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EXPERIMENTAL ARTICLES

Teichoic Acids in the Cell Walls of *Microbispora mesophila* Ac-1953^T and *Thermobifida fusca* Ac-1952^T

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Abstract—The cell walls of *Microbispora mesophila* strain Ac-1953^T (the family *Streptosporangiaceae*) and *Thermobifida fusca* Ac-1952^T (the family *Nocardiopsaceae*) were found to contain teichoic acids of a poly(glycerol phosphate) nature. The teichoic acid of *M. mesophila* (formerly *Thermomonospora mesophila*) represents a 1,3-poly(glycerol phosphate) containing 5% of substituent 2-acetamido-2-deoxy-α-D-galactosaminyl residues. Teichoic acid of such a kind was found in actinomycetes for the first time. The cell wall of *T. fusca* (formerly *Thermonospora fusca*) contains two teichoic acids, namely, unsubstituted 1,3-poly(glycerol phosphate) and β-glucosylated 1,3-poly(glycerol phosphate).

Key words: cell wall, teichoic acid, NMR spectroscopy, poly(glycerol phosphate).

Recent investigations of the anionic polymers of new representatives of the order *Actinomycetales* have considerably contributed to our knowledge of cell-wall polymers, such as teichoic acids, acidic polysaccharides, and teichuronic acids [1]. The data obtained made it possible to put forward a hypothesis postulating taxonomic significance of the structure of teichoic acids. The species specificity of teichoic acids was confirmed by the DNA–DNA homology studies [1].

The order *Actinomycetales* contains about 30 families, each of which comprises several genera [2]. Only some of these genera (such as *Streptomyces, Glycomyces, Actinomadura, Nocardiopsis, Nocardioides, Herbidospora, Planotetraspora*, and *Spirilliplanes*) have been studied with respect to their cell-wall teichoic acids [1].

The aim of this work was to study the cell-wall teichoic acids of two representatives of two *Actinomycetales* genera, *Microbispora* and *Thermobifida*, which have not yet been investigated in this respect.

MATERIALS AND METHODS

Microbispora mesophila strain Ac-1953^T and *Thermobifida fusca* strain Ac-1952^T were grown aerobically at 28°C on a shaker in a peptone–yeast extract medium [3] to the midlogarithmic growth phase. To prepare cell walls, the mycelium was harvested by centrifugation, washed with 0.95% NaCl, and disrupted by sonication in a UZDN-1 ultrasonic disintegrator (44 kHz) for a total of 12 min in 2-min bursts. The latter and all of the subsequent preparation steps were performed at 4°C. The homogenate was centrifuged. The lower, dense, part of the pellet, which represented unbroken mycelium, was discarded. The upper, loose and white in color, layer of the pellet, which contained cell walls, was collected, resuspended in 2% SDS, boiled for 5 min, washed several times with distilled water, and lyophilized.

Teichoic acids were purified and the derived phosphate esters were separated by electrophoresis on Filtrak FN-13 paper (Germany) in pyridine–acetate buffer A (pH 5.5–5.6) at 20 V/cm for 3–4 h. Monosugars, glycerol, and glycosides were separated by descending chromatography on the same paper in an *n*-butanol–pyridine–benzene–H_2O (5 : 3 : 1 : 3) mixture (solvent system B). Amino sugars were analyzed by paper chromatography in a pyridine–ethylacetate–acetic acid–H_2O (5 : 5 : 1 : 3) mixture (solvent system C). Teichoic acids and the derived phosphate esters were visualized using the Isherwood reagent; monosugars, using aniline phthalate; and glycerol and monosugars, using a 5% solution of $AgNO_3$ in ammonia.

Teichoic acids were extracted from cell walls with a 10% (wt/vol) trichloroacetic acid at 4°C for 24 h. The mixture was centrifuged, and the precipitated mycelium was extracted again under the same conditions. The extracts were pooled, dialyzed against distilled water, and lyophilized. Cell wall and teichoic acid preparations were subjected to acid hydrolysis at 100°C for 3 h in 2 N HCl to determine constituent phosphate

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Table 1. The chemical shifts of carbon atoms in the ^{13}C NMR spectrum of the cell-wall teichoic acid of *M. mesophila* Ac-1953^T

Fragment	C1	C2	C3	C4	C5	C6	CH_3	CO
-1)-sn-Gro-(3-P- 2 α-D-Gal*p*NAc-1	66.6 98.4	77.1 51.2	65.9 69.1	69.9	72.5	62.5	23.3	175.8
-1)-sn-Gro-(3-P-	67.7	70.8	67.7					
sn-Gro-(3-P-	63.5	71.8	67.7					

esters and monosugars and in 6 N HCl to determine constituent amino sugars. The alkaline hydrolysis of teichoic acids was performed as described by Naumova *et al.* [4]. The molecular mass of teichoic acids was determined by gel-filtration chromatography on Sephadex G-50 using authentic samples of teichoic acids as standards [5].

The ^{13}C NMR spectra of teichoic acids were recorded at 40°C using a Bruker-AM-300 spectrometer (Germany) operated at 75 MHz. Chemical shifts were measured relative to the signal of the internal standard CH_3OH, which has a chemical shift of 50.15 ppm relative to the external standard MeSi. The results were expressed with respect to MeSi.

RESULTS AND DISCUSSION

The Cell Wall of M. mesophila Ac-1953^T

As is evident from the data of paper chromatography and electrophoresis, the acid hydrolysis of this cell wall gave rise to glycerol, its mono- and diphosphates, inorganic phosphate, and trace amounts of the monosugars glucose, galactose, and arabinose.

The electrophoresis of the teichoic acid preparation from the cell wall of *M. mesophila* Ac-1953^T showed that it contained one teichoic acid with an electrophoretic mobility $E_{GroP} = 1.14$. The acid hydrolysate of this teichoic acid contained glycerol, its mono- and diphosphates, inorganic phosphate, and galactosamine. The alkaline hydrolysate contained glycerol, its mono- and diphosphates, inorganic phosphate, a galactosamine-containing phosphodiester with an electrophoretic mobility $E_{GroP} = 0.92$, and diglycerol triphosphate. The presence of the two latter phosphate esters in the alkaline hydrolysate of teichoic acid suggests that it is a poly(glycerol phosphate) with a phosphodiester bond between the C1 and C3 atoms of glycerol [7]. The presence of some amount of the galactosamine-containing phosphodiester and diglycerol triphosphate in the hydrolysate was indicative of a low degree of substitution in the polymer backbone (see the formation pathways of these phosphate esters shown in Fig. 1). Taken together, these data made it possible to infer that the teichoic acid of *M. mesophila* Ac-1953^T is a 1,3-poly(glycerol phosphate) slightly substituted by galactosaminyl residues.

To prove this inference, the teichoic acid was analyzed by NMR spectroscopy. The ^{13}C NMR spectrum of this teichoic acid exhibited two intense signals with a ratio of their integral intensities equal to 2 : 1. One of these signals was a broadened singlet with a chemical shift of 67.7 ppm. The other signal, with a chemical shift of 70.8 ppm, was a triplet associated with the C1, C2, and C3 atoms of glycerol in the unsubstituted glycerol phosphate units of the teichoic acid (Fig. 2, Table 1).

The lower-intensity signals of the spectrum were assigned to the carbon atom of glycosaminide bound to the nitrogen atom (signal at 51.2 ppm), to the CH_3 group of acetamide (signal at 23.3 ppm), and to the anomeric carbon atom of pyranosides (signal at 98.4 ppm). Eight low-intensity signals were assigned to α-*N*-acetylgalactosaminyl residues. Among other signals, the one at 77.1 ppm was typical of glycerol substituted at the C2 atom. This signal was split like that of the C2 atom of unsubstituted glycerol and had a lower-field chemical shift due to the α-effect of glycosylation. Two other doublet signals at 66.6 and 65.9 ppm were assigned to the C1 and C3 atoms of glycerol, which were shifted to the lower-field side due to the β-effect of glycosylation at the C2 atom, whereas the difference in their chemical shifts resulted from the presence of a chiral carbon atom in the substituent. All signals of the carbon atoms of the *N*-acetylgalactosaminyl ring and those of the substituted glycerol units had comparable integral intensities. This confirms the supposition that some glycerol units in the 1,3-poly(glycerol phosphate) chain have substituent 2-acetamido-2-deoxy-α-D-galactosaminyl residues. As is evident from a comparison of the integral intensities of signals from the substituted and unsubstituted glycerol units, the degree of substitution at the carbon atoms of glycerol is about 5%.

The molecular mass of the teichoic acid of *M. mesophila* Ac-1953^T determined by gel-filtration chromatography on Sephadex G-50 turned out to be about 9 kDa.

Thus, the teichoic acid of *M. mesophila* Ac-1953^T represents 1,3-poly(glycerol phosphate), some of the glycerol phosphate units of which are substituted at the C2 atoms by 2-acetamido-2-deoxy-α-D-galactosaminyl residues.

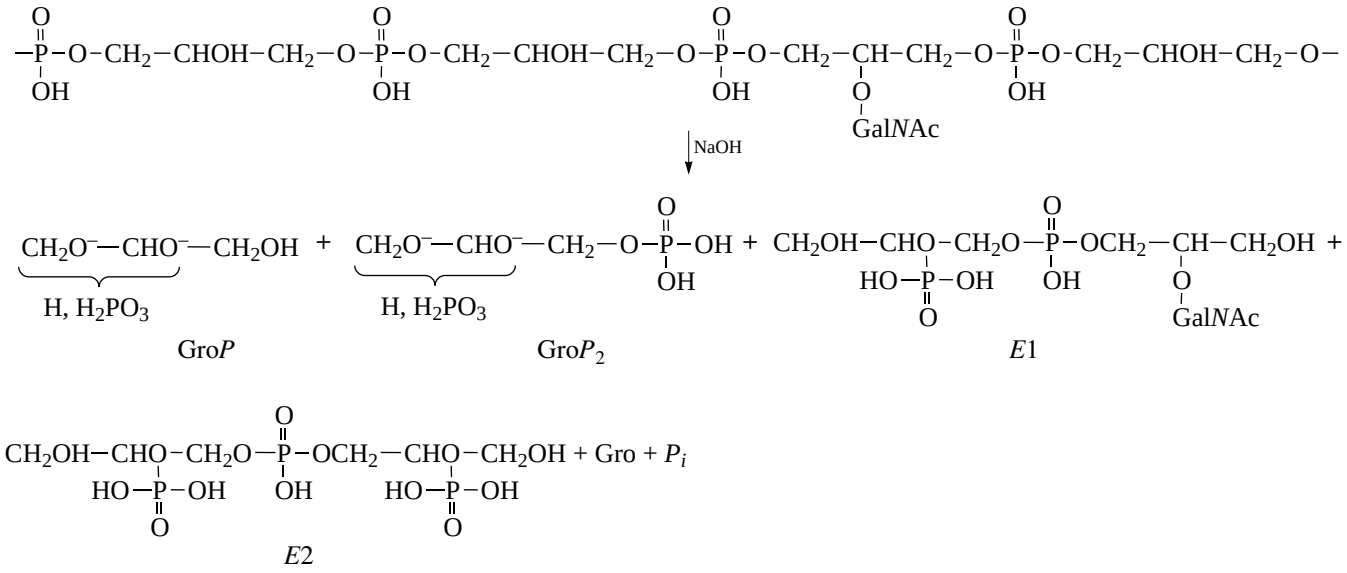

Fig. 1. The scheme of the alkaline hydrolysis of teichoic acid from the cell wall of *M. mesophila* Ac-1953^T. Gro*P* and GroP_2 are glycerol monophosphate and glycerol diphosphate, respectively.

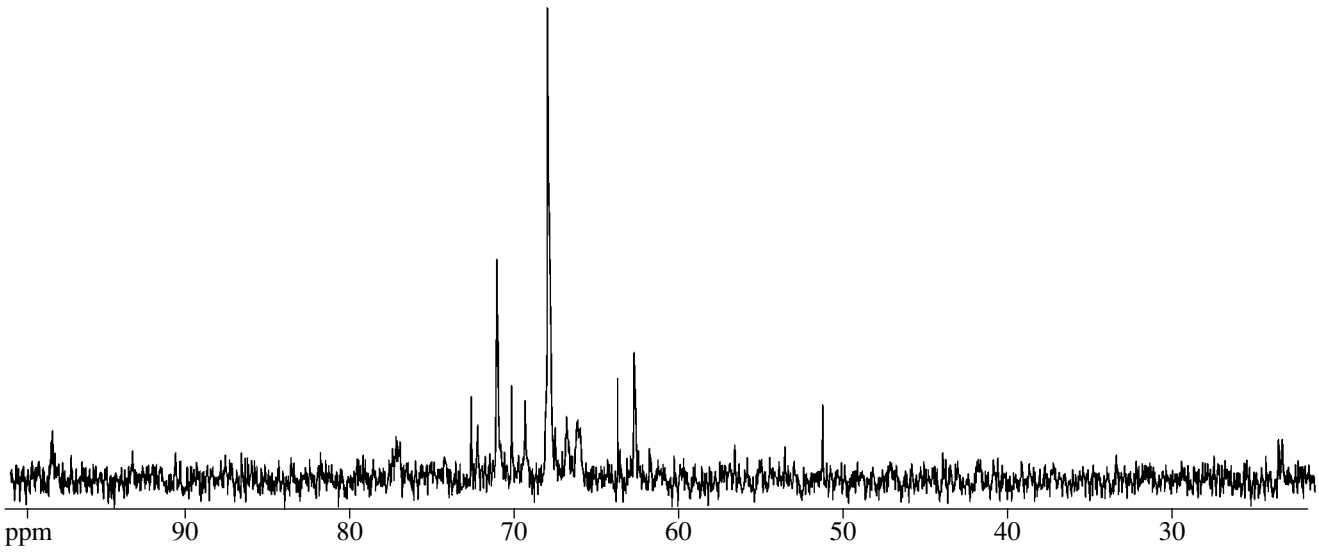

Fig. 2. The ^{13}C NMR spectrum of teichoic acid from the cell wall of *M. mesophila* Ac-1953^T.

The Cell Wall of T. fusca Ac-1952^T

When subjected to acid hydrolysis, this cell wall gave rise to glycerol, its mono- and diphosphates, inorganic phosphate, glucose, and galactose (in trace amounts).

The electrophoretic analysis of the teichoic acid preparation showed the presence of two phosphorus-containing polymers. The acid hydrolysis of one of these polymers, with an electrophoretic mobility E_{GroP} = 1.3, gave rise to only glycerol and its mono- and diphosphates. The alkaline hydrolysis of this polymer yielded the same phosphate esters and, in addition, diglycerol triphosphate. These data suggest that the polymer represents an unsubstituted 1.3-poly(glycerol phosphate) [7].

The acid hydrolysis of the second polymer (E_{GroP} = 1.07) yielded glycerol, its mono- and diphosphates, and glucose. The alkaline hydrolysis of this polymer could not digest it completely. These data suggest that the polymer represents a considerably substituted poly(glycerol phosphate) [7].

The type of the phosphodiester bond and the configuration of glycoside centers were determined by NMR spectroscopy. The ^{13}C NMR spectrum of the teichoic acid preparation exhibited two distinct sets of high-

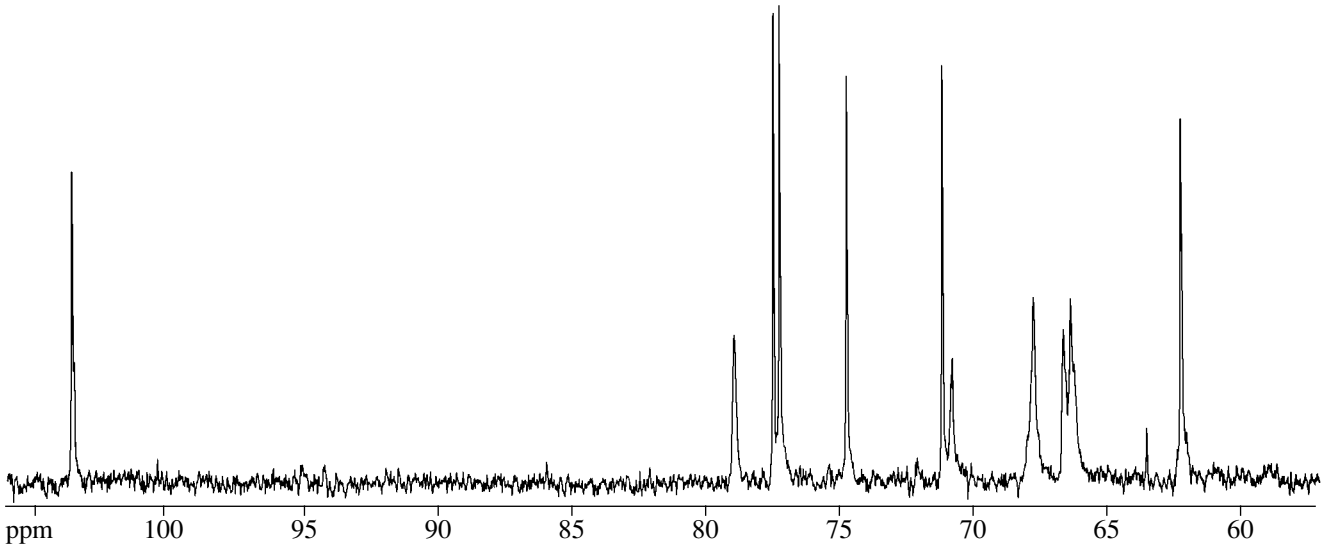

Fig. 3. The ^{13}C NMR spectrum of teichoic acid from the cell wall of *T. fusca* Ac-1952^{T}.

intensity signals and several lower intensity signals (Fig. 3). Sharp resonance peaks in the spectrum corresponded to the resonances of the carbon atoms of β-glucopyranose (Table 2). The presence of pyranose residues at the carbon atoms of glycerol usually leads to shifts of the signals of these carbon atoms. Specificaly, the signal of the carbon atom at which substitution occurs shifts to the lower field side (the so-called α-effect of substitution), whereas the signals of the adjacent atoms shift to the higher field side (the so-called β-effect of substitution) [8]. Bearing this in mind, the broadened low-field signal at 78.8 ppm and two higher field signals at 66.4 and 66.6 ppm can be assigned, respectively, to the C2, C1, and C3 atoms of the substituted glycerol units. The lower intensity signals at 67.7 ppm can be attributed to the C1 and C3 carbon atoms of the unsubstituted glycerol phosphate units and that at 70.8 ppm, to the C2 atom.

A comparison of the integral intensities of the resonances of the C2 atoms of the substituted and unsubstituted glycerol phosphate units showed that the substituted units comprise about 60%. It was difficult to determine the actual percent of substitution in the substituted teichoic acid because the teichoic acid preparation used for analysis contained some amount of unsubstituted poly(glycerol phosphate). It is obvious that the percent of substitution by glucose residues in the substituted teichoic acid is higher than 60% and may even reach 100%, as is evident from the low efficiency of the alkaline hydrolysis of this polymer [7].

Table 2. The chemical shifts of carbon atoms in the ^{13}C NMR spectrum of the cell-wall teichoic acid of *T. fusca* Ac-1952^{T}

Fragment	C1	C2	C3	C4	C5	C6
-1-sn-Gro-(3-P- 2 |	66.4	78.8	66.6			
β-D-Glc*p*1	103.6	74.7	77.1	71.1	77.3	62.3
-1)-sn-Gro-(3-P-	67.7	70.8	67.7			
sn-Gro-(3-P-	63.5	72.0	67.7			

Thus, the chemical and nuclear magnetic resonance studies showed that the cell wall of *T. fusca* Ac-1952^{T} contains two teichoic acids, unsubstituted 1,3-poly(glycerol phosphate) and β-glucosylated 1,3-poly(glycerol phosphate).

In conclusion, the cell walls of *M. mesophila* strain Ac-1953^{T} (the family *Streptosporangiaceae*) and *T. fusca* Ac-1952^{T} (the family *Nocardiopsaceae*) are found to contain teichoic acids of a poly(glycerol phosphate) nature. The teichoic acid of *M. mesophila* (formerly *Thermomonospora mesophila* [9]) represents a poly(glycerol phosphate) containing 5% *N*-acetylgalactosaminyl residues. This is the first time teichoic acid of such a kind has been found in actinomycetes. The cell wall of *T. fusca* (formerly *Thermonospora fusca* [9]) contains two teichoic acids, namely, unsubstituted 1,3-poly(glycerol phosphate) and β-glucosylated 1,3-poly(glycerol phosphate). A similar polymer was found in the cell wall of another member of the family *Nocardiopsaceae*, *Nocardiopsis trehalosi* [10]. The data presented provide further evidence that teichoic acids are widely distributed in the cell walls of actinomycetes.

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