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Structure of a phosphorylated polysaccharide from *Shewanella putrefaciens* strain S29

Alexander S. Shashkov [a,*], Sof'ya N. Senchenkova [a], Evgeny L. Nazarenko [b], Vladimir A. Zubkov [b], Natal'ya M. Gorshkova [b], Yuriy A. Knirel [a], Raisa P. Gorshkova [b]

[a] *N.D. Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences, Moscow 117913, Russian Federation*

[b] *Pacific Institute of Bioorganic Chemistry, Far East Branch of the Russian Academy of Sciences, Vladivostok 690022, Russian Federation*

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Abstract

A phosphorylated polysaccharide was isolated from the aqueous layer of the phenol–water extract of a non-halophilic bacterium *Shewanella putrefaciens* strain S29. The glycosyl phosphate linkage in the polysaccharide was split under mild acid conditions to give, after borohydride reduction, a phosphorylated oligosaccharide-alditol. On the basis of sugar analysis and ^{1}H, ^{13}C and ^{31}P NMR spectroscopy, including 2D COSY, relayed COSY, rotating-frame NOE spectroscopy (ROESY), heteronuclear $^{13}C,^{1}H$ COSY, and H-detected heteronuclear $^{1}H,^{31}P$ multiple-quantum coherence (HMQC), it was concluded that the polysaccharide is built up of tetrasaccharide-phosphate repeating units having the following structure:

4)-α-L-Qui*p*NAc-(1 → 3)-α-D-Glc*p*NAc-(1 → 3)-β-D-Qui*p*4NAc-(1 → 3)-α-D-Gal*p*-1-*P*-(O →

where QuiNAc and Qui4NAc are 2-acetamido-2,6-dideoxyglucose and 4-acetamido-4,6-dideoxyglucose, respectively. © 1997 Elsevier Science Ltd.

Keywords: *Shewanella putrefaciens*; Polysaccharide structure; Galactosyl phosphate; 4-Acetamido-4,6-dideoxyglucose

1. Introduction

Shewanella (*Alteromonas*, *Pseudomonas*) *putrefaciens* [1] is an aquatic Gram-negative bacterium which is found in many environments, such as sediments, oil fields and foods [2–5]. It has been reported to associate with bacteremic infections and isolated from feces, urine and peritoneal dialysis fluid, as well as from patients with skin ulcers, ear infections and intraabdominal infections [6–16]. *S. putrefaciens* is often isolated together with other bacterial species [7] and its true clinical significance remains unknown. This is a heterogeneous species; most marine isolates are psychrotrophic, while clinical isolates mesophilic

* Corresponding author.

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and halotolerant, with mol% guanine plus cytosine ~ 45 and 52–56%, respectively [5,17–19]. Composition and structures of surface polysaccharides of *S. putrefaciens*, including lipopolysaccharides, which may have a chemotaxonomic significance, are studied scarcely [20,21]. In this study we established the structure of a water-soluble polysaccharide from a non-halophilic strain *S. putrefaciens* S29.

2. Results and discussion

Bacterial cells were extracted with aq 45% phenol, and an acidic polysaccharide was recovered from the aqueous phase by dialysis and studied without further purification.

Sugar analysis of the polysaccharide using GLC–MS of derived alditol acetates revealed the presence of 2-amino-2,6-dideoxyglucose (QuiN), 4-amino-4,6-dideoxyglucose (Qui4N), and 2-amino-2-deoxyglucose in the ratios 0.3:1:1, as well as Gal and trace amounts of Glc and Man. Determination of the absolute configurations of the monosaccharides using GLC of acetylated glycosides with optically active alcohols [22,23] showed that Gal and GlcN have the D-configuration and QuiN has the L-configuration. The D-configuration of Qui4N was determined, and the L-configuration of QuiN confirmed, by analysis of the glycosylation effects in the ^{13}C NMR spectrum of the polysaccharide (see below).

The ^{13}C NMR spectrum of the polysaccharide (Table 1) contained signals for four anomeric carbons in the region δ 96.8–104.7, two CH_3–C groups of 6-deoxy sugars at δ 17.8 (a signal with the double integral intensity), two $HOCH_2$–C groups (C-6 of Gal and GlcN) at δ 61.6 and 62.3, three carbons bearing nitrogen in the region δ 54.5–57.8, 13 other sugar ring carbons in the region δ 68.2–81.8, and three *N*-acetyl groups (CH_3 at δ 23.4–23.6, CO at δ 174.7–175.3).

The ^1H NMR spectrum of the polysaccharide contained, inter alia, signals for four anomeric protons in the region δ 4.67–5.59, two methyl groups of 6-deoxy sugars at δ 1.16 and 1.29, and three *N*-acetyl groups at δ 1.95, 1.97 and 2.03. The ^{31}P NMR spectrum of the polysaccharide contained a signal for one phosphate group at δ −1.5.

Therefore, the polysaccharide has a phosphorylated tetrasaccharide repeating unit containing one residue each of D-galactose, 2-acetamido-2-deoxy-D-glucose, 2-acetamido-2,6-dideoxy-L-glucose, and 4-acetamido-4,6-dideoxy-D-glucose. A lower proportion of QuiN detected in sugar analysis of the polysaccharide was due to phosphorylation of this monosaccharide (see below). The absence from the ^{13}C NMR spectrum of any signals for non-anomeric sugar carbons in lower field than δ 82 indicated the pyranoid form of all sugar residues [24].

The ^1H NMR spectrum of the polysaccharide was assigned using sequential, selective spin-decoupling, 2D COSY and relayed COSY (Table 2), and then the ^{13}C NMR spectrum was assigned using 2D heteronuclear $^{13}C,^1H$ COSY (Fig. 1, Table 1). Assignment of the ^1H spin-systems for the amino sugars was confirmed by correlation of the protons H-2 (for GlcNAc and QuiNAc) and H-4 (for Qui4NAc) to the corresponding carbons bearing nitrogen (δ 54–58) revealed by a 2D heteronuclear $^{13}C,^1H$ COSY experiment.

Analysis of the coupling constant values (Table 2) confirmed the pyranoid form of the sugar residues

Table 1
^{13}C NMR data (δ, ppm)

Sugar residue	Carbon					
	C-1	C-2	C-3	C-4	C-5	C-6
Polysaccharide **1**						
-*P*-(O → 4)-α-L-Qui*p*NAc-(1 →	97.9	54.5	71.6	81.8 [a]	68.2	17.8
→ 3)-α-D-Glc*p*NAc-(1 →	98.3	54.7	77.2	69.3	72.8	61.6
→ 3)-β-D-Qui*p*4NAc-(1 →	104.7	74.1	78.6	57.8	72.4	17.8
→ 3)-α-D-Gal*p*-1-	96.8 [a]	68.7 [a]	79.7	70.3	73.0	62.3
Oligosaccharide-alditol **2**						
P-(O → 4)-α-L-Qui*p*NAc-(1 →	97.8	54.7	71.7	80.7 [b]	68.0 [b]	17.6
→ 3)-α-D-Glc*p*NAc-(1 →	98.2	54.8	76.9	68.9	72.6	61.2
→ 3)-β-D-Qui*p*4NAc-(1 →	104.2	74.0	78.4	57.6	72.3	17.6
→ 3)-D-Gal-ol	63.5	72.1	78.7	70.6	71.3	63.9

[a] A broadened signal due to coupling to phosphorus.
[b] $J_{C,P}$ 6 Hz.

Table 2
^{1}H NMR data (δ, ppm; J, Hz)

Sugar residue	Proton					
	H-1	H-2	H-3	H-4	H-5	H-6
Polysaccharide **1**						
-*P*-(O → 4)-α-L-Qui*p*NAc-(1 →	5.00	3.97	3.82	3.88	4.25	1.29
	$J_{1,2}$ 4	$J_{2,3}$ 9	$J_{3,4}$ 9	$J_{P,4}$ 7	$J_{4,5}$ 9	$J_{5,6}$ 6
→ 3)-α-D-Glc*p*NAc-(1 →	5.01	4.04	3.84	3.61	4.22	3.81
	$J_{1,2}$ 4	$J_{2,3}$ 10	$J_{3,4}$ 10	$J_{4,5}$ 9	$J_{5,6}$ 6 [a]	
→ 3)-β-D-Qui*p*4NAc-(1 →	4.67	3.47	3.71	3.74	3.50	1.16
	$J_{1,2}$ 8	$J_{2,3}$ 9	$J_{3,4}$ 10	$J_{4,5}$ 10	$J_{5,6}$ 6.5	
→ 3)-α-D-Gal*p*-1-	5.59	4.04	3.98	4.21	4.14	3.73
	$J_{1,P}$ 9	$J_{1,2}$ 4	$J_{2,3}$ 10	$J_{3,4}$ 4	$J_{4,5}$ < 2	$J_{5,6}$ 6 [a]
Oligosaccharide-alditol **2**						
P-(O → 4)-α-L-Qui*p*NAc-(1 →	5.00	3.98	3.85	3.77	4.27	1.28
	$J_{1,2}$ 4	$J_{2,3}$ 10	$J_{3,4}$ 9	$J_{P,4}$ 7	$J_{4,5}$ 9	$J_{5,6}$ 6
→ 3)-α-D-Glc*p*NAc-(1 →	5.03	4.04	3.84	3.63	4.20	3.84
	$J_{1,2}$ 4	$J_{2,3}$ 10	$J_{3,4}$ 10	$J_{4,5}$ 9	$J_{5,6}$ 6 [a]	
→ 3)-β-D-Qui*p*4NAc-(1 →	4.52	3.49	3.71	3.79	3.53	1.19
	$J_{1,2}$ 8	$J_{2,3}$ 9	$J_{3,4}$ 10	$J_{4,5}$ 10	$J_{5,6}$ 6.5	
→ 3)-D-Gal-ol	3.78	4.05	3.88	3.82	4.11	3.69
	$J_{1,2}$ 7 [b]	$J_{2,3}$ 1.5	$J_{3,4}$ 8	$J_{4,5}$ 1.5	$J_{5,6}$ 7 [a]	

[a] $1/2(J_{5,6a} + J_{5,6b})$.
[b] $1/2(J_{1a,2} + J_{1b,2})$.

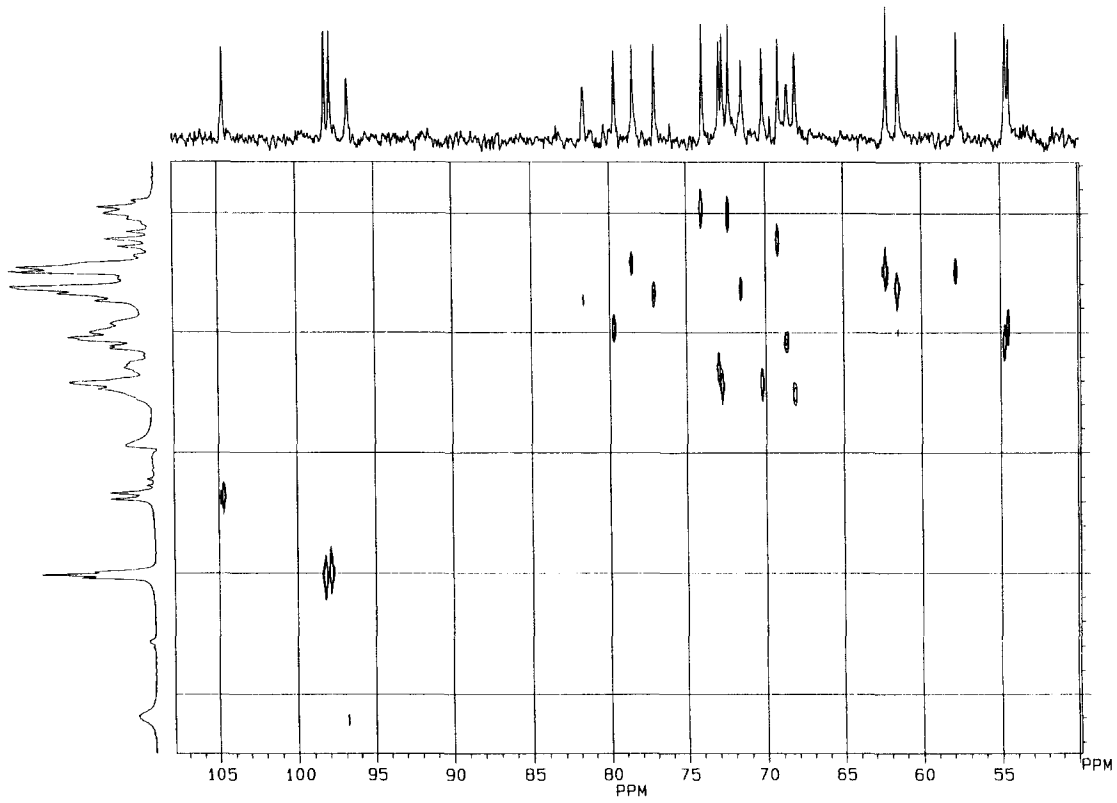

Fig. 1. Part of a 2D heteronuclear ^{13}C,^{1}H COSY spectrum of *S. putrefaciens* S29 polysaccharide. The corresponding parts of ^{1}H and ^{13}C NMR spectra are displayed along the vertical (*F1*) and horizontal (*F2*) axes, respectively.

and showed that Qui4NAc is β-linked ($J_{1,2}$ 8 Hz) and the other sugar residues are α-linked ($J_{1,2}$ 4 Hz) [25]. Splitting of the signals for Gal H-1 and QuiNAc H-4 due to coupling to phosphorus ($J_{H,P}$ 9 and 7 Hz, respectively) demonstrated the sites of phosphorylation in the polysaccharide. These were confirmed by a 2D $^{1}H,^{31}P$ HMQC experiment which revealed cross-peaks Gal H-1/P at δ 5.59/−1.5 and QuiNAc H-4/P at δ 3.88/−1.5. Therefore, the oligosaccharide repeating units are connected via the phosphate group and the sequence α-D-Gal*p*-1-*P*-(O → 4)-α-D-Qui*p*NAc is present in the polysaccharide.

Further sequence and linkage analysis of the polysaccharide was carried out using a 2D ROESY experiment which revealed the following interresidue cross-peaks: QuiNAc H-1, GlcNAc H-3 at δ 5.00/3.84, GlcNAc H-1, Qui4NAc H-3 at δ 5.01/3.71, and Qui4NAc H-1, Gal H-3 at δ 4.67/3.98. No interresidue cross-peak was observed for Gal H-1.

The substitution pattern was confirmed by downfield displacements, due to the α-effects of glycosylation, of the signals for C-3 of GlcNAc, Qui4NAc, and Gal from δ 72.0, 74.8 and 70.4 in the ^{13}C NMR spectra of the corresponding unsubstituted monosaccharides [26,27] to δ 77.2, 78.6, and 79.7, respectively, in the spectrum of the polysaccharide. The signal for QuiNAc C-4 was shifted downfield from δ 77.1 in α-QuiNAc [28] to δ 81.8 in the polysaccharide due to the α-effect of phosphorylation.

Analysis of the β-effects of glycosylation in the ^{13}C NMR spectrum of the polysaccharide showed that Qui4NAc has the same absolute configuration as D-GlcNAc whereas QuiNAc has the opposite configuration. This followed from a relatively small β-effect (−0.35 ppm) on Qui4NAc C-4 and a relatively large (by the absolute value) β-effect (−2.1 ppm) on GlcNAc C-4, caused by their glycosylation at position 3 by α-GlcNAc and α-QuiNAc, respectively [26,29].

Therefore, on the basis of the data obtained, it was concluded that the water-soluble polysaccharide from *S. putrefaciens* S29 has the structure **1**.

4)-α-L-Qui*p*NAc-(1→3)-α-D-Glc*p*NAc-(1→3)-β-D-Qui*p*4NAc-(1→3)-α-D-Gal*p*-1-*P*-(O→

1

This structure was confirmed by analysis of a phosphorylated oligosaccharide-alditol obtained from the polysaccharide by mild acid hydrolysis followed by borohydride reduction. GLC–MS analysis of derived alditol acetates revealed the same components as in the polysaccharide, while the same analysis without borohydride reduction of the monosaccharides revealed galactitol as the only sugar alditol. Hence, the oligosaccharide-alditol has a galactitol residue (Gal-ol) at the reducing end and was, thus, derived by the selective cleavage of the galactosyl phosphate linkage in the polysaccharide.

The ^{31}P NMR spectrum of the oligosaccharide-alditol contained a signal for one phosphate group at δ 0.8. The ^{1}H and ^{13}C NMR spectra contained the same signals as the corresponding spectra of the polysaccharide, except for that the signals for Gal-ol appeared instead of the signals for α-Gal-1-*P* (Tables 1 and 2). 2D NMR spectroscopic studies using the same experiments as for the polysaccharide, indicated that the oligosaccharide-alditol has the structure **2** which is consistent with the structure of the polysaccharide **1**.

P-(O→4)-α-L-Qui*p*NAc-(1→3)-α-D-Glc*p*NAc-(1→3)-β-D-Qui*p*4NAc-(1→3)-D-Gal-ol

2

The polysaccharide studied belongs to polymers having oligosaccharide-phosphate repeating units. Such polymers have been known as extracellular bacterial polysaccharides and cell-wall polysaccharides of Gram-positive bacteria [30]. It is worthy noting that the polysaccharide contains also two rare 6-deoxyacetamido sugars, 2-acetamido-2,6-dideoxy-L-glucose (*N*-acetyl-L-quinovosamine) and 4-acetamido-4,6-dideoxy-D-glucose (*N*-acetylviosamine), which have been found hitherto in a few polysaccharides of Gram-negative bacteria [31,32].

3. Experimental

Bacterial strain, growth, and isolation of the polysaccharide.—*S. putrefaciens* strain S29 was grown in the Youschimizu–Kimura medium [33]. Wet bacterial cells were extracted with hot aq 45% phenol as described [34], the resulting mixture was centrifuged, the aqueous layer dialysed, freed from insoluble contaminations by centrifugation, concentrated in vacuum, and freeze-dried to yield 800 mg of the polysaccharide from 20 L of the cultural fluid.

NMR spectroscopy.—^{1}H NMR spectra were recorded on a Bruker WM-250 spectrometer and ^{13}C

and ^{31}P NMR spectra on a Bruker AM-300 spectrometer for solutions in D_2O 60 °C (internal acetone, δ_H 2.225, δ_C 31.45, or external aq 85% H_3PO_4, δ_P 0). Selective spin-decoupling was performed in the difference mode modified as described [35]. Standard Bruker software was used to obtain 2D spectra. A ROESY experiment was carried out on a modified Bruker WM-250 spectrometer using the proposed pulse sequence [36] and a mixing time of 0.23 s; HDO signal was suppressed by presaturation during 1 s.

GLC and GLC–MS.—GLC was performed on a Hewlett–Packard 5890 instrument equipped with a glass capillary column (13 m × 0.25 mm) coated with DB-5 stationary phase using a temperature program of 160 °C (3 min) to 250 °C at 10 °C/min. GLC–MS (EI) was carried out with the same chromatograph equipped with a Hewlett–Packard 5970 mass spectrometer.

Sugar analysis.—Hydrolysis of the polysaccharide (0.5 mg) was performed with 2 M CF_3CO_2H at 120 °C for 2 h, after borohydride reduction and full acetylation, alditol acetates derived were analysed by GLC and GLC–MS. The absolute configurations of the monosaccharides were determined by the published method [22,23] modified as follows: the polysaccharide was hydrolysed as above, the hydrolysate evaporated, amino sugars were *N*-acetylated with aceticanhydride in an aq saturated $NaHCO_3$ solution at ambient temperature, the solution was deionised with an IR-120 (H^+) cation-exchange resin, evaporated to dryness, the residue heated with (*S*)-2-butanol (for GlcN) or (*S*)-2-octanol (for Gal and QuiN) in the presence of conc CF_3CO_2H at 130 °C for 4 h, the mixture evaporated to dryness, acetylated with aceticanhydride in pyridine, and analysed by GLC using the authentic samples prepared from the corresponding monosaccharides of the D-series and the corresponding (*S*)- and (*R*)-2-alkanols.

Mild acid hydrolysis.—The polysaccharide (35 mg) was hydrolysed with aq 1% CH_3COOH at 100 °C for 30 min, the resulting oligosaccharide was isolated by GPC on TSK HW-50 (F) in aq 0.3% CH_3COOH and reduced with $NaBH_4$ to give, after desalting by GPC, the oligosaccharide-alditol **2** (20 mg).

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