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STRUCTURE OF TEICHOIC ACID FROM THE MARINE PROTEOBACTERIUM *Sulfitobacter brevis* KMM 6006

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A glycopolymer was isolated from the marine gram-negative bacterium Sulfitobacter brevis *strain KMM 6006 and found to be teichoic acid containing ribitol, glycerine, and* N*-acetyl-D-glucosamine. The polymeric chain consisted of alternating 1,5-poly(4-*N*-acetyl-*β*-D-glucosaminylribitophosphate) and 1,3-poly(glycerophosphate) based on* ^{13}C *and* ^{31}P *NMR spectroscopy of the native polymer and the glycoside obtained by its dephosphorylation.*

Key words: *Sulfitobacter brevis*, NMR spectroscopy, teichoic acid, glycoside.

The genus *Sulfitobacter* was established in 1995 for marine gram-negative heterotrophic bacteria isolated from the H_2S/O_2 zone of the Black Sea. It included initially bacteria of a single species, *S. pontiacus*, which have the unique capability to grow in the presence of high sulfite concentrations [1]. The four species of sulfitobacteria that were described in the following years were isolated from various sources, e.g., *S. mediterraneus*, from samples of Mediterrean Sea water [2]; *S. brevis*, from a saline lake of eastern Antarctica [3]; *S. delicatus*, from tissue of the starfish *Stellaster equestris* (South China Sea); and *S. dubius*, from leaves of the marine grass *Zostera marina* (Troits Bay, Peter the Great Bay, Sea of Japan) [4]. These microorganisms belong phylogenetically to the cluster Roseobacter—Ruegeria—Sulfitobacter of class Alphaproteobacteria [5].

A recent study in October—November 2000 of the distribution, abundance, and taxonomic makeup of heterotrophic saprophytic bacterial populations in the waters and sediments near the nuclear accident in Chasma Bay (Sea of Japan) isolated several strains that were phenotypically, genotypically, and phylogenetically similar to Roseobacter—Ruegeria—Sulfitobacter [6]. It is noteworthy that a community of heterotrophic saprophytic bacteria, including sulfitobacteria, that are resistant to natural and man-made factors, including accidental radioactive contamination, has formed over the last 15 years in the waters and sediments of the bay.

Herein we present results of a structural analysis of teichoic acid isolated from one of these strains, KMM 6006, assigned to the species *S. brevis* because the nucleotide sequence of the 16S tRNA gene of this strain was 98% homologous with that of the type strain *S. brevis* ATCC BAA-4^T. The work is a continuation of the study of carbohydrate-containing biopolymers of marine gram-negative microorganisms, which sometimes contain noncarbohydrate substituents [7]. We previously established the structure of a glycerophosphate-containing *O*-specific polysaccharide from the *Pseudoalteromonas* sp. KMM 639 [8].

Microbial biomass was produced in a previously described medium. Lipopolysaccharide (LPS) was isolated by phenol:water extraction [9]. Mild acidic dehydration of LPS followed by gel chromatography produced teichoic acid (TA). TA appeared in SDS—PAAG electrophoresis [10] using standard dextrans of various molecular weights as a single band near molecular weight ~20 kDa. The homogeneity of the preparation and the value of its molecular weight were confirmed by high-efficiency gel chromatography.

A single monosaccharide, glucosamine, was found by paper chromatography in the TA hydrolysate. GC—MS of the hydrolysate and methanolysate of TA as polyol and methylglucoside acetates identified glycerine, ribitol, and glucosamine. Based on the specific optical rotation (+70.1°), glucosamine had the D-configuration. The absolute configuration of ribitol was not determined in this instance.

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TABLE 1. ^{13}C NMR Spectra of Teichoic Acid from *S. brevis* and the Glycoside in Model Compounds, δ, ppm

Fragment	C1	C2	C3	C4	C5	C6	CH_3	CO
				Teichoic acid				
→1,4)-Rib-ol-(5P→	67.7	72.2	72.4	80.7	66.2			
β-D-Glc*p*NAc-(1→	102.6	56.9	75.1	71.2	77.0	61.9	23.7	176.2
→1)-Gro-(3P→	67.4	70.7	67.4					
				Glycoside				
→ 4)-Rib-ol	64.0	72.8	72.9	82.4	61.8			
β-D-Glc*p*NAc-(1→	102.6	57.1	75.0	71.2	77.0	61.9	23.5	176.2
Gro	63.4	73.4	63.4					
				Model compounds				
Rib-ol	63.2	72.9	72.3	72.9	63.2			
β-OMe-Glc*p*NAc	103.0	56.7	75.2	71.3	77.1	62.1	23.5	175.8

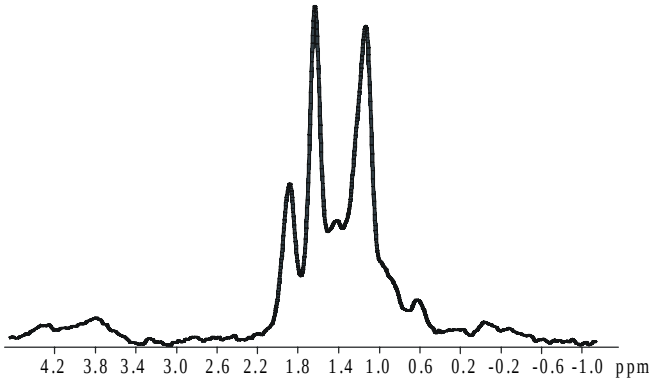


Fig. 1. ^{31}P NMR spectrum of native TA from *S. brevis*

Furthermore, the ^{31}P NMR spectrum of TA (Fig. 1) recorded at pD 5 exhibited a broad resonance at +1.44 ppm, which was consistent with a disubstituted monophosphate in a polymer chain [11]. However, the spectrum of the polymer recorded at pD 10 had three resonances at 1.13, 1.63, and 1.88 ppm with a 3:2:1 ratio of integrated intensities. The presence of three resonances of different integrated intensity can be explained by a nonstoichiometric distribution of glucosamine units in the polymer chain. These data taken together suggest that the obtained glycopolymer is teichoic acid with different types of phosphoric acid substitution.

The ^{13}C NMR spectrum of the polymer showed 16 resonances for C atoms (Table 1 and Fig. 2b). The resonance of the anomeric C atoms appeared at 102.6 ppm; two resonances of acetamides, at 23.6 (CH_3) and 176.2 (C=O); resonances of C bound to N, at 56.8; and twelve resonances, in the range 61.9-80.6. The spin—spin coupling constant (SSCC) $J_{C,H}$ of the anomeric C of 163 Hz was determined from the ^{13}C NMR spectrum of TA recorded without gate decoupling and indicated that the glucosamine had the β-configuration [12]. The set of chemical shifts and the SSCC of narrow resonances of C atoms in the range 61.9-77.0 ppm also agreed with those of an *N*-acetyl-β-D-glucopyranoside [13] with the exception of the resonance of C1, which was shifted to strong field (α-effect of various aglycons) in the spectrum of the polymer [14]. A broad singlet at 67.4 ppm (C2) and a triplet at 70.7 ppm with $J_{C,H}$ = 7.0 Hz (C1, C3) were consistent with glycerines in unsubstituted glycerophosphate units [15]. Broad resonances at 67.7 and 66.2 ppm, a singlet at 72.4, and doublets at 72.2 and 80.2 ($J_{C,P}$ = 7.0 Hz) were typical of a ribitol unit substituted at C1 and C5 by phosphoric acid and at C4(C2) by a glycopyranose [15].

The 2D heteronuclear $^1H/^{31}P$ HMBC spectrum [16] gave the following correlations: 3.90-4.03 ppm (^1H)/1.13 ppm (^{31}P); 4.03-4.16 ppm (^1H)/1.63 ppm (^{31}P) and 4.03; 4.10-4.15 ppm (^1H)/1.88 ppm (^{31}P). It is known that protons of glycerine CH_2 and CH groups resonate in the range 3.90-4.05 ppm [17]. Thus, the resonance at 1.13 ppm in the ^{31}P NMR spectrum belonged to a phosphate on the glycerines [15]. The resonance at 1.63 ppm belonged to 1,5-poly(4-*N*-acetyl-β-D-glucosaminylribitophosphate); the resonance at 1.88 ppm, to glycerophosphate-poly(4-*N*-acetyl-β-D-glucosaminylribitol).

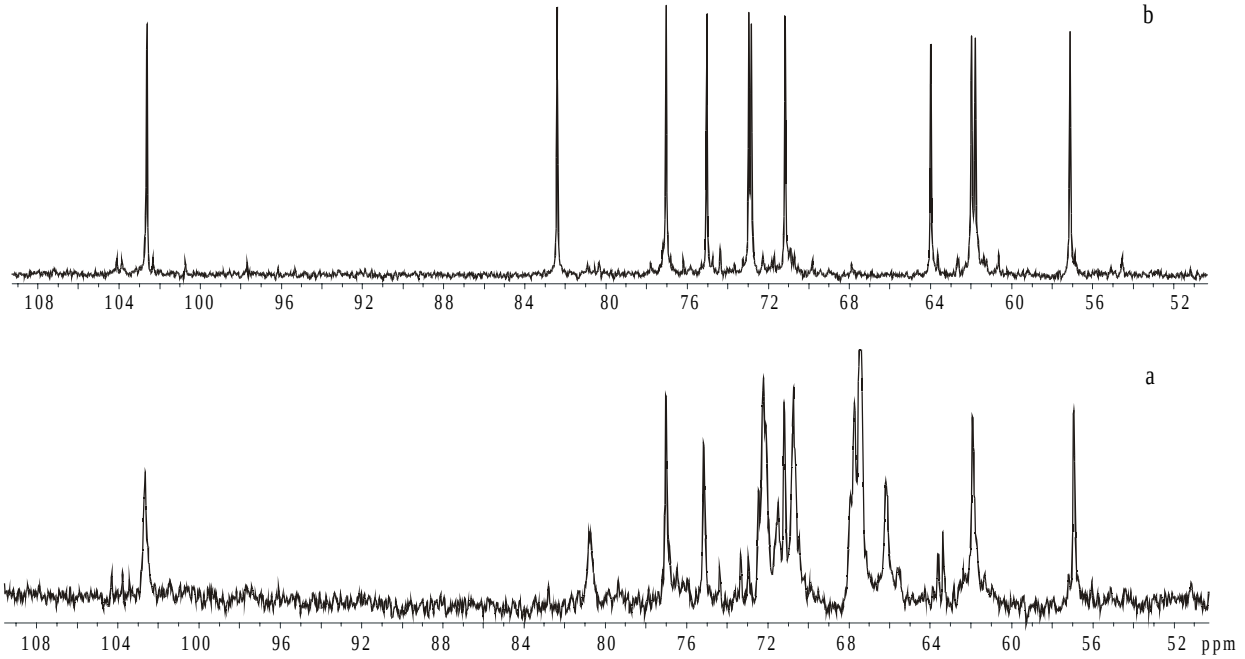
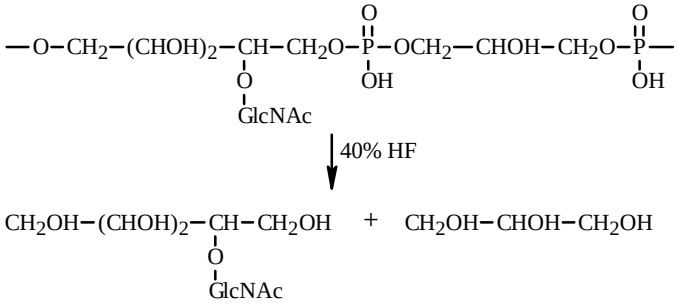


Fig. 2. ^{13}C NMR spectra of native TA from *S. brevis* (a) and the glycoside (b). Additional resonances of NHAc groups are at 23.5 and 23.7 ppm (CH_3) and 176.2 (C=O).

Dephosphorylation of TA followed by gel chromatography produced a glycoside containing ribitol and glucosamine in addition to glycerine.



A comparison of the ^{13}C NMR resonances of native TA and the glycoside (Table 1 and Fig. 2a) showed that the chemical shifts of the glucosamine were the same. Therefore, this monosaccharide does not form phosphodiester bonds. This was also consistent with the lack of broadening or splitting of the resonances for the glycopyranosyl unit. The resonances for ribitol in these same spectra did shift from 67.7 and 66.2 ppm in the spectrum of the polysaccharide to strong field by ~4 ppm in the spectrum of the glycoside (α-effect from formation of a phosphodiester bond) [18]. This fact confirmed that ribitol in TA was substituted at C1 and C5 by phosphoric acid. Similar shifts of the resonances for C1 and C3 of glycerine at 67.4 ppm (Table 1) also indicated that it was phosphorylated in these positions.

Thus, the results established that the glycopolymer from *S. brevis* strain KMM 6006 is TA, the polymer chain of which is constructed of alternating 1,5-poly(4-*N*-acetyl-β-D-glycosaminylribitophosphate) and 1,3-poly(glycerophosphate).

The studied glycopolymer is a so-called mixed TA that contains in the chain a poly(glycosylpolyolphosphate) unit and a poly(glycerophosphate) [18]. Such polymers are known mainly as components of cell walls of gram-positive microorganisms [19, 20] and are not characteristic of gram-negative bacteria. The TA obtained in this instance contained poly(glycosylribitophosphate) and poly(glycerophosphate), which were identified for the first time in the cell wall of gram-negative microorganisms.

EXPERIMENTAL

General Methods. ^{13}C NMR spectra in D_2O at 60°C were recorded on a Bruker Avance DRX-500 instrument using methanol (δ_C 50.15 ppm) as an internal standard. ^{31}P NMR spectra were recorded on the same instrument using H_3PO_4 (85%, δ_P 0 ppm) as an external standard. Descending chromatography was performed on Filtrak FN-15 paper using *n*-BuOH:C_5H_5N:H_2O (6:4:3) with development of monosaccharides by alkaline silver nitrate. Gel chromatography was performed over columns of gels TSK HW 50(F) (2.5 × 100 cm) in acetic acid (0.3%) and TSK HW 40(F) (1.5 × 80 cm) in water. Elution curves were constructed using an RIDK 101 (Czech Rep.) differential refractometer. GC was carried out in an Agilent 6850 chromatograph in a capillary column (30 m × 0.25 mm) with HP-5MS (5%) in the temperature range 170→230°C with He carrier gas. GC—MS was performed under these same conditions. SDS—PAAG electrophoresis was performed in polyacrylamde gel (12.5%) by the Laemmli method [10] with development of the gel by $AgNO_3$ [21]. High efficiency gel chromatography was performed on an Agilent 1100 instrument over a column (7.6 × 300 mm) of Shodex Asahipak GS 620HQ in NaCl (0.9%) at 40°C. The standards for SDS—PAAG electrophoresis and gel chromatography were dextrans (10, 20, 40, 60, and 110 kDa). The relative specific rotation of glucosamine (+70.1°) was determined on a Perkin—Elmer 141 polarimeter (+72.5° [22]).

The work used strain *S. brevis* KMM 6006 from the PIBOC FED RAS Collection of Marine Microorganisms. Bacteria were isolated from soil samples from Chasma Bay (Strelok Gulf, Sea of Japan) at 10-15 cm depth [6]. Bacteria were cultivated in Yoshimizu—Kimura liquid medium [23]. LPS and TA were isolated and carbohydrate analysis was carried out as before [8].

Dephosphorylation. TA (25 mg) was treated with HF (40%, 2 mL, 4°C, 72 h). Acid was removed in vacuo over NaOH. Chromatography over TSK HW 40(F) gel isolated the glycoside (12 mg) and glycerine (10 mg).

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