APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 1996, p. 3265–3269 Vol. 62, No. 9
0099-2240/96/$04.00+0


# Chemical Composition of Two Exopolysaccharides from *Bacillus thermoantarcticus*

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Received 1 April 1996/Accepted 22 June 1996

**The thermophilic bacterium *Bacillus thermoantarcticus* produces two exocellular polysaccharides (EPS 1 and EPS 2), which can be obtained from the supernatant of liquid cultures by cold-ethanol precipitation, in yields as high as 400 mg liter$^{-1}$. The EPS fraction was produced with all substrates tested, although a higher yield was obtained with mannose as the carbon and energy source. The EPS content was proportional to the total biomass. On a weight basis, EPS 1 and EPS 2 represented about 27 and 71%, respectively, of the total carbohydrate fraction. EPS 1 is a sulfate heteropolysaccharide containing mannose and glucose in a relative molar proportion of 1.0 and 0.7, respectively. EPS 2 is a sulfate homopolysaccharide containing mannose as the major component. The absolute configurations of hexoses were shown to be D for both EPSs. Nuclear magnetic resonance spectra confirmed the presence of α-D-mannose and β-D-glucose in EPS 1 and only α-D-mannose in EPS 2. In addition, $^1$H nuclear magnetic resonance analysis and chemical analysis indicated the presence of pyruvic acid in EPS 2.**

Microbial exopolysaccharides (EPSs) are of widespread occurrence and can readily be prepared by fermentation. Increasing attention is being paid to these molecules because of their bioactive role and their wide range of commercial applications, which include uses as food additives (xanthan, alginate, dextran, glucomannan) and in nonfood applications such as viscosity control, gelation, and flocculation (xanthan, agar, glucan) (17).

Studies of the chemical structure of these molecules, substituent identification, and chemical and physical properties are essential for understanding their possible applications and eventually to improve them by using fermentation technology and genetically altered microbial strains (18).

A few examples of extremophiles that produce exopolysaccharides include *Methanosarcina* species and some species of *Haloferax*, *Haloarcula*, and *Sulfolobus* (1, 11, 15). In this paper, we report the production and partial characterization of two exopolysaccharides from *Bacillus thermoantarcticus*, a new thermophilic bacterium isolated close to the crater of Mount Melbourne, Antarctica (10).

## MATERIALS AND METHODS

**Microorganism and culture conditions.** *B. thermoantarcticus* was grown in a 1-liter static culture (batch culture) and in a 3-liter fermentor (Chemap) with low-level mechanical agitation (100 rpm) and aeration of 20 ml min$^{-1}$ liter of broth$^{-1}$ (fermentor culture) at 65°C and pH 6.0. The culture medium contained 3 g of NaCl liter$^{-1}$, 6 g of glucose, mannose, or sucrose liter$^{-1}$, and 0.1 g of yeast extract liter$^{-1}$. The glucose medium supplemented with 20 g of agar liter$^{-1}$ was used for the preparation of agar plates. The colonies, grown on plates, were observed with a Leic-a Wild M 8 stereomicroscope, which showed the presence of a mucilaginous layer.

**Production of EPSs.** Microbial growth and EPS production were monitored quantitatively for both batch and fermentor cultures by sampling 10 ml of culture broth at 0, 8, 16, 24, and 48 h. Biomass production was monitored by reading the $A_{450}$. EPS production on cell-free culture broth was tested by the phenol-sulfuric acid method with glucose as the standard (3).

**Isolation and purification of EPSs.** Cells were harvested by centrifugation (9,800 × *g* for 20 min). The liquid phase was treated with 1 volume of cold ethanol added dropwise with stirring. The alcoholic solution was kept at −18°C overnight and then centrifuged at 15,300 × *g* for 30 min. The pellet was dissolved in hot water (1/10 initial volume), and the soluble fraction accounted for 95% of the total polymers. The solubilization procedure was repeated. The final aqueous solution was dialyzed against tap water (for 48 h) and distilled water (for 20 h), lyophilized, and weighed. The sample was tested for carbohydrate, protein, and nucleic acid contents. The polysaccharide was purified by gel chromatography (Sephadex G-50; 2.5 by 50 cm) with $H_2O$-pyridine-acetic acid (500:5:2, by volume) as the eluant, with 5-ml fractions collected at a flow rate of 6 ml h$^{-1}$; the fractions were subjected to anion-exchange chromatography (DEAE-Sepharose CL-6B; 1.5 by 40 cm) and eluted (10-ml fractions) with 0.1 liter of $H_2O$ and 1 liter of a NaCl gradient from 0 to 1 M at a flow rate of 12 ml h$^{-1}$. The fractions were tested qualitatively for carbohydrate by a spot test on thin-layer chromatography involving an α-naphthol spray and quantitatively by the method of Dubois et al. (3). The α-naphthol-positive fractions were pooled, exhaustively dialyzed against water, freeze-dried, and weighed. This material was used for all analytical work.

**Colorimetric assay.** The carbohydrate content was measured by the method of Dubois (3), by monitoring the $A_{490}$ with glucose as a standard. The total protein content was estimated by using the Bradford reagent (Bio-Rad) with bovine serum albumin as a standard. The nucleic acid content was tested spectrophotometrically by monitoring the $A_{260}$. Pyruvate was detected after polysaccharide hydrolysis (100°C for 3 h) with a 0.5% (wt/vol) solution of 2,4-dinitrophenylhydrazine in 2 M HCl (4). The presence of sulfate was identified by the method of Silvestri et al. (14).

**Molecular weight estimation.** Molecular weight was estimated by two methods. The first was gel filtration on a Sepharose CL-6B column (1 by 80 cm) with $H_2O$-pyridine-acetic acid (500:5:2, by volume) as the eluant. Fractions were collected at 3.7 ml h$^{-1}$ and tested by a spot test on thin-layer chromatography involving an α-naphthol spray. The second method was density gradient centrifugation (12) with a sucrose gradient from 0 to 50% (wt/vol) at 130,000 × *g* for 16 h. Centrifuge tubes were fractionated in 0.2-ml fractions diluted with water, dialyzed against water for 72 h, and tested for the presence of carbohydrate as reported above. In both experiments, 10 mg of EPS and a mixture of dextrans for calibration curves (10 mg each of T-700 [molecular weight, 670,000], T-400 [410,000], and T-150 [154,000]) were used.

**Sugar analysis.** Hydrolysis of EPSs was performed with 2 M trifluoroacetic acid at 120°C for 2 h. Sugar components were identified by thin-layer chromatography and high-pressure anion-exchange pulsed amperometric detection (HPAE-PAD) with sugar standards for identification and calibration curves. The thin-layer chromatogram was developed with the following solvent systems: acetone-butanol-$H_2O$ (8:2:2 by volume) for neutral sugars; butanol-$H_2O$-acetic acid (3:1:1 by volume) for acidic sugars; and butanol-ethanol-$H_2O$ (5:3:2 by volume) for oligosaccharides (16). Sugars were visualized by spraying the plates with α-naphthol. A Carbopac PA1 column equipped with a Dionex (Sunnyvale, Calif.) pulsed amperometric detector was eluted isocratically with 15 mM NaOH for neutral sugars and 100 mM NaOH and 150 mM sodium acetate for acidic sugars (2).

**Methylation analysis.** Polysaccharide methylation was carried out by previ-

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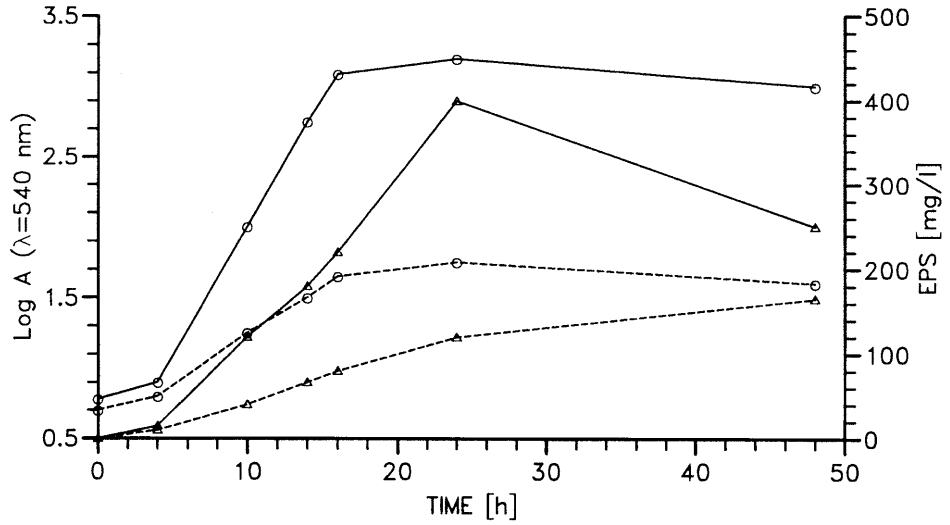


FIG. 1. Growth curves (○) and polysaccharide production (△) by *B. thermoantarcticus* in batch culture (– – – –) and fermentor culture (——). EPS represents polysaccharide dissolved in the culture medium.

ously described methods (6, 19). The methylated material (0.5 mg) was hydrolyzed with 2 M trifluoroacetic acid at 120°C for 2 h and then transformed in partially methylated alditol acetates by reduction with sodium borohydride, followed by acetylation with acetic anhydride-pyridine (1:1 [vol/vol]) at 120°C for 3 h. Unambiguous identification of sugars was obtained by gas-liquid chromatography and gas chromatography-mass spectrometry with sugar standards. Gas-liquid chromatography runs were performed on a Hewlett-Packard 5890A instrument, fitted with a flame ionization detector and equipped with an HP-5-V column and with a $N_2$ flow of 100 ml $min^{-1}$. The temperature program used was 170°C for 1 min, from 170 to 180°C at 1°C $min^{-1}$, 180°C for 1 min, and from 180 to 210°C at 4°C $min^{-1}$. Gas chromatography-mass spectrometry was performed on a Hewlett-Packard 5890-5970 instrument equipped with an HP-5-MS column and with a $N_2$ flow of 50 ml $min^{-1}$; the temperature program used was 170°C for 1 min and from 170 to 250°C at 3°C $min^{-1}$.

**Absolute configuration.** The absolute configuration of the sugars was determined as described by Leontein et al. (8), with optically active (+)-2-butanol, by gas-liquid chromatography of their acetylated-(+)-2-butyl glycosides (5). For gas-liquid chromatography runs, the same instrument and conditions described for the methylation analysis were used. Retention times were determined by comparison of the sample with authentic standards.

**Spectroscopic analysis.** The infrared spectra of polysaccharides (100-mg KBr tablet) were recorded at room temperature with a Fourier transform infrared Bio-Rad spectrometer. UV spectra of EPSs were obtained by reading the $A_{350}$ to $A_{210}$ of aqueous solutions (3 mg $ml^{-1}$) on a Varian DMS-90 instrument. The optical rotation value was obtained on a Perkin-Elmer 243 B polarimeter at 25°C in water. Nuclear magnetic resonance (NMR) spectra were obtained on a Bruker AMX-500 instrument (500.13 MHz for $^1H$ NMR and 125.75 MHz for $^{13}C$ NMR) at 70°C. Before the analysis, EPS samples were exchanged twice in $D_2O$ with intermediate lyophilization and then dissolved in 500 μl of $D_2O$ to a final concentration of 40 mg $ml^{-1}$. Chemical shifts were reported in parts per million relative to sodium 2,2,3,3-$d_4$-(trimethylsilyl)propanoate for $^1H$ NMR and $CDCl_3$ for $^{13}C$ NMR. The $^1J_{H\text{-}1,C\text{-}1}$ values were determined by heteronuclear multiple quantum coherence inverse detected experiments (9, 13).

## RESULTS AND DISCUSSION

**EPS production.** EPS production was conducted in a 3-liter fermentor and in a 1-liter batch culture. The production curves are shown in Fig. 1. The EPS production in fermentors reached 400 mg $liter^{-1}$ in the presence of mannose as the carbon source; this level was 2.4-fold higher than that obtained in batch culture. In the latter case, the curve reached its maximum during the late stationary phase, while in fermentors the EPS production increased with increasing cell density and reached its maximum at the beginning of the stationary phase. It is noteworthy that the highest production of EPS in fermentors was obtained when the principal physiologic parameters ($O_2$, stirring, and pH control) were also at the optima for biomass production.

Table 1 shows the yields of EPS with different carbon sources in the medium. The best minimal medium for EPS formation was that containing mannose as the carbon source; with sucrose, the EPS content was half that obtained with mannose, and the worst was glucose. No influence on EPS composition was observed when different minimal media were used.

**EPS purification.** The polysaccharide fraction was tested for carbohydrate (95%), protein (2%), and nucleic acid (trace amounts) content. The polymeric fraction was desalted on Sephadex G-50 with a yield of 80% and then chromatographed on DEAE-Sepharose CL-6B with a yield of 73%. The elution profile is shown in Fig. 2. Three fractions were obtained: the first (EPS 1) was eluted only in water, the second (EPS 2) eluted at 0.2 M NaCl, and the third (EPS 3) eluted at 0.7 M NaCl. On a weight basis, EPS 1 and EPS 2 represented about 27 and 71% of the total polysaccharide, respectively; EPS 3 was a minor component, constituting less than 2% of the starting material. The three fractions were assayed for carbohydrate and protein content: EPS 1 and EPS 2 contained at least 96% sugars and trace amounts of proteins (less than 0.2%), while the EPS 3 fraction showed a trace amount of carbohydrate.

**Characteristics of polymers.** The molecular weight of EPS 2 was estimated from a calibration curve of standard dextrans obtained by gel filtration on Sepharose CL-6B and also by

TABLE 1. Production of EPS in batch and fermentor culture[a]

| Carbon source | Batch culture | | Fermentor culture | |
|---|---|---|---|---|
| | Amt of EPS (mg $liter^{-1}$) | $OD_{540}$[b] | Amt of EPS (mg $liter^{-1}$) | $OD_{540}$ |
| Mannose | 165 | 0.750 | 400 | 1.600 |
| Glucose | 65 | 0.620 | 150 | 1.300 |
| Sucrose | 100 | 0.690 | 250 | 1.450 |

[a] The total carbohydrate fraction was determined by the phenol-sulfuric acid method (3).

[b] $OD_{540}$, optical density at 540 nm.

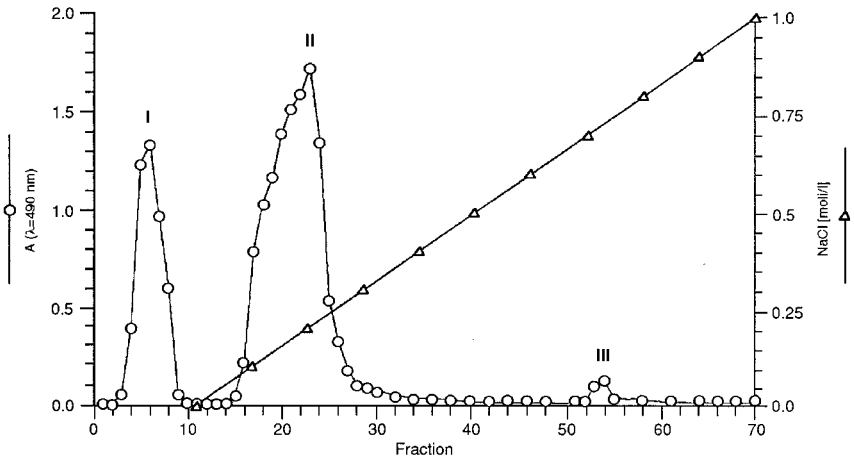


FIG. 2. Chromatography of polysaccharide fractions on Sepharose DEAE CL-6B. The bed dimension was 1.5 by 40 cm. The eluant was $H_2O$ with a linear salt gradient to 1 M NaCl. The flow rate was 12 ml $h^{-1}$, and the fraction volume was 10 ml.

density gradient centrifugation (12). In both cases, the molecular weight was approximately $3.0 \times 10^5$. The infrared spectrum of EPS 2 is similar to those of other bacterial polysaccharides reported in the literature (13). A broad absorption band attributable to S=O was observed at 1,240 $cm^{-1}$. The presence of sulfate groups in EPS 2, as well as in EPS 1, was confirmed by a positive color reaction with sodium rhodizonate (14).

The UV spectra of EPS 1 and EPS 2 did not indicate any strong absorption peaks in the range of 350 to 210 nm.

The presence of pyruvate in EPS 2 was detected in trace amounts by the method of Duckworth (4) and was confirmed by the presence of a minor signal at δ 1.50 in the $^1H$ NMR spectrum. The quantities of pyruvate varied with different preparations.

The optical rotation of EPS 2 was $[\alpha]_D^{20} = -90°$ (concentration of 5 mg ml of $H_2O^{-1}$).

**EPS composition.** Hydrolysis of EPS 1 with 2 M trifluoroacetic acid yielded mannose and glucose as principal constituents in a relative proportion of 1:0.7; hydrolysis of EPS 2, performed under the same conditions as EPS 1, yielded mainly mannose and a trace amount of glucose.

Analysis of the partially methylated alditol acetates, obtained from the permethylated EPSs after acid hydrolysis, revealed for EPS 1 the presence of a terminal glucose; the chain sugars were 1,2-linked mannose, 1,4-linked glucose, 1,3-linked mannose, and 1,6-linked mannose, and 1,3,4-linked glucose and 1,2,6-linked mannose represented branch points in the molecule. The relative proportions of these sugars were 0.9: 0.5:0.1:0.2:0.5:0.1:1.0. In an analogous manner, this analysis revealed, for EPS 2, the presence of a terminal mannose; the chain sugars were 1,2-linked mannose, 1,4-linked mannose, and 1,6-linked mannose, and the branch point was a 1,2,6-linked mannose. Their relative proportions were 1.0:0.5:0.2: 0.1:0.8 (Table 2).

The absolute configuration of hexoses in EPS 1 and EPS 2 was shown to be D when analyzed as their respective acetylated (+)-2-butyl glycosides from methylation analysis and NMR spectra. It was evident that the sugar residues in both EPSs were pyranoids.

**NMR analysis.** The $^1H$ and $^{13}C$ NMR spectra of both polysaccharides were complex. The $^1H$ spectrum of EPS 1 showed, inter alia, seven anomeric signals at δ 5.27 ($J_{1,2}$ n.r. [not resolved]), δ 5.10 ($J_{1,2}$ n.r.), δ 5.07 ($J_{1,2}$ n.r.), δ 4.92 ($J_{1,2}$ n.r.), δ 4.73 ($J_{1,2}$ 8 Hz), δ 4.59 ($J_{1,2}$ 8.1 Hz), and δ 4.52 ($J_{1,2}$ 8 Hz), while for EPS 2 the observable signals were at δ 5.31 ($J_{1,2}$ n.r.), δ 5.15 ($J_{1,2}$ n.r.), δ 5.12 ($J_{1,2}$ n.r.), and δ 5.08 ($J_{1,2}$ n.r.). To determine the anomeric configuration, the chemical shifts of anomeric signals in $^1H$ and $^{13}C$ NMR spectra of EPS 1 and EPS 2 were analyzed together with their $^3J_{H\text{-}1,H\text{-}2}$ and $^1J_{H\text{-}1,C\text{-}1}$. Sugar components of EPS 1 were labelled from A to G, and those of EPS 2 were labelled from A to D, in both cases with respect to decreasing chemical shifts (Table 3).

TABLE 2. Gas chromatography-mass spectrometry analysis of the permethylated EPS from *B. thermoantarcticus*

| Retention time (min) | Methylated sugar | Proportion of sugar (%) in: | |
|---|---|---|---|
| | | EPS 1 | EPS 2 |
| 5.3 | 2,3,4,6-Tetra-*O*-MeGlc | 28.0 | |
| 5.30 | 2,3,4,6-Tetra-*O*-MeMan | | 27.79 |
| 7.15 | 3,4,6-Tri-*O*-MeMan | 17.70 | 17.40 |
| 7.20 | 2,4,6-Tri-*O*-MeMan | 6.30 | |
| 7.45 | 2,3,6-Tri-*O*-MeMan | | 6.40 |
| 7.50 | 2,3,6-Tri-*O*-MeGlc | 4.20 | |
| 7.90 | 2,3,4-Tri-*O*-MeMan | 16.80 | 17.70 |
| 8.90 | 2,6-Di-*O*-MeGlc | 2.50 | |
| 10.80 | 3,4-Di-*O*-MeMan | 24.40 | 29.20 |

[a] Retention time of the corresponding alditol acetate under the conditions described in Materials and Methods.

TABLE 3. Chemical shifts and coupling constants of anomeric signals in $^1H$ and $^{13}C$ NMR spectra of EPS(s) from *B. thermoantarcticus*

| Residue | EPS 1 | | | EPS 2 | | |
|---|---|---|---|---|---|---|
| | δ H-1/C-1 | $^3J_{H\text{-}1,H\text{-}2}$[a] | $^1J_{H\text{-}1,C\text{-}1}$[a] | δ H-1/C-1 | $^3J_{H\text{-}1,H}$ | $^1J_{H\text{-}1,C\text{-}1}$ |
| A | 5.27/101.1 | (bs) | 168 | 5.31/101.1 | (bs) | 170 |
| B | 5.10/98.9 | (bs) | 171 | 5.15/102.4 | (bs) | 173 |
| C | 5.07/102.6 | (bs) | 173 | 5.12/99.0 | (bs) | 171 |
| D | 4.92/100.1 | (bs) | 168 | 5.08/102.6 | (bs) | 168 |
| E | 4.73/103.4 | (d) 8.0 | 165 | | | |
| F | 4.59/103.2 | (d) 8.1 | 160 | | | |
| G | 4.52/103.5 | (d) 8.0 | 162 | | | |

[a] Coupling constants are in hertz. bs, broadened singlet; d, doublet.

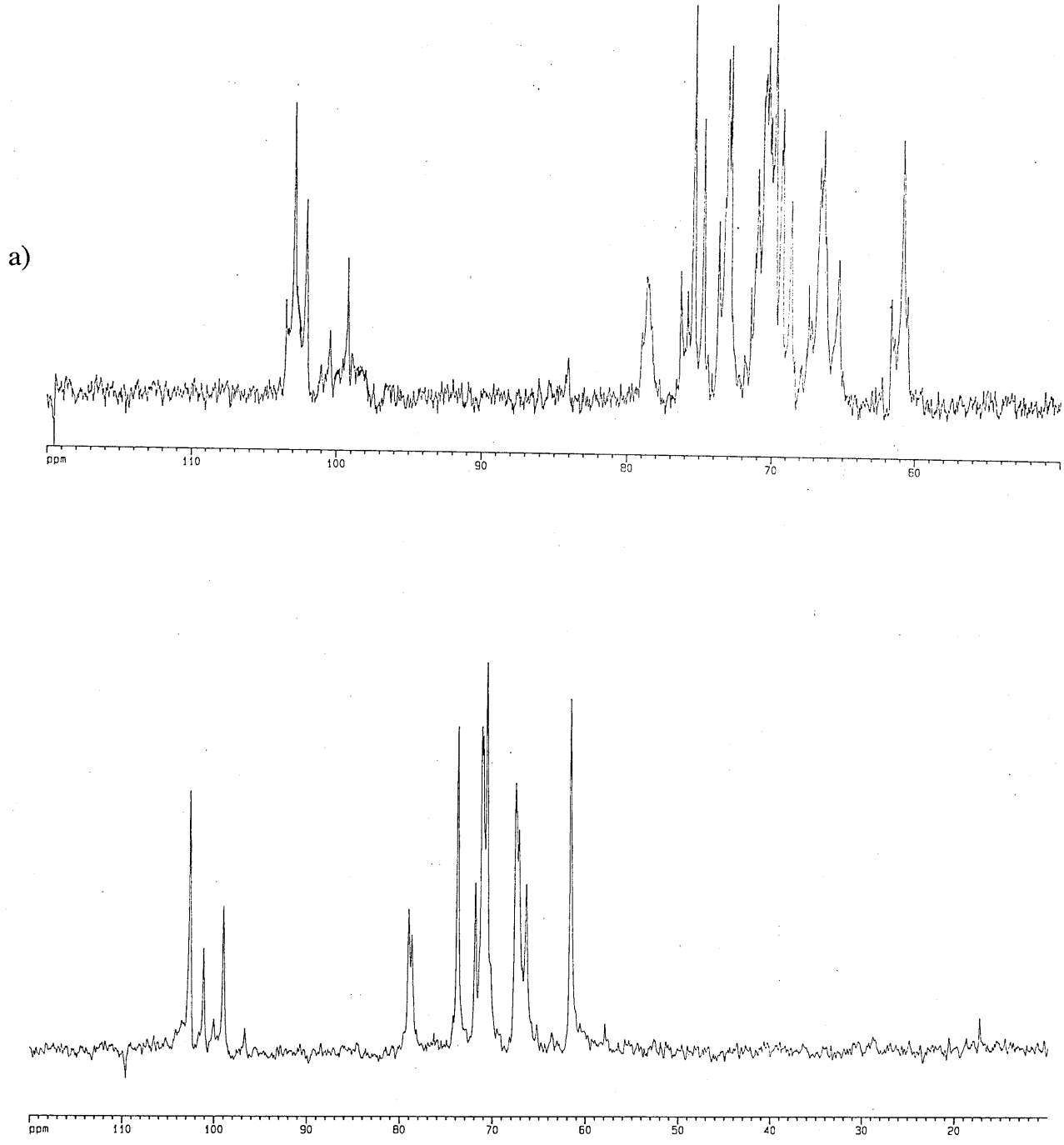


FIG. 3. $^{13}C$ NMR spectra of EPS 1 (a) and EPS 2 (b).

In EPS 1, residues from A to D exhibited an α-*manno* configuration because of their small $^{3}J_{H\text{-}1,H\text{-}2}$ and larger $^{1}J_{H\text{-}1,C\text{-}1}$ values (≈170 Hz), while residues E to G showed a β-*gluco*/*galacto* configuration because of their larger $^{3}J_{H\text{-}1,H\text{-}2}$ (≈8 Hz) and small $^{1}J_{H\text{-}1,C\text{-}1}$ values (≈160 Hz). In the same way, for EPS 2 all residues showed an α-*manno* configuration; in fact, $^{3}J_{H\text{-}1,H\text{-}2}$ were not measurable and $^{1}J_{H\text{-}1,C\text{-}1}$ were approximately 170 Hz (Fig. 3) (7).

There exists relatively little information about polysaccharide production by thermophiles, and until now it was not clear whether these organisms were likely to prove to be a useful source of polymers. However, the number of extremophiles is rapidly increasing, making available new resources for the exploitation of molecules with interesting properties. *B. thermoantarcticus* produces two different polysaccharides named EPS 1 and EPS 2, both containing sulfate groups. EPS 1 is a heteropolysaccharide whose repeating unit consists of four different α-D-mannoses and three different β-D-glucoses. It seems to be closely related to some xanthan polymers. In fact, as reported in the literature (18), some possible repeating units of xanthan molecules consist mainly of glucose and mannose, and some of them can be substituted with acetyl or pyruvate or

both. EPS 2 is a mannan with a molecular weight of ≈300,000, and four different α-D-mannoses were found as the repeating unit. It also showed trace amounts of pyruvic acid. More detailed information about EPS structures will be available when NMR spectroscopic studies, presently under way, are completed. Therefore, it will be of interest, after full structure elucidation, to obtain information about the physical properties of these new polysaccharides.

## ACKNOWLEDGMENTS

This research was conducted within the framework of the Italian National Program for Antarctic Research.

We are grateful to I.C.M.I.B.-N.M.R. Service of C.N.R. We thank I. Romano, V. Calandrelli, and E. Pagnotta for technical assistance; A. Panico for her skillful assistance in some experiments; and R. Turco for artwork.

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