Isolation and Characterization of Cell Wall Components of the Unicellular Cyanobacterium *Synechococcus* sp. PCC 6307

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Cell walls and outer membranes, free of thylakoids and cytoplasmic membranes, were isolated from the unicellular cyanobacterium *Synechococcus* sp. PCC 6307. Electron microscopy revealed C-shaped cell wall fragments, which were partially converted to outer membrane vesicles after removal of the peptidoglycan by lysozyme digestion. The major constituents of the outer membrane were proteins and lipopolysaccharide, while lipids and carotenoids were minor components. The polypeptide patterns of the outer membranes were dominated by two major proteins (M_r 52000 and 54000). Five strongly polar lipids (unidentified), free fatty acids and small amounts of sulpholipid were detected in extracts of partially purified cell wall fractions, but monogalactosyldiglyceride and digalactosyldiglyceride were not found. The peptidoglycan layer (10 nm thick) was also isolated. Its chemical composition indicated an A1 γ -type structure. The degree of cross-linkage was 57%. A polysaccharide, consisting of fucose, mannose, galactose and glucose, was bound to the peptidoglycan, most likely via muramic acid 6-phosphate.

INTRODUCTION

Cell walls of cyanobacteria are composed of a peptidoglycan layer and an outer membrane. Outer membrane constituents include proteins, lipids and carotenoids (Drews & Weckesser, 1982; Resch & Gibson, 1983; Omata & Murata, 1984; Jürgens & Weckesser, 1985; Benz & Böhme, 1985). Lipopolysaccharides have been found in some, but not all, cyanobacteria studied (Weckesser *et al.*, 1979; Schmidt *et al.*, 1980*a, b*; Raziuddin *et al.*, 1983). Proteins of apparent M_r 50000 to 70000 dominate the polypeptide patterns of cell walls from *Synechocystis* sp. PCC 6714 (Omata & Murata, 1984; Jürgens *et al.*, 1985), *Anacystis nidulans* (Golecki, 1977; Murata *et al.*, 1981; Resch & Gibson, 1983), *Synechococcus leopoliensis* and *Synechococcus* sp. (Resch & Gibson, 1983). The two major outer membrane proteins of *Synechocystis* sp. PCC 6714 are associated with peptidoglycan by strong ionic interactions (Jürgens *et al.*, 1985). Carotenoids and lipids are minor, but regular constituents of the outer membrane of *Synechocystis* sp. (Jürgens & Weckesser, 1985) and *Synechococcus* sp. (Murata *et al.*, 1981; Resch & Gibson, 1983). The peptidoglycan of *Synechocystis* sp. PCC 6714 has properties in common with that of Gram-positive bacteria (Jürgens *et al.*, 1983, 1985).

This paper describes the isolation and chemical characterization of the cell wall, the outer membrane and the peptidoglycan of the cyanobacterium *Synechococcus* sp. PCC 6307, the reference strain for the high GC group (Rippka *et al.*, 1979).

Abbreviations: A₂pm, diaminopimelic acid; GlcNAc, *N*-acetylglucosamine; GlcN-6-P, glucosamine 6-phosphate; ManNAc, *N*-acetylmannosamine; MurNAc, *N*-acetylmuramic acid; MurN-6-P, muramic acid 6-phosphate; Tyv, Tyvelose.

METHODS

Strain and growth conditions. *Synechococcus* sp. PCC 6307, obtained from the Pasteur Culture Collection (PCC), Paris, France, was grown photoautotrophically in BG-11 medium pH 7.5 (Rippka *et al.*, 1979) at 25 °C, illuminated with white fluorescent lamps (5000 lx). Mass cultures (10 l) were prepared in a Biostat E fermenter (Braun Melsungen) gassed continuously with air/CO₂ (99:1, v/v, at 250 l h⁻¹). Cells were harvested in the stationary growth phase by centrifugation (12000 g, 30 min), washed once with 20 mM-Tris/HCl buffer, pH 8.0 ('Tris-buffer', used throughout this study) and stored at -20 °C until use.

Isolation of cell walls. Cells were broken in Tris-buffer by means of a cooled (4 °C) vibrogen shaker (type Vi 2; E. Bühler) for 20 min at full speed using a ratio of cells to glass beads (0.17–0.18 mm in diameter) of 1:2 (w/w). The cell homogenate was passed through a glass filter (type G-1; Schott) to remove glass beads. Whole cells were separated by low-speed centrifugation (300 g, 10 min). Cell envelopes were obtained from the supernatant after centrifugation at 12000 g for 30 min. They were washed with Tris-buffer until the final supernatant was colourless. The cell envelope fraction (5 ml suspension, 2 mg protein ml⁻¹) was loaded onto a discontinuous sucrose gradient [10 ml each of 55, 50 and 40% (w/w) sucrose and 5 ml of 30% (w/w) sucrose in Tris-buffer] and centrifuged in a swing-out bucket rotor (AS 4.13, Kontron) for 12 h at 16000 g. Cell walls were isolated from the 55% sucrose band and washed free of sucrose by resuspension in Tris-buffer and centrifugation (48000 g, 30 min). Sucrose gradient centrifugation was repeated twice, yielding the gradient-purified cell wall fraction 'CW I' as the final pellet. For further purification the CW I fraction was resuspended in 5 ml Tris-buffer containing 10 mM-MgCl₂. After the addition of the same volume of 4% (w/w) Triton X-100 in Tris-buffer, Triton-soluble components were extracted from the cell walls by stirring for 20 min at 23 °C (Schnaitman, 1971). Cell walls were recovered by centrifugation (48000 g, 30 min) and washed several times with Tris-buffer containing 10 mM-MgCl₂ until the supernatant was free of detergent (lack of absorbance at 280 nm; Garewal, 1973). The final pellet contained the Triton-insoluble cell wall fraction 'CW II'.

Isolation of outer membranes. The CW I or CW II fraction was resuspended in 20 mM-ammonium acetate buffer, pH 6.5, and digested with lysozyme [from hen egg white, 53000 units (mg protein)⁻¹, Sigma] in an enzyme/substrate ratio of 1:25 (w/w) at 37 °C for 24 h. Outer membranes (OM I and OM II, respectively) were collected by centrifugation (48000 g, 1 h) and were further purified by sucrose gradient centrifugation (as given above). Sucrose was removed by repeated centrifugation (48000 g, 1 h) and resuspension in Tris-buffer containing 10 mM-MgCl₂.

Isolation of peptidoglycan. Gradient-purified cell walls (fraction CW I, 1 g wet weight, see above) were suspended in 4–5 ml water and added dropwise into 50 ml of a boiling 4% (w/v) SDS solution containing 0.1% 2-mercaptoethanol. After heating to 100 °C for 15 min and centrifugation (48000 g, 15 °C, 1 h), the extraction was repeated with the sediment (the supernatant was discarded). The final sediment ('rigid layer') was washed with distilled water for a complete removal of SDS (Braun & Rehn, 1969; Jürgens *et al.*, 1983). Protein was removed from the rigid layer by incubation with pronase [from *Streptomyces griseus*, specific activity 8 units (mg protein)⁻¹; 40 units per mg rigid layer protein were used] while stirring at 37 °C for 24 h. After centrifugation at 48000 g (15 °C, 20 min), the sediment (peptidoglycan-polysaccharide complex) was boiled in 4% (w/v) SDS for 15 min and subsequently freed from SDS by washing with distilled water. For hydrofluoric acid (HF) treatment, 20 mg peptidoglycan-polysaccharide complex was suspended in 2 ml ice-cold HF and incubated at 0 °C for 48 h. Peptidoglycan was then sedimented at 12000 g (4 °C, 15 min), washed several times with ice-cold distilled water and lyophilized.

Isolation and separation of lipids and carotenoids. Lipids and carotenoids were extracted from cell wall and outer membrane fractions with trichloromethane/methanol (1:1, v/v) under dim light (Bligh & Dyer, 1959). Phase separation was achieved by the addition of 2.5 vols trichloromethane and 1% (w/w) NaCl followed by centrifugation (7000 g, 5 min). The methanol/water phase was re-extracted four times with an equal volume of trichloromethane. The trichloromethane phases containing lipids and carotenoids were combined and evaporated to a final volume of 0.25–0.5 ml and stored in sealed tubes under a nitrogen atmosphere at -20 °C. Lipids and carotenoids were separated as described previously (Jürgens *et al.*, 1985).

Electron microscopy and SDS-PAGE. Embedding and ultrathin sectioning techniques were as described by Golecki (1977). SDS-PAGE was performed as cited by Jürgens & Weckesser (1985), using 15% (w/v) polyacrylamide slab gels.

Analytical chemical methods. Amino acids and amino sugars were determined after hydrolysis with 4 M-HCl (105 °C, 18 h) in an automatic amino acid analyser, model LC 6001 (Biotronik). Neutral sugars were liberated by treatment with 0.1 M-HCl (100 °C, 48 h) and quantified by gas-liquid chromatography (GLC) as their alditol acetates using a Varian Aerograph (Varian, model 1440) and a glass column (length 150 cm, internal diameter 3 mm; 3% ECNSS-M on Gas-chrom Q, 100–200 mesh; isothermal column temperature 180 °C; injector and detector temperature 240 °C). Fatty acids were quantified after methyl-esterification in methanol/concentrated HCl (5:1, v/v) at 85 °C for 18 h and determined by GLC on a glass column (15% EGSS-X on Gas-chrom P, 100–200 mesh; column size and temperatures as for the estimation of neutral sugar derivatives, see above). Organic

phosphorus was determined by the method of Lowry *et al.* (1954). The purity of the cell wall and outer membrane fractions was confirmed by measuring the degree of chlorophyll *a* contamination, using the methods of extraction and determination described by Mackinney (1941).

RESULTS

Fine structure of the cell wall

Gradient-purified and Triton-insoluble cell wall fractions (CW I and CW II, respectively) appeared as C-shaped fragments in ultrathin sections, showing an electron-dense peptidoglycan layer (10 nm thick) and a double-track-structured outer membrane (8 nm thick) (Fig. 1*a*). In ultrathin sections of outer membrane preparations the peptidoglycan layer was not detectable, demonstrating the efficient digestion of this layer by lysozyme. Furthermore, the C-shaped cell wall fragments were partially converted into outer membrane vesicles after the lysozyme treatment (Fig. 1*b*).

Chemical composition of the cell wall and outer membrane

As evidenced by the chemical composition (Table 1) of unfractionated preparations, cell walls of *Synechococcus* sp. PCC 6307 contained proteins, lipopolysaccharide, polysaccharide, lipids and carotenoids as well as the peptidoglycan-specific components MurNAc and A₂pm. The fatty acids β -C₁₄OH and β -C₁₆OH, the amino sugars GlcN and ManN, and the neutral sugars Rha, Fuc, Man, Gal, Glc and Tyv were constituents of the lipopolysaccharide (Schmidt *et al.*, 1980*a*). Part of the GlcNAc, ManNAc and Man comprised components of the peptidoglycan-bound polysaccharide (see below). Fatty acids other than the β -hydroxy fatty acids are representative of lipids of the outer membrane other than those contained in the lipopolysaccharide. Some loss of lipopolysaccharide and lipids was observed after Triton X-100

Fig. 1. Ultrathin sections of gradient-purified cell walls CW I (*a*) and outer membranes OM II (*b*) from *Synechococcus* sp. PCC 6307. Bars, 0.2 μ m. For terminology of CW I and OM II see Methods. PG, peptidoglycan; OM, outer membrane.

Table 1. *Chemical composition of gradient-purified cell walls (CW I), of Triton-insoluble cell walls (CW II), and of outer membranes (OM II) from Synechococcus sp. PCC 6307*

Compound	Content (nmol per mg fraction dry weight)*		
	CW I	CW II	OM II
Glu	377	383	229
Ala	537	558	353
A ₂ pm	178	197	17
Other amino acids	1834	1850	2574
GlcNAc	352	336	122
MurNAc	124	134	6
ManNAc	101	95	41
Rha	257	290	627
Fuc	78	70	189
Man	88	126	111
Gal	63	39	133
Glc	42	34	67
Tyv	+	+	+
Phosphate	+	+	+
C _{14:0}	26	2	3
C _{16:0}	122	30	27
C _{16:1}	11	3	3
C _{18:0}	11	1	2
C _{18:1}	18	3	5
C ₁₄ OH	80	27	28
C ₁₆ OH	64	40	47
Unknown fatty acids	184	39	46
Carotenoids	+	+	+

* Values are the mean of three determinations. +, Present, but not quantified.

extraction of gradient-purified cell walls, as indicated by a decrease of the fatty acid contents (Table 1). Carotenoids were detected in cell wall (CW I and CW II) and outer membrane (OM II) fractions (amounts below 1% of fraction dry weight). The purified outer membrane fraction (OM II, Table 1) contained very few peptidoglycan components, due to efficient removal of the peptidoglycan by lysozyme digestion. Furthermore, the recovery of constituents of the peptidoglycan-bound polysaccharide (see below) in the supernatant of lysozyme-treated cell walls (after centrifugation at 176 000 g, 4 °C, 1 h) indicated that this enzymic digestion not only removed the peptidoglycan but also its specific polysaccharide (data not shown).

Outer membrane proteins

The polypeptide patterns of cell envelopes, gradient-purified cell walls (CW I), and Triton-insoluble cell walls (CW II) of *Synechococcus* sp. PCC 6307 were dominated by two outer membrane proteins of M_r 52 000 and 54 000 (Fig. 2, lanes B, C and D, respectively). A few other proteins were present in only minor quantities. Purified outer membrane fractions OM I and OM II (Fig. 2, lanes E and F, respectively) showed protein patterns identical to those of the corresponding cell wall fractions CW I and CW II, but were enriched in a high- M_r protein (M_r 97 000) and allowed the detection of a protein of M_r 37 000, not visible prior to purification.

Outer membrane carotenoids

Cell walls and purified outer membrane fractions of *Synechococcus* sp. PCC 6307 were intensely yellow coloured due to the presence of carotenoids. Absorption spectra showed maxima at 443, 462, and 491 nm (Fig. 3). At least four carotenoids (not identified) were detected in pigment extracts of the Triton-insoluble fraction, CW-II (Fig. 4b) and in purified outer membranes, OM-II (data not shown). Two carotenoids (Fig. 4b, bands 3 and 4) dominated the pigment pattern. Echinone and β -carotene, carotenoids indicative of the thylakoid/cytoplasmic membrane fraction (Jürgens & Weckesser, 1985), were absent from CW II and OM-II fractions. Chlorophyll *a*, a typical component of the cyanobacterial thylakoid membrane, was

Fig. 2. Polypeptide patterns of cell envelopes (B), gradient-purified cell walls (C), Triton-insoluble cell walls (D), and outer membrane fractions OM I (E) and OM II (F). Lane A contains standard proteins. Samples containing 30 μ g protein were boiled in SDS-PAGE buffer for 5 min and subjected to electrophoresis in a 15% polyacrylamide slab gel, at a constant current of 10 mA. For details on respective fractions see Methods.

Fig. 3

Fig. 4

Fig. 3. Absorption spectrum of the pigment extract of the Triton-insoluble cell wall fraction (CW II) in trichloromethane, recorded at room temperature.

Fig. 4. Thin-layer chromatography of pigment extracts from CW I (a) and CW II (b) fractions on silica aluminium foils (5 $\times$ 10 cm). The solvent system (Stransky & Hager, 1970) was light petroleum (b.p. 40–60 $^{\circ}$ C)/2-propanol/water (100:11:0.5, by vol.). Pigments: 1–4, unknown carotenoids; 5, echinenone; 6, chlorophyll *a*; 7, β -carotene.

Fig. 5. Separation of lipid extracts from the gradient-purified (CW I) and the Triton-insoluble (CW II) cell wall fractions by two-dimensional thin-layer chromatography (separation and detection were as described by Jürgens *et al.*, 1985). Lipids: 1-5, unknown polar lipids; 6, digalactosyldiglyceride; 7, sulpholipid; 8, phosphatidylglycerol; 9, unknown lipid; 10, monogalactosyldiglyceride; 11, free fatty acids.

also absent, as confirmed by thin-layer chromatography and spectrophotometric analysis. In contrast, fraction CW I, not Triton purified, contained in addition to carotenoids 1-4, small amounts of echinenone, chlorophyll *a* and β -carotene (Fig. 4*a*, bands 5, 6, and 7, respectively), due to some contamination by thylakoid membranes, and possibly also by cytoplasmic membranes.

Table 2. Chemical composition of the rigid layer (SDS-insoluble cell wall fraction), of the peptidoglycan-polysaccharide complex, and of the isolated peptidoglycan of *Synechococcus* sp. PCC 6307

Compound	Content (nmol per mg fraction dry weight)*		
	Rigid layer	Peptidoglycan-polysaccharide complex	Peptidoglycan
Glu	567	674	677
Ala	908	1037	906
A ₂ pm	507	620	753
Gly	83	6	7
Compound X†	57	42	32
Other amino acids	340	—	—
GlcNAc	982	935	786
MurNAc	465	558	559
MurN-6-P	+	+	—
ManNAc	220	128	40
Fuc	14	22	18
Man	321	182	72
Gal	7	12	11
Glc	11	8	16
Phosphate	8	11	—

* Values are the mean of three determinations. —, Absent; +, present, but not quantified.

† Co-migrating with His on the amino acid analyser.

Outer membrane lipids

The Triton-insoluble cell wall fraction CW II, but not the CW I preparation, lacked monogalactosyldiglyceride and digalactosyldiglyceride, galactolipids typical of thylakoid membranes; this clearly demonstrates the value of the Triton purification step (Fig. 5). Five unidentified strongly polar lipids, free fatty acids, and small amounts of sulpholipid were detected in the CW II fraction. One of the polar lipids (spot 5 in Fig. 5) seemed to be a unique constituent of the cell wall since it was absent from cytoplasmic/thylakoid membrane fractions (data not shown).

'Rigid layer' and peptidoglycan

The rigid layer (SDS-insoluble cell wall fraction) was obtained in a 43% yield (net weight basis) from CW I and CW II preparations. It was enriched in the peptidoglycan-specific components MurNAc and A₂pm, and contained amino acids and neutral sugars (Table 2). Pronase treatment of the rigid layer fraction (and removal of the enzyme by SDS) yielded the peptidoglycan-polysaccharide complex. The peptidoglycan constituents A₂pm, MurNAc, GlcNAc, Ala and Glu were found in a molar ratio of 1.0:0.9:1.5:1.7:1.1 (Table 2). In addition, MurN-6-P was identified on the amino acid analyser; GlcN-6-P was absent. The fraction contained non-peptidoglycan compounds such as neutral sugars, Fuc, Man, Gal and Glc. Fatty acids were not found. MurN-6-P was confirmed by two-dimensional thin-layer electrophoresis/chromatography separation of a partial acid hydrolysate (4 M-HCl, 100 °C, 30 min) of the complex. The residual peptide pattern was essentially identical to that of *Synechocystis* sp. PCC 6714 (Jürgens *et al.*, 1983). The peptidoglycan-polysaccharide complex was treated with 48% aqueous HF in the cold in order to cleave possible phosphodiester bonds at MurN-6-P between peptidoglycan and polysaccharide. The peptidoglycan fraction, recovered in the pellet after centrifugation at 12000 *g*, amounted to a 66% yield (dry weight basis). The peptidoglycan constituents were confirmed in the expected molar ratios (Table 2). Neutral sugars and phosphate were not completely removed (indicating incomplete separation), but their contents had decreased significantly and MurN-6-P was no longer detectable by two-dimensional thin-layer electrophoresis/chromatography of partial acid hydrolysates (experimental conditions as for the peptidoglycan-polysaccharide complex).

DISCUSSION

Cell walls and outer membranes of *Synechococcus* sp. PCC 6307 were isolated free of cytoplasmic and thylakoid membranes as shown by electron microscopy, SDS-PAGE and comparison of pigment and lipid patterns. The intense yellow colour of cell walls was due to the presence of carotenoids localized in the outer membrane as described for *Synechocystis* sp. PCC 6714 (Jürgens & Weckesser, 1985). The stability of cell walls against non-ionic detergents was as high, as with other unicellular cyanobacteria (Resch & Gibson, 1983; Jürgens *et al.*, 1985). The polypeptide patterns of the gradient-purified and Triton X-100-insoluble cell walls were very similar. Thus, proteins were not removed from the outer membrane by the detergent. Similar observations have been made with *Escherichia coli* (Schnaitman, 1971). However, as shown for *E. coli* (Schnaitman, 1971), Triton X-100 extraction partially removed lipids and some lipopolysaccharide, although such loss should in principle be prevented by stabilization of the outer membrane by the addition of magnesium chloride. The sugar spectrum of the outer membrane of *Synechococcus* included typical O-specific sugars of the isolated lipopolysaccharide, such as tyvelose or rhamnose (Schmidt *et al.*, 1980a; Weckesser *et al.*, 1979). These sugars (and consequently the lipopolysaccharide) were removed by extraction of the cell wall with SDS (2%, w/v, 90 °C, 5 min). It should be noted that SDS extraction at lower temperature did not remove the lipopolysaccharide. The phosphorus content of the outer membrane of *Synechococcus* sp. PCC 6307, although relatively low, and the abundance of strongly polar lipids, suggests the presence of phospholipids as well as glycolipids other than monogalactosyldiglyceride and digalactosyldiglyceride. A peptidoglycan-protein-polysaccharide complex was obtained from the rigid layer (SDS-insoluble cell wall fraction). The sugar spectrum found in this fraction (Fuc, Man, Gal, ManNAc, GlcNAc; the GlcNAc to be ascribed partly to the peptidoglycan) was different from that of the lipopolysaccharide of *Synechococcus* sp. PCC 6307 (Schmidt *et al.*, 1980a). Fatty acids and also the neutral sugars rhamnose and tyvelose, characteristic of the lipopolysaccharide of this strain, were lacking in the peptidoglycan-polysaccharide complex. A different composition of the lipopolysaccharide compared to that of the polysaccharide bound to the peptidoglycan has also been observed in cell walls of *Synechocystis* sp. PCC 6714 (Jürgens *et al.*, 1985; Jürgens & Weckesser, 1986).

The peptidoglycan of *Synechocystis* sp. PCC 6714 (Jürgens *et al.*, 1983) is of the A₁ type according to the classification by Schleifer & Kandler (1972). The comparable chemical composition of the peptidoglycan of *Synechococcus* sp. PCC 6307 suggests a similar structure. In both these cyanobacteria, the degree of cross-linkage is in the range of that determined for the peptidoglycans of Gram-positive bacteria. Another similarity to Gram-positive bacteria is that the polysaccharide of the peptidoglycan-polysaccharide complex of *Synechococcus* sp. PCC 6307 is most probably bound via phosphodiester bridges to MurN of the peptidoglycan. (Insufficient separation of the polysaccharide from the peptidoglycan observed after HF treatment might have two explanations: either incomplete cleavage during HF treatment or co-sedimentation of the polysaccharide with peptidoglycan due to a higher polymeric structure of the polysaccharide. The lack of MurN-6-P in the peptidoglycan fraction favours the latter explanation.) Fragments confirming the binding mechanism proposed above were recently identified with *Synechocystis* sp. PCC 6714 (Jürgens & Weckesser, 1986).

It is concluded that the genetically only distantly related cyanobacteria, *Synechococcus* sp. PCC 6307 and *Synechocystis* sp. PCC 6714 share common characteristics with respect to cell wall structure. Both strains synthesize a lipopolysaccharide-containing outer membrane and a relatively thick (10–12 nm) peptidoglycan layer. The latter is composed of about eight single layers (Jürgens *et al.*, 1985) and is complexed to a specific polysaccharide. Peptidoglycans of this type are characteristic of Gram-positive bacteria; however, these bacteria also contain teichoic acids, not encountered in the peptidoglycan-polysaccharide complex of *Synechocystis* sp. PCC 6714 (Jürgens & Weckesser, 1986) and *Synechococcus* sp. PCC 6307. These observations support the theory (Jürgens *et al.*, 1983; Jürgens & Weckesser, 1986) that cyanobacteria might have developed a unique cell wall organization combining structural elements typical of both Gram-negative and Gram-positive bacteria. However, representatives of other genera will have to be studied to prove the generality of this proposal.

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