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Polysaccharide Covalently Linked to the Peptidoglycan of the Cyanobacterium *Synechocystis* sp. Strain PCC6714

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A polysaccharide was found to be covalently linked to the peptidoglycan of the unicellular cyanobacterium *Synechocystis* sp. strain PCC6714 via phosphodiester bonds. It could be cleaved from the peptidoglycan-polysaccharide (PG-PS) complex by hydrofluoric acid (HF) treatment in the cold (48% HF, 0°C, 48 h) yielding a pure, HF-insoluble peptidoglycan fraction and an HF-soluble polysaccharide fraction. The PG-PS complex was isolated from the Triton X-100-insoluble cell wall fraction by hot sodium dodecyl sulfate treatment and digestion with proteases. Digestion of the complex with *N*-acetylmuramidase released the glycopeptide-linked polysaccharide, which was further purified by dialysis and gel filtration on Sephadex G-50 and G-200. The polysaccharide consisted of glucosamine, mannosamine, galactosamine, mannose, and glucose and had a molecular weight of 25,000 to 30,000. Muramic acid-6-phosphate was identified as the binding site of the covalently linked, nonphosphorylated polysaccharide as revealed by chemical analysis of linkage fragments of the PG-PS complex.

Peptidoglycan fractions from cyanobacteria, as thus far studied, contain the constituents of the A1 γ-type of peptidoglycan (35), glucosamine (GlcN), muramic acid (MurN), glutamic acid (Glu), alanine (Ala), and diaminopimelic acid (A_2pm) (10, 19). The peptidoglycan layer of cyanobacteria is thicker (10 nm or more) than that of gram-negative bacteria. It seems to be composed of about eight layers in *Synechocystis* sp. strain PCC6714 (21). Although cyanobacteria have an outer membrane, they react positively in the Gram reaction (8, 21). Also, the degree of cross-linkage (56%) of *Synechocystis* sp. strain PCC6714 peptidoglycan (19) is in the range of that for gram-positive bacteria (31).

In addition, nonpeptidoglycan components, such as amino and neutral sugars, are found in peptidoglycan fractions of many cyanobacteria, such as *Phormidium uncinatum* (13), *Anacystis nidulans* (9, 14, 17), *Aphanothece halophytica* (18), *Synechococcus* sp. strain PCC6716 (36), and *Synechocystis* sp. strain PCC6714 (19, 20). The present paper reveals, at least for *Synechocystis* sp. strain PCC6714, that these nonpeptidoglycan components are constituents of a polysaccharide which is covalently linked to muramic acid 6-phosphate (MurN-6-P) of peptidoglycan via phosphodiester bonds.

MATERIALS AND METHODS

Cultivation of cyanobacteria. *Synechocystis* sp. strain PCC6714 was kindly provided by the Pasteur Culture Collection, Paris, and cultivated photoautotrophically in BG-11 medium, pH 7.5, at 25°C (30). Mass cultures were prepared in a 12-liter fermentor (Jungkeit, Göttingen, FRG) and gassed by air and carbon dioxide at flow rates of 250 and 2.5 liters/h, respectively, with illumination from white fluorescent lamps at a constant light intensity of 5,000 lux.

Preparation of the PG-PS complex. Cell walls of *Synechocystis* sp. strain PCC6714 were prepared as described previously (19, 21). The Triton X-100-insoluble cell wall fraction was extracted three times by boiling in 4% (wt/vol) sodium dodecyl sulfate (SDS) at 100°C for 15 min. Rigid layers (peptidoglycan plus covalently linked polymers) were collected by ultracentrifugation (176,600 × *g*, 1 h) and washed with distilled water until they were free of SDS (16). Nonpeptidoglycan amino acids were removed from the rigid layer fraction by digestion with pronase (from *Streptomyces griseus*, 6 U/mg of protein; Sigma Chemical Co., St. Louis, Mo.) in 20 mM Tris buffer, pH 7.4, at 37°C for 12 h. After repeated SDS extractions (4% SDS, 100°C, 15 min) and removal of the detergent (as above), the peptidoglycan-polysaccharide (PG-PS) complex was collected by ultracentrifugation (176,600 × *g*, 1 h) and lyophilized.

Isolation of the glycopeptide-linked polysaccharide. The lyophilized PG-PS complex was ultrasonicated for 10 min and digested with *N*-acetyl muramidase (from hen egg white; 53,000 U/mg of protein) in 20 mM ammonium acetate buffer, pH 6.5, at 37°C for 24 h. The digest was controlled by measuring the decrease in absorbance at 578 nm of the suspension and by determining the formation of reducing groups (28) during the incubation. The incubation mixture containing the water-soluble glycopeptide-bound polysaccharide and the free glycopeptides of the peptidoglycan was dialyzed against distilled water for 12 h, removing the free glycopeptides. The glycopeptide-linked polysaccharide fraction was evaporated to a small volume (2 ml) and chromatographed on a Sephadex G-50 (fine; Pharmacia, Upsala, Sweden) column (2.6 by 9 cm).

Preparation of the liberated polysaccharide. Ice-cold 48% hydrofluoric acid (HF; 1 ml) was added to the lyophilized PG-PS complex (up to 30 mg), and the mixture was kept at 0°C for 48 h (12, 19). The HF-insoluble peptidoglycan was removed by centrifugation (12,500 × *g*, 0°C, 30 min), and the supernatant containing the HF-soluble polysaccharide was dialyzed against distilled water in the cold and lyophilized.

Isolation of MurN-6-P. MurN-6-P was liberated from the PG-PS complex by partial acid hydrolysis (6 M HCl, 110°C, 1 h) (25) and separated from peptides, disaccharides, and monomers formed after hydrolysis by cation exchange chromatography on Dowex 50 WX-8 (H^+ form, 0.9 by 20 cm column) with a gradient of 0 to 2 M HCl. MurN-6-P eluted

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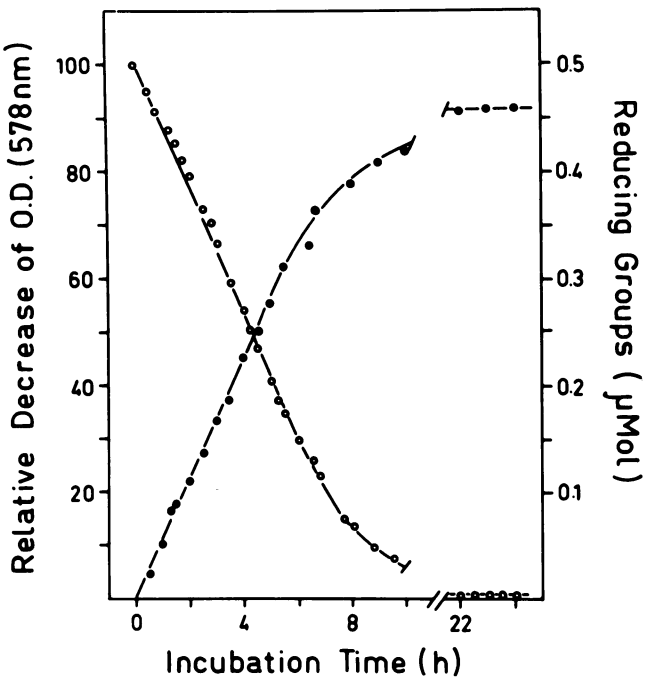

FIG. 1. Digestion of the PG-PS complex from *Synechocystis* sp. strain PCC6714 with *N*-acetylmuramidase (EC 3.2.1.17, 53,000 U/mg of protein). The PG-PS complex (50 mg) was ultrasonicated for 10 min, and the suspension in 100 ml of 20 mM ammonium acetate buffer, pH 6.5, was incubated in an enzyme-substrate ratio of 1:50 (wt/wt) at 37°C for 24 h. The digest was controlled by measuring the decrease in optical density at 578 nm of the suspension (○) and by determining the reducing groups (28) formed during the digestion (●).

with the water fraction and was evaporated to dryness and lyophilized.

Isolation of fragments from the PG-PS complex. The lyophilized PG-PS complex (50 mg) was degraded by partial acid hydrolysis (1 M HCl, 100°C, 1 h) and evaporated to dryness. The residue was dissolved in distilled water (final concentration, 50 mg/ml), and 500 μg of the hydrolysate was separated by high-voltage paper electrophoresis in pyridine-acetic acid-water (5:2:43, vol/vol/vol), pH 5.3, at a constant voltage of 3,000 V for 90 min. The fragments were detected by staining with ninhydrin (0.1% in acetone) and alkaline silver nitrate (Trevelyan procedure [39]) or by staining with fluorescamine (0.05% in acetone) for preparative purposes.

Molecular weight determination of the glycopeptide-linked polysaccharide. The glycopeptide-linked polysaccharide (30 mg, lyophilized fraction) was dissolved in a small volume (1 to 2 ml), applied to a Sephadex G-200 column (1.5 by 90 cm), and separated by gel filtration. The column was eluted with distilled water at a constant flow rate of 10 ml/h. In a parallel run dextrans with molecular weights of 9,000, 18,100, 42,500, 65,600, and 150,000 (Sigma) were chromatographed on the same column under identical conditions. The dextrans and the glycopeptide-linked polysaccharide were detected by the phenol-sulfuric acid method (11). The molecular weight of the glycopeptide-linked polysaccharide was calculated from the elution volume compared with those of the dextrans of known molecular weights.

Analytical methods. Amino acids and amino sugars liberated by 4 M HCl (105°C, 18 h) were analyzed with an automatic amino acid analyzer, model LC 6001 (Biotronik, Munich, FRG). The amino sugars mannosamine (ManN) and galactosamine (GalN), neutral sugars, and acetyl groups were determined by gas-liquid chromatography as previously described (19). Organic phosphorus was estimated by the method of Lowry et al. (26). Reducing sugars were determined by the Park and Johnson procedure (28). Amino groups of peptidoglycan degradation products were determined by ninhydrin reaction (27) and aldehyde groups by the diphenylamine reaction (7). Reduction of neutral sugars and amino sugars was carried out in 0.2 M sodium borohydride at pH 8.5 for 12 h (38).

Chemical treatments and extractions. The PG-PS complex was treated with 0.1 M HCl at 60°C for 16 h (2), 10% trichloroacetic acid (TCA) at 65°C for 7 h (34), 0.1 M EDTA at 4°C for 24 h (24), and 48% HF at 0°C for 48 h (12, 19), or extracted with 4% SDS at 100°C for 15 min (4), 99% formamide at 150°C for 20 min (29), and 50% phenol at 68°C for 30 min (40).

RESULTS

Stability of the PG-PS complex. Various treatments were tested to remove the polysaccharide moiety from the peptidoglycan. The PG-PS complex was found to be stable against extraction with hot 4% SDS (100°C, 15 min), 50% phenol (68°C, 30 min), and 0.1 M EDTA (4°C, 24 h). In addition, treatment with 10% TCA (65°C, 7 h) had no significant effect. However, the polysaccharide was partially removed from the PG-PS complex by treatment with hot 99% formamide (150°C, 20 min). Treatment of the PG-PS complex with 0.1 M HCl (60°C, 16 h) released the polysaccharide components from the complex, while the peptidoglycan remained insoluble and retained the phosphate groups. Complete cleavage of the PG-PS complex was achieved by HF treatment in the cold (48% HF, 0°C, 48 h). This treatment yielded an HF-soluble, nonphosphorylated polysaccharide and an HF-insoluble peptidoglycan fraction which was dephosphorylated and free of nonpeptidoglycan components.

Isolation of the glycopeptide-linked polysaccharide. The glycopeptide-linked polysaccharide was prepared from the PG-PS complex of *Synechocystis* sp. strain PCC6714 by

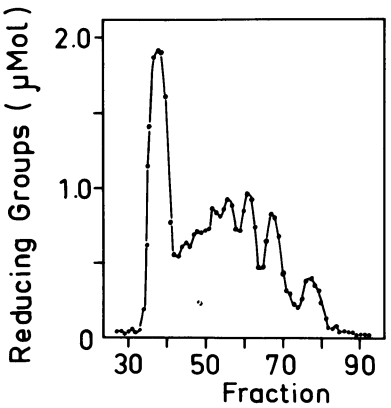

FIG. 2. Separation of lysozyme degradation products from the PG-PS complex of *Synechocystis* sp. strain PCC6714 by gel filtration. The enzymatically degraded PG-PS complex (30 mg in 20 mM ammonium acetate buffer, pH 6.5) was concentrated to a small volume (1 to 2 ml) and applied to a column (2.6 by 90 cm) of Sephadex G-50 (fine). Gel filtration was performed at a flow rate of 50 ml/h. Fractions of 5.4 ml were collected, and a portion (50 μl) of each fraction was tested for reducing sugars (28).

TABLE 1. Chemical composition of the glycopeptide-linked and liberated, HF-soluble polysaccharide fractions from the PG-PS complex of *Synechocystis* sp. strain PCC6714

Component	Amt in PS[a] fraction (nmol/mg of fraction [dry wt])	
	Glycopeptide-linked PS	HF-liberated PS
Amino sugars		
MurN	397	—[b]
GlcN	744	627
ManN	203	330
GalN	14	22
Amino acids		
Ala	904	—
Glu	429	—
A_2pm	441	—
Neutral sugars		
Man	284	567
Glc	30	57
Phosphorus	108	—

[a] PS, Polysaccharide.
[b] —, None.

digestion with *N*-acetyl muramidase. By this enzymatic treatment the PG-PS complex could be slowly but completely solubilized after about 20 h of incubation (Fig. 1). The degradation products (glycopeptide-linked polysaccharide and free glycopeptides) were separated by gel filtration on Sephadex G-50 (Fig. 2). The glycopeptide-linked polysaccharide eluted with the void volume (fractions 32–42), while glycopeptides free of polysaccharide components and phosphate eluted in fractions 43 through 85. The fractions 32 through 42 (Fig. 2) were pooled, dialyzed, and further chromatographed on Sephadex G-200 in parallel to dextrans of known molecular weights under identical conditions. From this run a molecular weight of about 25,000 to 30,000 was calculated for the glycopeptide-linked polysaccharide.

Chemical composition of the glycopeptide-linked and the liberated, HF-soluble polysaccharide. The glycopeptide-linked polysaccharide obtained by gel filtration of *N*-acetylmuramidase degradation products from the PG-PS complex consisted of the peptidoglycan components MurN, GlcN, L-Ala, D-Glu, meso-A_2pm (m-A_2pm), D-Ala, and the polysaccharide components GlcN, ManN, GalN, mannose (Man), and glucose (Glc) (Table 1), similar in chemical composition to the undigested PG-PS complex (19). In addition, phosphate was present as MurN-6-P (see below). Nonpeptidoglycan amino acids were completely absent, as revealed by amino acid analysis. The liberated polysaccharide prepared by HF treatment of the PG-PS complex in the cold (48% HF, 0°C, 48 h) and dialysis was free of peptidoglycan components (Table 1). Polysaccharide components were present in the HF-soluble fraction of the HF-treated PG-PS complex, but no phosphate.

Identification of MurN-6-P. MurN-6-P was isolated from the PG-PS complex by partial acid hydrolysis and cation exchange chromatography on Dowex 50 WX-8 (see Materials and Methods). MurN-6-P eluted in fractions 5 to 10 (Fig. 3), while ninhydrin-positive degradation products (peptides, disaccharides, and monomers) lacking phosphate remained bound to the resin (fractions 15 to 60 in Fig. 3). Fractions 5

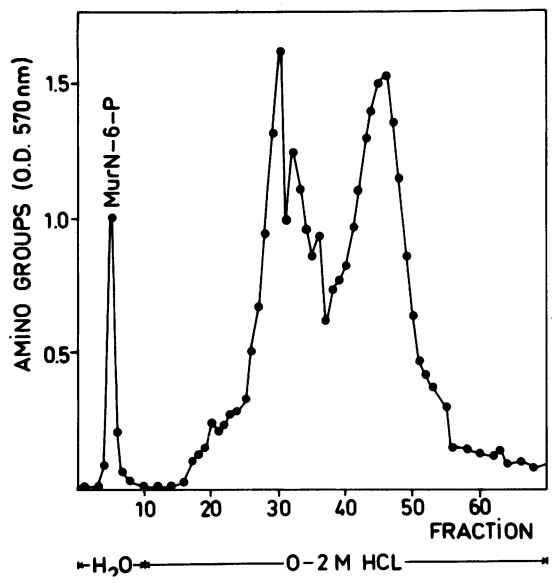

FIG. 3. Cation exchange chromatography of a partial acid hydrolysate (6 M HCl, 110°C, 1 h) of the PG-PS complex from *Synechocystis* sp. strain PCC6714. The partial acid hydrolysate (50 mg) was applied to a column (0.9 by 20 cm) of Dowex 50 WX-8 (H^+ form) and eluted with distilled water (fractions 1 through 10) followed by a gradient of 0 to 2 M HCl (fractions 11 through 70) at a flow rate of 40 ml/h. Fractions of 2 ml were collected and analyzed for amino groups (27) and phosphate (26) and by amino acid analysis. Fractions 3 through 10, MurN-6-P; fractions 15 through 60, monomers, peptides, and disaccharides.

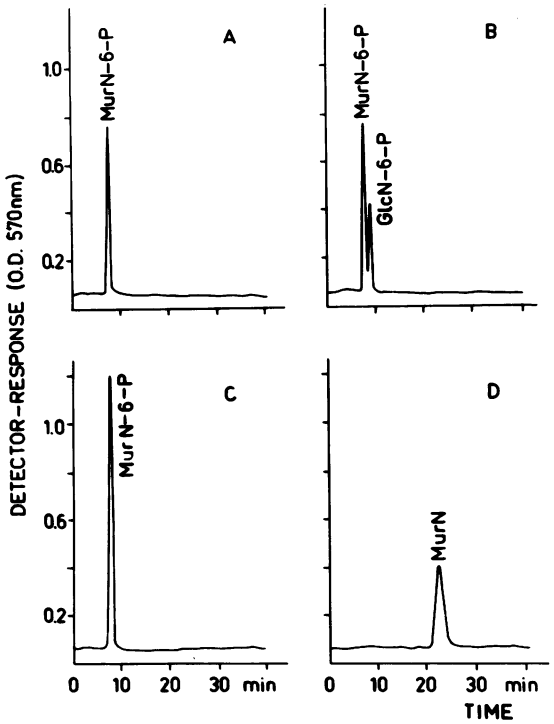

FIG. 4. Identification of MurN-6-P from the PG-PS complex of *Synechocystis* sp. strain PCC6714. Fractions 3 through 10 (see Fig. 3) were pooled and analyzed by amino acid analysis without hydrolysis (A), after the addition of authentic GlcN-6-P (B), after the addition of MurN-6-P from *Bacillus cereus* AHU1356 or *Bacillus subtilis* 168 peptidoglycan (C), and after total acid hydrolysis (4 M HCl, 105°C, 18 h) (D).

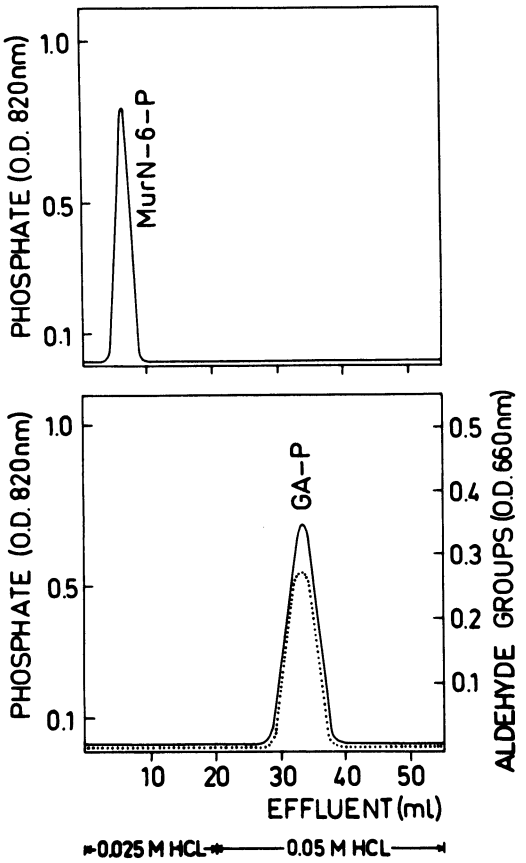

FIG. 5. Anion exchange chromatography of MurN-6-P (fractions 3 through 10 in Fig. 3) before and after oxidation with sodium *meta*-periodate (0.2 M $NaIO_4$, 25°C, 12 h) on Dowex 1-X8 (Cl^- form, 0.9 by 10 cm). The column was eluted with 0.025 M HCl (fractions 1 through 20) followed by 0.05 M HCl (fractions 21 through 50) at a flow rate of 10 ml/h. Fractions of 1 ml were collected and analyzed for phosphate (solid lines; 26) and aldehyde groups (dotted line; 7).

to 10 were pooled and analyzed by amino acid analysis, yielding a single peak of MurN-6-P (Fig. 4A). MurN-6-P from *Synechocystis* sp. strain PCC6714 peptidoglycan eluted differently from authentic glucosamine-6-phosphate (GlcN-6-P) (Fig. 4B), but identically with MurN-6-P isolated from the peptidoglycans of *Bacillus cereus* AHU1356 and *Bacillus subtilis* 168 (Fig. 4C) on the amino acid analyzer. After total acid hydrolysis (4 M HCl, 105°C, 18 h) of fractions 5 to 10 (Fig. 3), a single peak identical with muramic acid (MurN) was detected by amino acid analysis (Fig. 4D). The total hydrolysate (4 M HCl, 105°C, 18 h) of MurN-6-P contained MurN and phosphate in equal amounts.

To determine the position of the phosphate group of MurN, fractions 5 to 10 (Fig. 5) were oxidized by sodium meta-periodate (0.2 M $NaIO_4$, 12 h, pH 8.5). The oxidation products were separated by anion exchange chromatography on Dowex 1-X8 (Cl^- form, 0.9 by 10 cm column; the eluent was 0.025 M to 0.05 M HCl) (Fig. 5). The eluted fractions were tested for aldehyde groups by the diphenylamine reaction (7) and for phosphate by the method of Lowry et al. (26). Fractions 30 to 40 reacted positively by both methods. They cochromatographed with glycolaldehyde-phosphate (GA-P), which is produced by periodate oxidation only when MurN has a phosphate group in the C-6 position, proving that MurN-6-P is a component of the PG-PS complex of *Synechocystis* sp. strain PCC6714 (Fig. 5). MurN-6-P eluted in fractions 3 to 10 when a preparation not oxidized with $NaIO_4$ was used (Fig. 5).

Fragments from the PG-PS complex. Fragments of the PG-PS complex were formed by partial acid hydrolysis (1 M HCl, 100°C, 1 h) and separated by high-voltage paper electrophoresis (Fig. 6). The fragments were detected by ninhydrin and alkaline silver nitrate staining. For preparative purposes the fragments reacting positively with both staining procedures were stained in a parallel run with a fluorescent dye, fluorescamine, and analyzed for amino sugars, neutral sugars, and phosphate. Three fragments were found to contain peptidoglycan and polysaccharide components in addition to phosphate (Table 2). The MurN-6-P-containing fragments (1, 3, and 4 in Fig. 6 and Table 2) represent the linkage region of the PG-PS complex and prove the covalent linkage of the polysaccharide to the peptidoglycan (Fig. 7).

DISCUSSION

Cyanobacteria are believed to have a gram-negative cell wall type due to the presence of an outer membrane. However, polysaccharide covalently linked to peptidoglycan in the cell wall of *Synechocystis* sp. strain PCC6714 (Fig. 7), a high degree of cross-linkage (56% [19]), a rather thick peptidoglycan layer (12 nm, presumably eight-layered [21]), and a positive Gram reaction are all properties typical of gram-positive bacterial peptidoglycan. Polysaccharide covalently linked to peptidoglycan via phosphodiester bonds is also found in streptococci, *Micrococcus luteus*, *Mycobacterium butyricum* (25), *Staphylococcus aureus* (23, 31), and

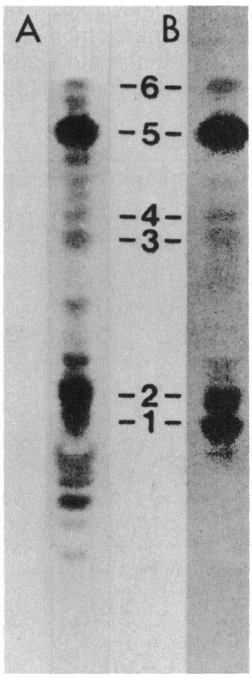

FIG. 6. Separation of fragments from the PG-PS complex of *Synechocytis* sp. strain PCC6714 by high-voltage paper electrophoresis. A portion (500 μg) of a partial acid hydrolysate (1 M HCl, 100°C, 1 h) of the PG-PS complex was separated on Whatman 3 MM paper in pyridine-acetic acid-water (5:2:43, vol/vol/vol), pH 5.3, at a constant voltage of 3,000 V for 90 min. Fragments were detected with ninhydrin (A) and alkaline silver nitrate (B) (39). Fragment numbers are indicated (see Table 2). Top, cathode; bottom, anode. The origin is indicated below fragment 1.

TABLE 2. Chemical composition of fragments from the PG-PS complex of *Synechocystis* sp. strain PCC6714[a]

Fragment	Molar ratio of component (mol/mol)									
	MurN-6-P	MurN	Glu	Ala	A_2pm	GlcN	ManN	GalN	Man	Glc
1	0.9	—[b]	4.4	2.6	2.0	4.1	—	—	0.5	—
2	—	1.2	0.8	1.7	1.0	—	—	—	—	—
3	1.2	—	1.2	2.6	1.0	4.2	1.1	—	1.6	1.9
4	1.1	—	2.3	3.4	1.0	6.2	1.2	0.6	3.0	2.8
5	—	—	—	—	—	8.2	1.0	—	—	0.6
6	—	—	—	—	—	3.1	1.0	—	0.9	1.0

[a] For details of procedure and identification of fragments, see Fig. 6 and legend.
[b] —, None.

various bacilli (2, 41), but has never been detected in gram-negative bacteria (25). MurN-6-P is the binding site for teichoic acids in *Staphylococcus aureus* (37), *Staphylococcus lactis* (5), and *Bacillus cereus* (33), for teichuronic acids in *Bacillus licheniformis* (31) and *Micrococcus luteus* (32), for neutral polysaccharides in *Lactobacillus acidophilus* (6), *Lactobacillus casei* (15), *Streptococcus* spp., and *Mycobacterium butyricum* (25), and for a charged polysaccharide in *Bacillus cereus* AHU1356 (1). The polysaccharide of *Bacillus cereus* AHU1356 has a molecular weight of 33,000 and is composed of GlcN, ManN, GalN, and glucose (Glc) in molar ratios of 4:2:1:1 (1). The polymer amounts to 50% of the cell wall fraction dry weight. The peptidoglycan-linked polysaccharide of *Synechocystis* sp. strain PCC6714 has a similar chemical composition (GlcN, ManN, GalN, Man, and Glc). Here, the polysaccharide moiety amounted to 44% of the PG-PS complex (dry weight basis) and had a molecular weight of about 25,000 to 30,000. The peptidoglycan-linked polysaccharide of *Synechocystis* sp. strain PCC6714 seems not to be localized in the outer membrane, since the polysaccharide components ManN and GalN were found to be absent from outer membrane fractions (22).

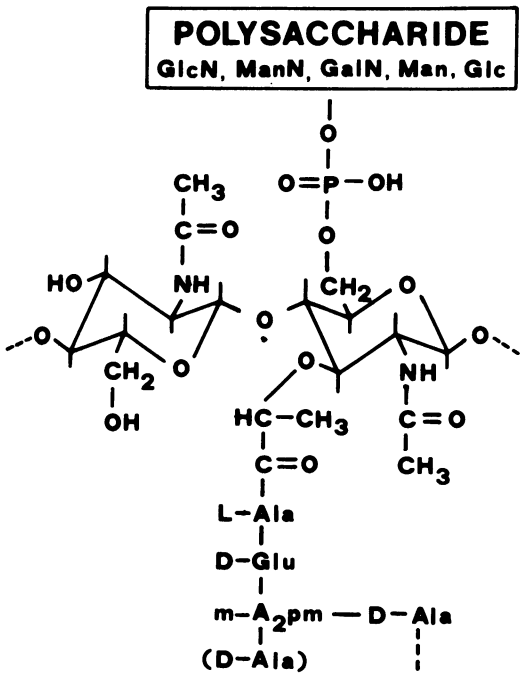

FIG. 7. Proposed structure of the PG-PS complex from *Synechocystis* sp. strain PCC6714.

From our studies on the PG-PS complex of *Synechocystis* sp. strain PCC6714, we can conclude that treatment with HF in the cold (48% HF, 0°C, 48 h) is an excellent method for cleaving the PG-PS complex into an HF-soluble polysaccharide and an HF-insoluble peptidoglycan fraction. Both polymers remained intact after this treatment. Although the polysaccharide components could be removed also from the PG-PS complex by mild acid treatment (0.1 M HCl, 60°C, 16 h), *N*-acetyl groups were partially removed from the amino sugars of the peptidoglycan backbone and the polysaccharide. This purification is suitable only if a phosphorylated peptidoglycan fraction is to be isolated. *N*-Acetyl groups can be reintroduced by treatment with a mixture of acetic anhydride-pyridine (1:1, vol/vol) at 100°C for 30 min (3). However, treatment with hot formamide (99%, 150°C, 2 h) removed only part of the polysaccharide components. This drastic method seems to be disadvantageous, since cleavage of the polysaccharide is not complete. In addition, *N*-formyl groups are introduced into the peptidoglycan, which decreases sensitivity to *N*-acetyl muramidases (29). Furthermore, partial destruction of the amino sugars cannot be excluded when using such drastic conditions.

Teichoic acids are not present in the PG-PS complex of *Synechocystis* sp. strain PCC6714, since neither glycerol nor ribitol was detected in this fraction. In addition, the polysaccharide moiety did not contain any phosphate. Phosphate groups were present in the PG-PS complex only as MurN-6-P, representing the binding site for the peptidoglycan-linked polysaccharide of *Synechocystis* sp. strain PCC6714.

We conclude from the data that *Synechocystis* sp. strain PCC6714 has a cell wall type which combines structural elements from both the gram-negative cell wall type (presence of an outer membrane composed of lipopolysaccharide, lipids, proteins, and carotenoids) (22) and the gram-positive cell wall type (thick and highly cross-linked, eight-layered peptidoglycan network [19] with covalently linked polysaccharide).

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