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A mannitol teichoic acid containing rhamnose and pyruvic acid acetal from the cell wall of *Brevibacterium permense* VKM Ac-2280

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Abstract

The cell wall of *Brevibacterium permense* VKM Ac-2280 contains two teichoic acids. The major polymer represents a 1,6-poly(mannitol phosphate) substituted wirh either L-rhamnose (~ 70%, unit A) or (*S*)-acetal of pyruvic acid (~ 30%, unit B) with the overall chain length ~ 10 mannitol phosphate units.

A -1-D-Man-ol-6-*P*-
3
↑
1
α-L-Rha*p*

B -1-D-Man-ol-6-*P*-
4 5
\ /
(*S*)-Pyr

The other polymer is an unsubstituted 1,3-poly(glycerol phosphate). The structures of the polymers were established using chemical degradations and NMR spectroscopy. The data obtained may be helpful in determination of the species-specific status of newly isolated *Brevibacterium* strains.

Keywords: Poly(mannitol phosphate); Pyruvic acid acetal; L-Rhamnose; *Brevibacterium*

1. Introduction

The cell wall of bacteria of the genus *Brevibacterium* from the order *Actinomycetales* contains teichoic acids with diverse structures as the dominant non-peptidoglycan polymers. These are glycerol, ribitol and mannitol teichoic acids with various glycosyl residues attached to a poly(alditol phosphate) backbone.[1,2]

The physiological role of teichoic acids is thought to be in ion exchange and control of the activity of autolytic enzymes, which are important for growth and division of bacterial cells.[3] The interest in these polymers shown in recent years stems from their taxonomic significance for Gram-positive bacteria, especially actinomycetes. It was shown that structures of teichoic acids are valuable taxonomic markers for species and subspecies of certain genera from the order *Actinomycetales*.[4] Presumably, the structure of the cell-wall teichoic acid is useful as an additional chemotaxonomic marker for attribution of new species of Gram-positive bacteria to the genus *Brevibacterium*. However, the teichoic acid structure has been established only in one strain, *Brevibacterium iodinum* N.C.T.C. 9742.[5]

In the present work, we describe the structure elucidation of a new mannitol teichoic acid that contains L-rhamnose and pyruvic acid acetal linked to a 1,6-poly(mannitol phosphate). The polymer was isolated from the cell wall of *Brevibacterium permense* VKM Ac-2280, which belongs to a recently described *Brevibacterium* species.[6]

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✠ Dedicated to the memory of Dr Naumova who sadly passed away 18 August 2003.

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2. Results and discussion

Hydrochloric acid degradation of the cell wall of *B. permense* VKM Ac-2280 afforded rhamnose, glycerol and an alditol with the chromatographic mobility R_{Glc} 1.1 on PC in system 2. In addition to glycerol mono- and bisphosphates, two alditol phosphates were identified among phosphoric esters.

On PC in systems 1 and 2, the alditol coincided with mannitol. Additional identification of the alditol was carried out following subsequent hydrolysis of the cell wall with aqueous 40% HF and 2 M HCl. The resulting alditol was isolated by preparative PC in system 2 and identified as mannitol based on the coincidence of the signals in the ^{13}C NMR spectrum with those of the authentic sample[7] (Tables 1 and 2). The D configuration of mannitol was determined by the specific optical rotation value $[\alpha]_D^{20}$ +26° (*c* 1, 0.033 M borax) (compare published data[8] $[\alpha]_D^{20}$ +24° for D-mannitol).

Preliminary analysis of the cell wall suggested the presence of at least two teichoic acids containing different alditols or one mixed polymer. In an attempt to separate the polymers, the whole extract of the cell wall with aqueous 10% CCl_3CO_2H was fractionally precipitated with 2 and 4 vol ethanol to give preparations I and II, respectively. These preparations differed in composition, particularly in the content of mannitol phosphates and glycerol phosphates released by hydrolysis with hydrochloric acid. The preparation I afforded mainly mannitol phosphates and rhamnose, whereas glycerol phosphates were present in trace amounts.

Table 1
^{13}C NMR data (δ, ppm)

Residue	C-1	C-2	C-3	C-4	C-5	C-6
D-Man-ol	64.6	72.2	70.7	70.7	72.2	64.6
-1-Man-ol-6-*P*-[a]	68.7	70.8	69.8	69.8	70.8	68.7
Glycoside G1						
D-Man-ol 3 ↑ 1	63.4 (-1.2)	72.15 (0)	77.7 (+7.0)	70.6 (-0.1)	71.0 (-1.2)	63.9 (-0.7)
α-L-Rha*p*	101.3	71.55	72.4	72.9	70.25	17.45
Mannitol teichoic acid (preparation I)						
-1-D-Man-ol-6-*P*- 4 5 \ / (*S*)-Pyr	67.5 (-1.2) 177.0	71.9 (+1.1) 106.5	69.2 (-0.6) 23.0	77.6 (+8.7)	79.0 (+8.2)	66.4 (-2.3)
-1-D-Man-ol-6-*P*- 3 ↑ 1	68.3 (-0.4)	71.3 (+0.5)	79.4 (+9.6)	71.2 (+1.4)	70.8 (0)	68.7 (0)
α-L-Rha*p*	103.2	72.1	71.6	73.4	70.3	18.2
D-Man-ol-6-*P*- 3 ↑ 1	64.7	72.4	79.3	71.2	70.9	68.7
α-L-Rha*p*	103.2	72.1	71.6	73.4	70.3	18.2

Effects of glycosylation are given in parentheses. (*S*)-Pyr stands for (*S*)-acetal of pyruvic avid.
[a]Ref. 19.

Table 2
^{1}H NMR data (δ, ppm)

Residue	H-1	**H-1'**	H-2	H-3	H-4	H-5	H-6	**H-6'**
D-Man-ol	3.87 $J_{1,1'}$ 11.8	3.68 $J_{1',2}$ 6.1	3.76	3.80	3.80	3.76	3.87	3.68
Glycoside G1								
D-Man-ol 3 ↑ 1	3.83	3.68	3.99	4.02	3.80	3.72	3.87	3.69
α-L-Rha*p*	5.01		4.04	3.82	3.48	3.85	1.29	
Mannitol teichoic acid (preparation I)								
-1-D-Man-ol-6-*P*- 4 5 \ / (*S*)-Pyr	4.15	4.03	3.88	3.75 1.67	4.57	4.55	4.18	4.18
-1-D-Man-ol-6-*P*- 3 ↑ 1	4.15	4.03	4.05	4.07	3.89	3.83	4.16	4.06
α-L-Rha*p*	5.00		4.06	3.82	3.45	3.86	1.29	
D-Man-ol-6-*P*- 3 ↑ 1	3.71	3.69	3.85	4.06	3.90	3.92	3.97	3.93
α-L-Rha*p*	5.00		4.06	3.82	3.45	3.86	1.29	

(*S*)-Pyr stands for (*S*)-acetal of pyruvic avid.

Analysis by paper electrophoresis showed that each of the preparations I and II contained two polymers. The polymer 1 with the electrophoretic mobility M_{GroP} 0.97 was the major component of the preparation I and proved to be mannitol teichoic acid. The electrophoretic mobility of the polymer 2 (M_{GroP} 1.22) corresponded to that of an unsubstituted poly(glycerol phosphate).[9] This was the main component of the preparation II. The much smaller content of glycerol teichoic acid in the preparation I compared to the preparation II was in keeping with their alditol phosphate composition determined by acid hydrolysis.

Alkaline hydrolysis of the preparation I yielded the major phosphate (P1) with the electrophoretic mobility M_{GroP} 0.51 and a neutral glycoside (G1) with the mobility R_{Glc} 0.6 on PC in system 2. Upon acid hydrolysis, the phosphate P1 afforded mannitol monophosphate (P2) and rhamnose. The *P*: Man-ol: Rha ratios in P1 were close to equimolar. Treatment of P1 with alkaline phosphatase (phosphomonoesterase) resulted in complete dephosphorylation and formation of a glycoside, which coincided with G1 on PC in system 2. This glycoside gave a characteristic colour when stained with alkaline silver nitrate but could not be stained with aniline hydrogen phthalate.

The glycoside G1 was isolated by preparative PC following treatment of the preparation I with aqueous 40% HF. On acid hydrolysis, G1 yielded mannitol and rhamnose in the ratio 0.9:1. Upon periodate oxidation, G1 gave two moles of formaldehyde per mole of

rhamnose, which indicated that positions 2 and 5 of mannitol are free and, hence, the glycosyl substituent is attached to the hydroxyl group at C-3 or C-4 of mannitol. Determination of the absolute configuration of rhamnose showed that it belongs to the L series. Taking account of these and NMR spectroscopic data (see below), the phosphate P1 was identified as an α-L-rhamnopyranosyl-(1→3)-D-mannitol phosphate.

In addition to rhamnose, mannitol, pyruvic acid and inorganic phosphate, acid hydrolysis of the preparation I yielded two major phosphates, P2 (M_{GroP} 0.72) and P3 (M_{GroP} 1.16). Upon action of phosphomonoesterase, P2 afforded mannitol and inorganic phosphate in the ratio 0.96:1. P2 was rather acid-stable towards acid hydrolysis giving only small amounts of mannitol and inorganic phosphate. Treatment of P3 with phosphomonoesterase resulted in complete dephosphorylation and formation of mannitol and inorganic phosphate in the ratio 1:2.2. Therefore, P2 is a mannitol monophosphate and P3 is a mannitol bisphosphate.

The polymer 2 was isolated from the preparation II by preparative paper electrophoresis. On alkaline hydrolysis, it afforded glycerol mono- and bisphosphates as well as a diglycerol trisphosphate, which were identified by paper electrophoresis using the corresponding authentic samples. No glycosyl substituent was detected. These data together suggest that the polymer 2 is an unsubstituted 1,3-poly(glycerol phosphate).[10]

2D COSY, TOCSY, ROESY, $^{1}H,^{13}C$ HSQC, HMQC-TOCSY and HMBC experiments were employed to assign the 1D NMR spectra of the preparation I. The major signals in the ^{13}C NMR spectrum (Table 1) corresponded to a rhamnosylated mannitol, whereas minor signals belonged to a mannitol residue that bears a pyruvic acid acetal. Determination of the glycosylation site on mannitol by 2D NMR spectroscopy failed owing to too close positions of the signals for H-1′, H-2 and H-3 of the rhamnosylated mannitol residue in the ^{1}H NMR spectrum. However, glycosylation of mannitol at O-2 could be excluded based on the chemical shift difference of −0.4 ppm for the major C-1 signal in the ^{13}C NMR spectrum, which is too small to be a β-effect of glycosylation.

The ^{13}C NMR spectrum of the glycoside G1 (Table 1) contained twelve signals, including one signal for the anomeric carbon (δ 101.3), two signals for unsubstituted C–CH_2OH groups (δ 63.4 and 63.9) and one signal for a C–CH_3 group (δ 17.45). The other signals were observed in the resonance region of substituted (δ 77.7, one signal) and unsubstituted methine carbons of rhamnose and mannitol (δ 70.25–72.9, seven signals). The ^{1}H NMR spectrum (Table 2) contained characteristic signals for H-1 (δ 5.01, d, $J_{1,2}$ 2 Hz) and H-6 (δ 1.29, d, $J_{5,6}$ 6.5 Hz) of α-L-Rha*p* as well as signals for the other protons at δ 3.48–4.04.

The 1D NMR spectra of the glycoside G1 were assigned using 2D COSY, TOCSY, ROESY, $^{1}H,^{13}C$ HSQC and HMBC experiments. In the COSY spectrum, the proton at the linkage carbon of mannitol at δ 4.02 gave no cross-peaks with either of the protons of two CH_2OH groups and is thus either H-3 or H-4. This was confirmed by an α-L-Rha*p* H-1,Man-ol H-3(4) cross-peak in the ROESY spectrum and α-L-Rha*p* H-1,Man-ol C-3(4) and α-L-Rhap C-1,Man-ol H-3(4) cross-peaks in the HMBC spectrum.

Although no unambiguous proof of the position of glycosylation was obtained, we consider that mannitol in G1 is glycosylated at O-3 rather than at O-4. This conclusion is based on the biosynthesis data of a 1,5-poly(ribitol phosphate) from ribitol 5-phosphate,[11] which enabled the assumption that the repeating unit of the polymer 1 is mannitol 6-phosphate.

The ^{13}C NMR spectrum of the preparation I (Table 1) revealed the presence of mannitol residues of three types, viz.: (i) an internal mannitol 1,6-bisphosphate bearing α-L-Rha*p* at position 3 (∼60%); (ii) an internal mannitol 1,6-bisphosphate bearing a pyruvic acid acetal at positions 4 and 5 (∼30%); and (iii) a terminal mannitol 6-phosphate bearing α-L-Rha*p* at position 3 (∼10%). These data suggest that the average chain length of the polymer 1 is 10 mannitol phosphate units. No mannitol residue that bears both α-L-Rha*p* and the pyruvic acid acetal was found, which would give rise to additional signals in the ^{1}H and ^{13}C NMR spectra. Intense α-L-Rha*p* H-1,Man-ol H-3 and α-L-Rha*p* H-1,Man-ol C-3 cross-peaks in the ROESY and HMBC spectra, respectively, confirmed glycosylation of the terminal mannitol residue at position 3.

Glycosylation effects in the ^{13}C NMR spectra of oligo- and polysaccharides, which are due to interaction of the anomeric proton of glycon with protons of aglycon, are useful for determination of the relative absolute configurations of glycosidically linked monosaccharides.[12] They were applied also for determination of the absolute configuration of arabinitol in an α-L-Rha-(1→2)-D-Ara-ol fragment of the capsular polysaccharide from *Streptococcus pneumoniae* type 17F.[13] Analysis of the ^{13}C NMR chemical shifts of the polymer 1 (Table 1) showed that the β-effects of 3-O-glycosylation of mannitol with rhamnopyranose are positive on both C-2 and C-4 and, hence, they are inapplicable for determination of the relative absolute configurations of the glycosylating aldopyranose and mannitol. This is a consequence of a spatial remoteness of H-1 of Rha*p* from H-2 and H-4 of mannitol in the predominant conformer around the glycosidic bond, which is in keeping with the absence of α-L-Rha*p* H-1,Man-ol H-2 or α-L-Rha*p* H-1,Man-ol H-4 correlation peaks in the ROESY spectrum.

The assignment of the signals in the ^{1}H NMR spectrum of the preparation I (Table 2) enabled

determination of the absolute configuration of the pyruvic acid acetal. In case of the *S* configuration, the acetal methyl group is 1,3-trans oriented relative to H-4 and H-5 of mannitol, and it can come in proximity to these protons in no conformation of the five-membered acetal ring. In case of the *R* configuration, the situation is opposite. The ROESY spectrum revealed correlation peaks for the acetal methyl group at δ 1.67 with H-6,6′, H-3 and H-2 of mannitol at δ 4.18, 3.75 and 3.88, respectively, but no correlation peaks with H-4 and H-5 of mannitol, which suggests the *S* configuration at the pyruvic acid acetal carbon.

To sum up, the cell wall of *B. permense* VKM Ac-2280 contains two teichoic acids. The major polymer is a 1,6-poly(mannitol phosphate) with 70% of the mannitol units bearing an α-L-rhamnopyranosyl group at position 3 and 30% bearing a pyruvic acid acetal at positions 4 and 5. The other polymer is an unsubstituted 1,3-poly(glycerol phosphate). It is noteworthy that the cell wall of all *Brevibacterium* strains studied to date contains several teichoic acids that differ in structure of the constituent alditols, a poly(glycerol phosphate) being present in each strain.[1,2]

3. Experimental

The culture of *B. permense* VKM Ac-2280 was grown on a pepton-yeast medium[14] for 12–18 h on a shaker at 28 °C. The biomass was collected at the logarithmic growth phase. The cells were harvested by centrifugation, washed with 0.95% NaCl and used for preparation of cell walls.[15] The cell wall preparation was heated in aq 2% sodium dodecyl sulfate for 5 min at 100 °C, washed several times with water and freeze-dried.

Teichoic acids were isolated from cell walls by extraction with aq 10% CCl_3CO_2H for 24 h at 4 °C. The mixture was centrifuged, and the mycelium was repeatedly treated with aq 10% CCl_3CO_2H under the same conditions. The supernatants were combined, dialysed against distilled water, and freeze-dried. Fractional precipitation of teichoic acids from the crude preparation was carried out by adding EtOH (2 vol), and the resulting precipitate was centrifuged off. More EtOH (2 vol) was added to the supernatant, and the precipitate was collected by centrifugation. Both precipitates were dissolved in water, centrifuged, the supernatants were dialysed against distilled water and freeze-dried to give the preparations I and II from the precipitates with 2 and 4 vol EtOH, respectively.

Descending PC and electrophoresis were performed on Filtrak FN-13 paper. The following solvent systems were used in PC: (1) 3:1:5:3 Py–butanol–water for glycerol and sugars; (2) 4:1:5 butanol–AcOH–water for mannitol, glycerol, sugars and glycosides; (3) 7:3 propanol–aq 2 M NH_3; and (4) 1:1 pentanol–5 M HCO_2H for pyruvic acid. Paper electrophoresis was performed in a Py–acetate buffer pH 5.6.[16] The compounds were visualised on paper using the following spray reagents: the molybdate reagent for phosphorus-containing compounds; 5% $AgNO_3$ in aq NH_3 for alditols and sugars; aniline hydrogen phthalate for reducing sugars; and aniline xylose for pyruvic acid.

Rha was identified with a Biotronik LC-2000 sugar analyser, using a column (15 × 0.37 cm) of Dionex A × 8-II in 0.5 M sodium borate buffer pH 8.0 at 65 °C; detection was effected with copper 2,2-bicinchoninate.[17] The absolute configuration of Rha was determined by a modified method[18] using GLC of the acetylated glycosides with (*S*)-octan-2-ol. Mannitol was isolated by GPC on a column (75 × 1.3 cm) of TSK HW-4 (*S*) in aq 1% HOAc using a Knauer differential refractometer for monitoring. Optical rotations were measured with a PU-5 polarimeter in 0.033 M borax at 20 °C.[8]

Acid hydrolysis of the cell wall and teichoic acids was performed with 2 M HCl for 3 h at 100 °C and/or aq 40% HF for 24 h at 4 °C. Alkaline hydrolysis was performed with 1 M NaOH for 3 h at 100 °C. Hydrolysis with calf intestine alkaline phosphatase (EC 3.1.3.1, Fermentas, Ltd.) was performed in Tris–HCl buffer pH 7.8 for 2 h at 37 °C. The molar ratios of mannitol, rhamnose and phosphorus were determined as described previously.[16]

NMR spectra were recorded with a Bruker DRX-500 spectrometer for 2–3% solutions in D_2O at 30 °C with internal acetone (δ 2.225 for ^1H and δ 31.45 for ^{13}C) or aq 80% H_3PO_4 (δ 0). 1D ^1H NMR spectra were obtained with a pre-saturation of the HDO signal for 1 s. 2D NMR spectra were obtained using Bruker software with standard pulse sequences.

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