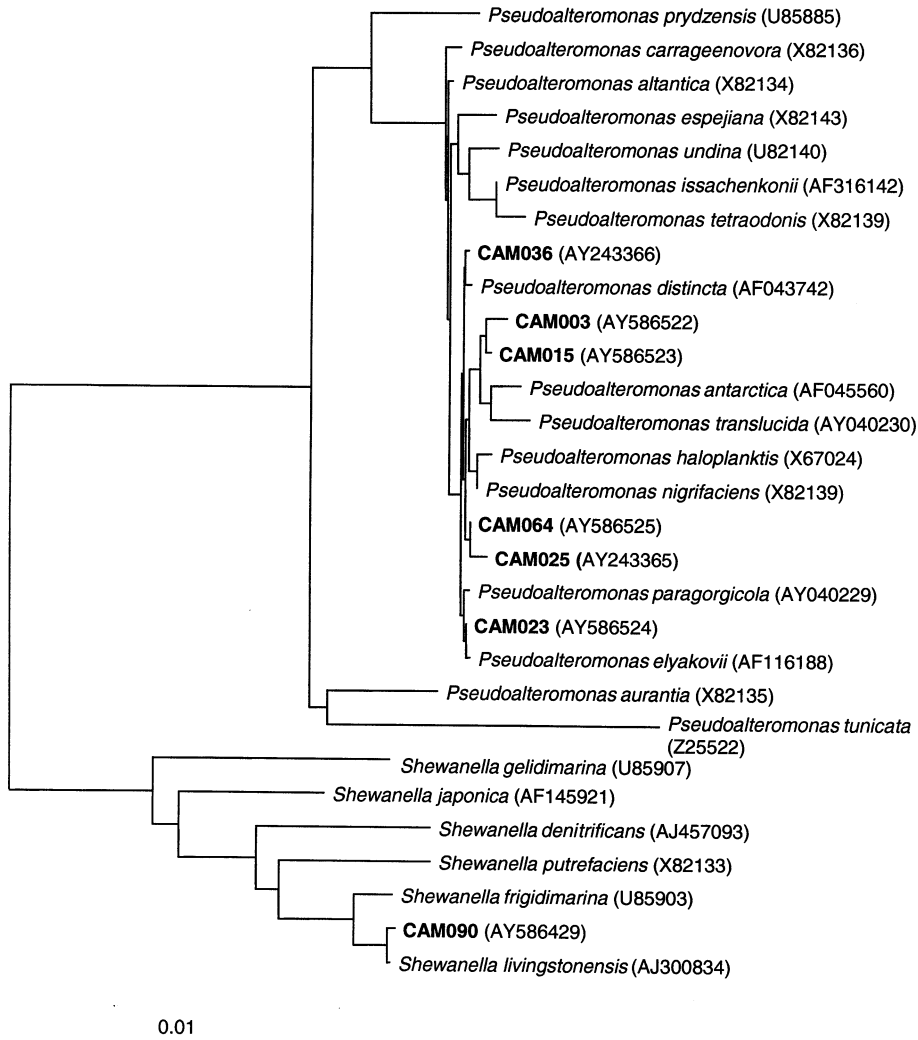


Figure 1. Phylogenetic relationship of Antarctic marine bacterial isolates (CAM036, CAM003, CAM015, CAM064, CAM025, and CAM090) in the order *Alteromonadaceae* based on 16S rRNA sequences. The tree was created using maximum-likelihood distanced clustered by the Neighbor-Joining method. Bar: 0.01 changes per mean nucleotide position. Numbers in parentheses are GenBank nucleotide accession numbers. Outgroup for the analysis was *Escherichia coli* (J01695).

acter-Bacteroides (CFB) phylum, and were dominated by branched chain fatty acids, including i15:1 ω 10c, i15:0, and β -OH i15:0. Differences were apparent between the whole-cell fatty acid profiles of the strains (Table 2). A study by Gosink et al. [33] showed that β -OH i15:0 was the most abundant (38%) whole-cell fatty acid in *Polaribacter irigensii*. We found β -OH i15:0 present at 20% of the total fatty acids and i15:0 (15%) as the second most abundant whole-cell fatty acid in CAM006, and this was in agreement with the earlier findings. In a study by Bernardet et al. [6] the most abundant whole-cell fatty acids of *Flavobacterium* species were reported and are consistent with the whole-cell fatty acid profile of *Flavobacterium odoratus* (since renamed *Myroides odoratus*) and were identified as i15:0 (39%) and β -OH i17:0 (9%). These were also abundant in CAM030: i15:0 (19%), β -OH i17:0 (4%). There were many minor whole-cell fatty acids common between the strains; however, one obvious difference was the presence in large amounts of i15:1 ω 10c in CAM030 (22%) and in the other *Flavobacterium* species reported in this study but the absence

of this fatty acid in *M. odoratus*. As 16S rRNA sequencing results showed that CAM030 was more closely related to *M. odoratus* than to any of the other genera in the *Flavobacteriaceae*, further work is necessary to identify this Antarctic marine isolate. Phylogenetically these three isolates, as members of family *Flavobacteriaceae*, are also part of the larger CFB phylum.

Chemical Characterization of EPS. Figure 3 shows the approximate contribution of neutral sugars, uronic acids, amino sugars, sulfates, and proteins to the EPS produced by each strain. Data are reported as percentages of the total EPS on a weight basis. Neutral sugars accounted for nearly half of all 10 EPS. Uronic acids made up the second most abundant fraction of EPS from CAM030, CAM023, CAM025, CAM036, CAM064, and CAM090. Protein was a major component of EPS from CAM005, CAM006, and CAM015. The protein content in EPS from CAM005 and CAM006 was 40% and 50%, respectively. Purification of these EPS included high-speed centrifugation, followed by pressure filtration,

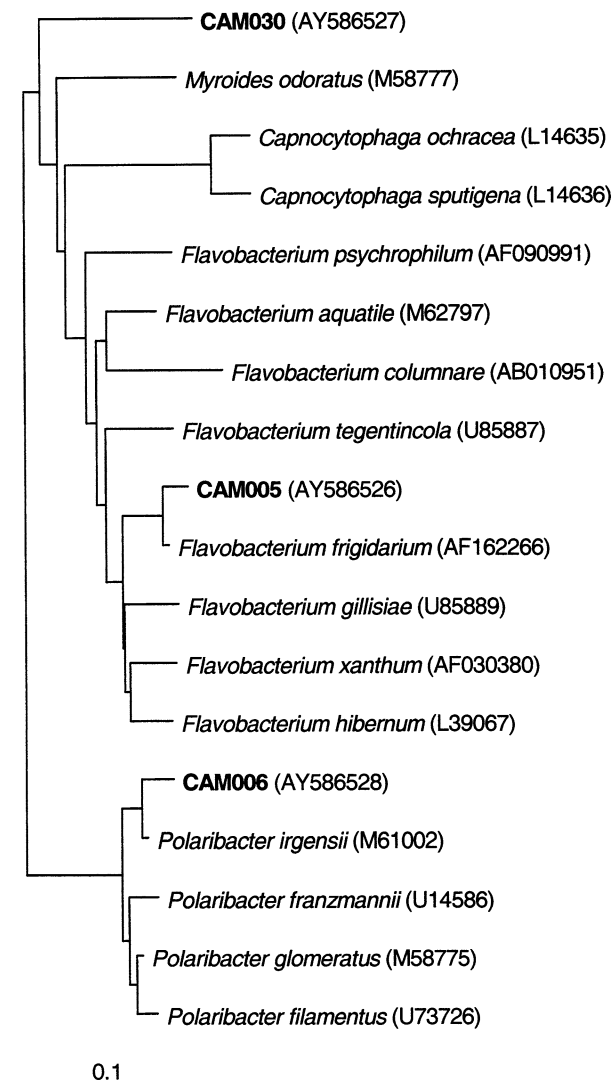


Figure 2. Phylogenetic relationship of Antarctic marine bacterial isolates (CAM030, CAM005, and CAM006) in the family *Flavobacteriaceae* based on 16S rRNA sequences. The tree was created using maximum-likelihood distance clustered by the Neighbor-Joining method. Bar: 0.1 changes per mean nucleotide position. Numbers in parentheses are GenBank nucleotide accession numbers. Outgroup for the analysis was *Escherichia coli* (J01695).

dialysis (0.1 MDa cut-off), and ethanol precipitation. These steps were expected to have removed cellular material and unbound proteins that otherwise would have contributed to elevated protein levels. The protein in these EPS may be bound to the polysaccharide, and further analyses are required to better understand these structures. Fourier transform–infrared analyses showed EPS from CAM003 to have approximately 15% sulfate, while EPS of strains CAM025, CAM036, and CAM064 contained lower percentages of sulfate. Amino sugars were present in EPS from several strains as determined by GC analysis (Fig. 3).

The abundances of monosaccharide components in EPS produced by each isolate were presented as percentage of total monosaccharides (Table 3). Mannose represented the most abundant neutral sugar in the EPS, especially in the EPS of strains CAM003, CAM005, CAM030, CAM006, CAM064, and CAM090. *Pseudoalteromonas* sp. CAM003 contained high percentages of both fucose and mannose, whereas closely related strains CAM023 and CAM025, included glucose as the most abundant neutral sugar. *Pseudoalteromonas* sp. CAM036 contained mannose and glucose as well as galacturonic acid in nearly equal amounts, on a percentage basis. The amino sugars, N-acetyl galactosamine and N-acetyl glucosamine were present in high amounts in CAM030 (approximately 10%). N-acetyl galactosamine was also present in CAM036 and CAM064 at 14% and 11%, respectively (Table 3).

Average molecular weights of EPS are presented in Table 4. Polydispersity gives an indication of the molecular weight distribution or range of size homogeneity within each polymer. As polydispersity increases, there is a larger difference between the sizes of the largest molecules and the smallest molecules. All polymers in this study had a polydispersity below 2.5 based on the molecular weight measurements. These data indicate that for each EPS analyzed, only one polymer was present and this polymer was relatively homogeneous with respect to molecular weight.

The EPS produced by strains CAM003, CAM005, CAM006, CAM015, CAM023, CAM025, and CAM036 had molecular weights greater than 1.7 MDa. CAM030, CAM064, and CAM090 produced EPS with molecular weights less than 0.2 MDa. Molecular weight measurements also indicated a wide range in the average molecular weight of EPS produced by the Antarctic marine isolates examined in this study. Large differences were apparent between the EPS produced by the closely related strains CAM025 and CAM064, with CAM025 producing the EPS with the highest molecular weight (5.7 MDa) and CAM064 producing one of the smallest (0.1 MDa).

Discussion

Characterization of Bacteria. Studies of the diversity of polar sea ice and seawater microbial communities have shown that the “*Gammaproteobacteria*” and CFBs are the dominant taxonomic groupings generally found in this environment, as determined by cultivation – and non-cultivation–dependent techniques [12, 17, 65, 66]. The 10 Antarctic marine bacterial isolates examined in this study belong to these phylogenetic groups, as demonstrated by results from 16S rDNA sequencing and whole-cell fatty acid analysis. Our data also indicated that 6 of the 10 strains investigated were in a clade within the genus *Pseudoalteromonas* particularly common in the Southern Ocean, especially in sea ice [9].

Table 2. Whole cell fatty acids* of ten Antarctic marine bacterial isolates reported as percentage of total fatty acids

Fatty acid [^]	Isolates									
	CAM005	CAM030	CAM006	CAM003	CAM015	CAM023	CAM025	CAM036	CAM064	CAM090
i13:0	1.0	tr [†]	3.0	3.6						18.9
13:0	tr	tr	1.6	3.1	tr	tr	tr	tr	3.4	4.0
β-OH i12:0				1.6	2.0	1.7	1.5	2.3	2.1	tr
14:0	tr	tr	tr	1.1	tr	tr	tr	tr	tr	2.1
β-OH 12:0				3.1	4.3	3.3	3.5	6.5	6.5	2.0
i15:1ω10c	9.3	21.9	13.7			2.4	1.9	2.1	3.5	
a15:1ω10c	1.7	7.9	tr							
i15:0	9.6	18.8	14.9							5.0
a15:0	3.9	4.0	1.1							
α-OH i13:0			2.4							
15:1ω8c				3.5	4.5	3.9	4.0	3.3	2.5	5.3
15:1ω6c			3.7							
α-OH i13:1	2.5	1.7								
15:1ω8t				1.7	tr	1.1	1.5	1.8	1.0	tr
15:1ω6t	1.8	2.1								
15:0	3.2	7.3	4.0	4.6	4.2	3.7	4.7	3.3	3.7	3.2
β-OH 13:0	tr	tr	tr	tr	2.0	2.2	2.2	3.3	2.6	1.7
i16:1ω6c	2.9	tr	tr							
i16:0	4.0	tr		1.1	tr	1.2	tr	tr	tr	
16:1ω9c	1.4			1.0	tr	tr	tr	tr	tr	1.2
16:1ω7c	5.3	1.3	8.3	31.1	26.3	18.8	21.4	26.2	21.7	38.2
16:1ω7t			1.7	7.8	3.8	8.6	7.5	6.7	7.9	
β-OH 14:1									tr	3.5
16:0	1.5	1.2	tr	9.1	9.2	7.6	7.5	7.3	8.6	9.0
β-OH 14:0										2.0
β-OH i15:0	12.2	10.4	20.6							
α-OH i15:0		6.8	14.4							
α-OH a15:0	2.0	1.7	2.1							
17:1ω8c				20.5	26.9	29.2	29.3	24.2	21.0	10.2
17:1ω8t				3.8	4.4	6.5	6.8	4.1	4.1	2.2
17:0	tr			4.5	6.5	5.7	4.8	5.6	5.1	3.2
β-OH 15:0	1.5	3.8	3.1							
α-OH 15:0			1.4							
α-OH i16:0	11.4	4.4	4.4							
18:1ω9c	tr			tr	tr	tr	tr		tr	1.7
18:1ω7c	1.3	tr		5.4	5.8	4.2	3.5	3.3	6.3	8.6
β-OH 16:0	7.3	tr	tr							
β-OH i17:0	12.9	4.4	1.2							

*listed in order of retention time.

[†]tr: <1% total area.

[^]Also present in trace amounts in the following strains: CAM005: i14:0, a17:1, i17:1, β-OH 17:1; CAM030: a17:1, i17:1, β-OH i17:1, β-OH 17:1; CAM006: i14:0, α-OH 13:0, β-OH i17:1; CAM003: i17:0, 18:0; CAM015: i17:0, 18:0; CAM023: i17:0, 18:0; CAM064: 18:0; CAM090: i14:0, 18:0.

Characterization of EPS. The EPS produced by 10 Antarctic strains were diverse with respect to crude chemical (Fig. 3) and monosaccharide (Table 3) composition. In a study of *Pseudomonas antarctica*, NF₃, [14, 16], crude chemical analysis of exopolymers produced by this strain showed a high protein content; individual monosaccharide data were not presented. Capsular material, generally high in proteins [44], was included in the fraction analyzed in the earlier study. As our study examines EPS excreted into the media, comparison of these two data sets is not possible. A study by Corsaro et al. [22] examined the exopolymers produced by another Antarctic marine isolate, *P. haloplanktis* TAC 125, and showed the polysaccharide component to consist of mannose with traces of glucose. Because the fraction

examined in this study also included the LPS layer, it is not possible to compare these results with those of our study. Excreted EPS produced by non-marine strains was analyzed in a study by Ford et al. [30]. Uronic acids made up less than 1% of the total carbohydrates. This is significantly lower than the uronic content of the EPS examined in the present study.

The EPS produced by several deep-sea hydrothermal vent bacterial isolates have been well characterized [60], including two from genus *Pseudoalteromonas*. Although the strains belong to two different species, the EPS produced by these deep-sea isolates were very similar with respect to crude chemical and monosaccharide composition. These results contrast with those presented in a previous study [55] and this one, in which EPS produced

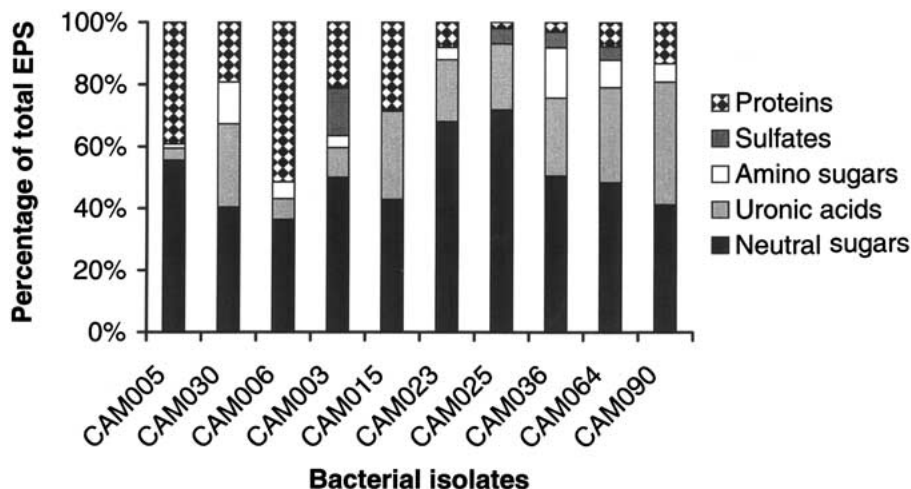


Figure 3. Crude chemical composition of exopolysaccharides produced by Antarctic marine bacteria. Neutral sugars, uronic acids, proteins were determined by colorimetric analysis. Sulfate content was estimated from Fourier transform–infrared spectra. Amino sugar content was determined by gas chromatography.

by closely related strains in the genus *Pseudoalteromonas* vary substantially. Sulfate content was noted as the only structural difference between the polymers in the two hydrothermal vent strains, and sulfate content may have had an influence on the intrinsic viscosity of these polymers, because it also varied. Another study found that two closely related hydrothermal vent bacteria from different subspecies of *Alteromonas macleodii*, and isolated from different sites, produced very different EPS under the same growth conditions [20]. These EPS show a high metal binding capacity [52]. This quality has been attributed to chemical characteristics also present in the EPS produced by the Antarctic marine isolates examined in this study, including carboxyl groups present in uronic acids, amides present in amino sugars, and sulfates—and to a lesser extent hydroxyl groups—which are abundant in all monosaccharides. These EPS are thought to aid in attaching bacteria to the hydrothermal chimney as well as lowering the concentrations of toxic heavy metals in the microenvironment.

Monosaccharide analyses of the 10 EPS examined in this study showed that pentoses (ribose and xylose), hexoses (glucose, galactose, mannose, rhamnose, and fucose), amino sugars (N-acetyl glucosamine and N-acetyl galactosamine), and uronic acids (glucuronic acid and galacturonic acid) were present, with glucose, galactose, or mannose being the most abundant monosaccharides. These sugars are typically found in bacterial EPS [44]. A survey of EPS produced by pure cultures of seawater isolates by Kennedy and Sutherland [45] shows that this is also the case for EPS produced by marine bacteria. Arabinose was present, to varying degrees, in all EPS examined in this study, and xylose was present as a minor component in CAM030, CAM090, and CAM090. Arabinose and xylose are not commonly found in bacterial EPS [44]. The significance of these findings requires further investigation.

The Ecological Role of EPS. In both sea ice and marine particulate material, bacteria are seen as an integral part of the trophodynamics and are credited with

Table 3. EPS monosaccharides from 10 Antarctic marine bacterial isolates reported as percentage of total sugars

Monosaccharides	Isolates									
	CAM005	CAM030	CAM006	CAM003	CAM015	CAM023	CAM025	CAM036	CAM064	CAM090
Ara*	5	6	2	4	10	12	3	3	4	13
Rib				2			1			
Rha		1		6	6		5			2
Fuc			11	29			1			
Xyl		2			1					2
Man	74	48	33	40	36	2	1	24	64	41
Gal	3	4	38		4	1	5	1	4	5
Glc	8	9	4	16	38	75	52	26	8	10
Gal A		2				5	30	30		
Glc A	8	10	6	1	3	3			6	20
N-A Gal		10	1	1	3	2	1	14	11	7
N-A Glc	1	8	4	1				1	2	

*Ara: arabinose; Rib: ribose; Rha: rhamnose; Fuc: fucose; Xyl: xylose; Man: mannose; Gal: galactose; Glc: glucose; Gal A: galacturonic acid; Glc A: glucuronic acid; N-A Gal: N-acetyl-galactosamine; N-A Glc: N-acetyl-glucosamine.

Table 4. Molecular weights of exopolysaccharides produced by Antarctic marine bacteria as determined by size exclusion chromatography

Isolate	Molecular weight (MDa)	I (polydispersity)
CAM005	1.81	1.249
CAM030	0.19	1.891
CAM006	2.10	1.224
CAM003	1.80	2.075
CAM015	2.80	1.143
CAM023	1.80	1.381
CAM025	5.70	2.496
CAM036	1.70	1.616
CAM064	0.10	1.172
CAM090	0.08	1.326

a critical importance in the organic carbon cycle [5, 35]. Bacterial polysaccharides form the fibrillar framework, act as glue in the ultrastructure and provide the structural network for microbial associations within marine aggregates [7, 24, 28, 39]. Studies of sea ice microbial communities also have found bacteria to be strongly particle associated [34, 67]. More recent results showed that microbial EPS was produced in high concentrations, and it has been suggested that it played an important role in cryoprotection [42, 47].

Exopolymers produced by marine bacteria generally contain 20–50% of the polysaccharide as uronic acid [45]. Uronic acids contain an acidic carboxyl group that is ionizable at seawater pH. This contributes a negative charge to the overall polymer [23]. Until recently, sulfate was thought to occur only in polysaccharides produced by Archaea and Cyanobacteria, and its presence in polymers produced by prokaryotes is seen as very uncommon [3] (Arias et al. 2003). When sulfate is present as a functional group, it also contributes to the anionic quality of these EPS in seawater [50]. The overall negative charge gives the molecule a “sticky” quality. The “stickiness” is important in terms of the affinity of these EPS for binding to cations such as dissolved metals [18]. Based on the data presented, the EPS from our isolates, which contain uronic acids as well as sulfates, would be in a highly polyanionic state in the marine environment.

The availability of iron (Fe^{+3}) as a trace metal is of critical importance in the Southern Ocean where it is known to limit primary production [62]. As much as 99% of dissolved iron in the ocean is bound to organic ligands [71]. Our results indicate that several of the EPS produced by Antarctic bacterial isolates derived from Southern Ocean particulate material included uronic acids and/or sulfates (CAM030, CAM006, CAM23, and CAM036; Fig. 3). These EPS may be acting as ligands for cations such as iron and other trace metals in the Southern Ocean environment. A study of Arctic sea ice demonstrated that photosynthesis rates by phytoplankton from under the ice were stimulated to similar levels by sea

ice extracts as they were by the chelator, ethylenediamine tetra-acetic acid and trace metals [1]. From those results, the authors suggested that a natural “conditioning agent” is produced within the bottom-ice algal layer that enhances phytoplankton growth. Sea ice bacterial communities and high amounts of EPS are concentrated in these layers [47, 48]. It is not clear whether dissolved iron is a limiting nutrient for primary production in sea ice microbial communities. Isolates derived from pack ice (CAM003 and CAM025) or from fast ice (CAM005, CAM064, and CAM090) produced EPS containing uronic acids and/or sulfates. These EPS would be negatively charged in the marine environment and could possibly have some role in enhancing primary production. The overall significance of this finding requires further investigation.

Exopolysaccharides produced by sea ice isolates CAM003, CAM005, and CAM025 were shown, by molecular weight analysis to be 1.8, 1.8, and 5.7 MDa, respectively. Most exopolysaccharides produced by marine bacteria have a molecular weight in the 0.1–0.3 MDa range [23], therefore the EPS produced in liquid culture by three sea ice isolates examined in this study are between 5 and 50 times larger than average. The structure and properties of EPS are influenced by the length of the polymer chain, that is, the molecular weight [21]. As the length of the polymer increases, there is a greater opportunity for complex entanglement of the chains and intramolecular associations, and these contribute to the tertiary structure and physical behavior of the polymer [68]. A fungal strain, *Phoma herbarum*, isolated from Antarctic soil produced a homosaccharide of glucose with a molecular weight of 7.4 MDa [63]. The authors of this study suggested the fungal EPS could provide a cryoprotective role in the harsh Antarctic environment where the availability of liquid water and temperatures are extremely low. Similarly, the freezing processes in sea ice result in brine channels where temperature is very low and salinity is high because of brine. Strain CAM025 was observed to produce 30 times as much EPS at -2° and 10°C compared to 20°C [55]. Whether this increased EPS production at low temperature is a cold adaptation mechanism for this strain, requires further investigation. Bacterial EPS production in brine channels and perhaps other cold, high brine ecosystems may provide a barrier against the environmental extremes experienced by the bacterial cell by modifying water properties near the cell.

An exopolysaccharide, known as muran, is produced by the halophilic bacterium, *Halomonas maura* [3]. When this strain was grown in media containing high salt concentration (2.5%, w/v), it produced a high molecular weight (4.5 MDa) EPS that contained glucose, mannose, and galactose as well as high amounts of glucuronic acid (8%, w/w) and sulfates (6.5%, w/w). This polysaccharide was able to bind a range of heavy metal cations. The authors

also noted the stability of muran under different stress conditions including high salt concentrations and during freezing/thawing. There are similarities between muran and several of the EPS produced by our Antarctic marine isolates in terms of chemical composition. Further work is necessary to more accurately define the structure of the Antarctic marine bacterial EPS and to relate these findings to the function of these molecules in the natural environment. As yet, it is unclear how these polysaccharides may be acting mechanistically as organic ligands, protectants against low temperature or high salinity, or whether the size of these EPS is related to their ecological role.

Biotechnological Potential. Polysaccharides with biotechnological potential have been derived from bacteria isolated from extreme environments such as hydrothermal vents and hypersaline habitats as mentioned above. Because of their physicochemical properties, which include stabilizing, thickening, gelling, coagulating, adhesion, and water retention, bacterial EPS have been used in many commercial applications for example in the pharmaceutical, food and environmental industries [29, 36, 69]. Exopolysaccharides produced by Antarctic bacteria examined in the current study appear to be diverse with respect to chemical composition. These EPS also share chemical characteristics such as high uronic acid content, sulfate content, and molecular weight found in polysaccharides that have been exploited commercially. A better understanding of the chemical structure of these complex molecules is the first step in the discovery of potential biotechnological uses, as well as their role in the Antarctic marine environment.

Conclusions

Bacteria living in Antarctic sea ice and particulate material in the Southern Ocean experience extreme ecological pressures. Exopolysaccharides are produced by many marine bacteria isolated from this environment. This study is a first step toward understanding the role of these substances in the Antarctic marine environment. A set of 10 isolates were found to belong to the “*Gamma-proteobacteria*” and CFBs, which are dominant taxonomic groups in this environment. Study of the chemical composition of EPS produced by these isolates shows that there are distinct differences in terms of crude chemical composition, size, and monosaccharide content, even for those EPS produced by closely related strains. Whether the specific ecological role for these bacterial EPS includes cryoprotection in the high-salinity, low-temperature brine channels in sea ice, or trace metal binding in marine aggregates remains to be elucidated. Increased knowledge of the role of Antarctic bacterial EPS will also provide insight into possible commercial uses for these novel polymers.

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