Isolation and Chemical Analysis of the Sheaths of the Filamentous Cyanobacteria *Calothrix parietina* and *C. scopulorum*

By JÜRGEN WECKESSER,¹* KARIN HOFMANN,¹ UWE J. JÜRGENS,¹
BRIAN A. WHITTON² AND BERND RAFFELSBERGER³

¹Institut für Biologie II, Mikrobiologie, der Albert-Ludwigs-Universität, Schänzlestr. 1,
D-7800 Freiburg i. Br., Federal Republic of Germany

²Department of Botany, University of Durham, Durham DH1 3LE, UK

³Gödecke AG, Berlin, Mooswald-Allee 1–9, D-7800 Freiburg i. Br., Federal Republic of Germany

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The sheaths of two species of cyanobacteria, *Calothrix parietina* (two strains) and *C. scopulorum* (one strain), were studied. Their fine structure showed osmiophilic fibres running parallel to the cell surface. Enriched sheath fractions were obtained from both species in high yields in the phenol/water interphase after hot phenol/water extraction of cell homogenates. The purified sheaths of the two species had similar chemical compositions, with about 50% (of sheath dry weight) neutral sugars (galactose, glucose, mannose, xylose, arabinose, rhamnose, fucose, a 2-*O*-methylhexose and an unidentified 2-*O*-methyl sugar), 5% amino acids and small amounts of glucosamine and galacturonic acid. The sheath composition was independent of changes in the iron or phosphate content of the culture medium. The sheath was insoluble in water, and its chemical composition remained essentially unchanged on application of drastic extraction methods, including boiling in 2% (w/v) SDS. The sheath bound heavy metals (up to at least 0.7% of sheath dry weight) with the effectiveness (measured as absolute quantities) Fe > Zn > Cu > Ni > Mn > Mo > Co. Ni, Cu, Zn and Fe were highly enriched relative to their concentration in the culture medium. The concentration factors for Mo, Mn, Ca, Na and K were low.

INTRODUCTION

Calothrix, which belongs to Section IV of cyanobacteria according to the classification of Rippka *et al.* (1979), has a complex developmental cycle, with hormogonia being formed from the ends of trichomes (Livingstone & Whitton, 1983). The unbranched trichomes are up to 1 mm long, typically tapering from base to apex, and are surrounded by a thick sheath. Hormogonia are formed by apical release of about ten cells which lack both a sheath and cyanophycin, and are enriched in gas vacuoles and polyphosphate granules. Absence of combined nitrogen leads to the formation of basally located heterocysts (Rai *et al.*, 1978), a thicker sheath and sometimes loss of tapering. 'Hairs' (long, thin, vacuolated, colourless cells at the end of the trichome) or false branches may be formed during starvation of phosphate, iron or (rarely) magnesium (Sinclair & Whitton, 1977; Livingstone & Whitton, 1983; Douglas *et al.*, 1986).

The chemical composition of the sheaths of the cyanobacteria *Chroococcus minutus* (Adhikary *et al.*, 1986), *Gloeotheca* (Weckesser *et al.*, 1987) and *Chlorogloeopsis* (Schrader *et al.*, 1982*a*) has been described. Except for the last, which produces filaments under some conditions, none of these are filamentous cyanobacteria. This paper describes a technically easy method for the isolation of the sheath of *Calothrix* species, and gives a chemical description of the sheath and data on metal binding.

METHODS

Organism and growth. *Calothrix parietina* strains D184 and D550 were from the culture collection of the Department of Botany, University of Durham, UK. *C. scopulorum* was obtained from the culture collection of the Institut für Pflanzenphysiologie der Universität, Göttingen, FRG.

The cyanobacteria were grown in two different media: medium I (low phosphate and high iron concentration), and medium II (high phosphate and low iron concentration). The media contained (mg l^{-1}): $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$, 7 (medium I) or 74 (medium II); $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 50; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 200; NaCl , 230; KCl , 19; $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 10 (medium I) or 2 (medium II). The iron was added as a Fe-EDTA complex, which was prepared by dissolving Na_2EDTA (12.7 g) in water (400 ml) before the addition of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (9.7 g); the volume was adjusted to 1 l and appropriate volumes were employed for media I and II. The final concentrations of trace elements (prepared as a separate stock solution) were (mg l^{-1}): $\text{MnSO}_4 \cdot 2\text{H}_2\text{O}$, 0.4; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.06; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.02; H_3BO_3 , 0.7; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.01; $\text{NiSO}_4 \cdot 6\text{H}_2\text{O}$, 0.01. HEPES (0.4 g l^{-1}) was added to the medium and the pH adjusted to 7.6 with 1 M-NaOH. K_2HPO_4 (740 mg l^{-1}) was autoclaved separately and added after cooling.

Cultures (300 ml) were grown in 1 l Erlenmeyer flasks illuminated with cool white fluorescent light (1500 lx), with shaking, at 25 °C for 3 weeks. The cyanobacteria were harvested by filtration through a filter paper, washed with distilled water and lyophilized.

Isolation of the sheath. Lyophilized cells (300 mg) were suspended in 40 ml 20 mM-Tris/HCl buffer, pH 8.0, and broken by passing twice through a French pressure cell at 83 MPa. The homogenate was treated with hen egg-white lysozyme (EC 3.2.1.17) (5 mg in 25 ml 20 mM-ammonium acetate buffer, pH 6.8, 37 °C, 12 h) and then washed three times with water (50 ml) by centrifugation at 20000 g, 22 °C, 30 min, removing the upper sheath layer each time by pipette. The sheath (in 20 ml water) was further purified by addition, dropwise, into 20 ml boiling 2% (w/v) SDS. After boiling for 15 min, cooling to 22 °C and centrifugation (10000 g, 22 °C, 30 min), the sediment was washed about seven times with 20 ml water before lyophilization.

Hot phenol/water extraction of the cell homogenate was performed by the method of Westphal *et al.* (1952). After cooling to about 10 °C and phase-separation by centrifugation (5000 g, 10 °C, 30 min), the phenol/water interphase was taken with a Pasteur pipette and dialysed extensively against running tap water and finally against distilled water.

Analytical chemical methods. Conditions for liberation of neutral and amino sugars, uronic acids, amino acids and fatty acids were as described by Schrader *et al.* (1982b). Neutral sugars were quantified by gas-liquid chromatography as alditol acetates, reduced with NaBD_4 for mass spectrometric fragmentation. The alditol acetates were separated on an ECNSS-M glass column (2 mm $\times$ 1.5 m, 3% on Gas-Chrom Q, 100–200 mesh) using a Varian Aerograph, model 1400-1. Injection and column temperatures were both 240 °C. Mass spectrometric analysis of the sugar alditol acetates was performed on a Finnigan MAT GC/MS automatic system, model 1020. Mass spectra were taken at 70 eV in the mass range m/e 43–400 in 1 s. Fatty acids were estimated by gas-liquid chromatography as methyl esters using an EGSS-X column, 15% on Gas-Chrom P, 100–200 mesh. Amino sugars and amino acids were separated on an automatic amino acid analyser (Evers *et al.*, 1986). Uronic acids were separated by high-voltage paper electrophoresis (Kickhöfen & Warth, 1968) and determined quantitatively by the carbazole method (Galambos, 1967). Phosphorus was determined by the method of Lowry *et al.* (1954).

For metal determination, 10–12 mg sheath (dry weight) was dissolved in 0.5 ml concentrated HCl containing 10 μl HF by heating to 160 °C for 2 h in an autoclave (5 MPa). Stock solutions of the corresponding metals were treated similarly. Samples were then diluted with de-ionized water to the appropriate volumes. Measurements of Cu, Co, Ni and Mo were made with a Perkin-Elmer 2380 atomic absorption spectrophotometer with hollow cathode lamps, together with a Perkin-Elmer HGA 76 graphite furnace and a deuterium background corrector. For determination of Zn, Fe, Mn, Ca, K and Na, a Perkin-Elmer 3030B flame atomic absorption spectrophotometer with hollow cathode lamps (air/acetylene or acetylene/dinitrogen monoxide flames) was used.

Electron microscopy. Freshly harvested cells were fixed in 1% (w/v) osmium tetroxide, dehydrated in alcohol and polymerized in Epon using standard procedures. Ultrathin sections were stained with uranyl acetate followed by lead citrate (Golecki, 1977). A Philips EM 400 microscope was used at 80 kV.

RESULTS

Sheath of C. parietina strains D184 and D550

In ultrathin sections, the sheath of *C. parietina* D550 showed an osmiophilic fine structure with the fibres running parallel to the cell surface (Fig. 1). The fibres were more densely packed in the outer area of the sheath. A large space between sheath and cell wall (presumably caused by shrinkage during embedding for electron microscopy) indicated that the sheath is not bound to the cell wall (Fig. 1).

Fig. 1. Ultrathin section of *C. parietina* D550 grown in medium I. Note the relatively thick peptidoglycan layer in the cell wall. Bar, 0.5 μ m.

Table 1. *Chemical composition of sheath fractions of C. parietina* D184 grown in medium I (low phosphate, high iron)

Each value is the mean of two determinations. +, Present, but not quantified.

Constituent	Content (% of fraction dry weight) after treatment with:				
	Lysozyme	Lysozyme + SDS	Phenol/ water	Phenol/ water + lysozyme	Phenol/ water + lysozyme + SDS
Rhamnose	1.2	1.2	1.3	1.3	1.3
Fucose	1.4	0.3	1.0	1.0	0.9
Arabinose	2.5	2.2	2.9	2.7	2.7
Xylose	3.0	2.5	3.4	3.1	3.1
2-O-Me-hexose	1.6	0.9	2.2	2.1	1.9
2-O-Me-sugar	+	+	+	+	+
Mannose	4.8	5.1	5.7	5.9	5.1
Galactose	9.4	9.5	12.9	11.1	10.2
Glucose	12.7	14.9	17.0	16.2	16.0
Fatty acids	0.1	0.2	0.1	0.2	0.2
Galacturonic acid	3.3	3.0	3.8	1.2	3.0
Phosphorus	0.04	0.01	0.03	0.03	0.02
Glucosamine	1.3	0.8	1.1	0.9	1.1
Muramic acid	+	+	+	+	+
Diaminopimelic acid	+	+	+	+	+
Amino acids (total)	17.2	9.0	15.6	17.6	14.4

Sheaths from *C. parietina* D184 and D550 were obtained from cell homogenates by lysozyme treatment, followed by differential centrifugation. Further purification was achieved by treatment with 2% (w/v) boiling SDS. The yield was 20–30% of cell dry weight with both strains. The neutral sugar content was about half of the sheath material dry weight, with galactose and glucose dominating over mannose, xylose, arabinose, rhamnose, fucose and two *O*-methyl sugars (data obtained with strain D184: Table 1). The *O*-methyl sugar having a retention time of

Table 2. *Metals in the isolated sheath (obtained from cell homogenates by boiling 2%, w/v, SDS treatment, 15 min) from C. parietina D550, grown in media with different iron and phosphate contents*

Values shown are the mean of three determinations.

Metal	Concentration in		
	Culture medium ($\mu\text{g l}^{-1}$)	Sheath fraction [$\mu\text{g (50 mg lyophilized sheath)}^{-1}$]*	
		Medium I†	Medium II†
Fe (medium I)	420	304	
Fe (medium II)	84		125
Mn	130	3	4
Mo	26	2	2
Zn	12	17	8
Cu	5	12	7
Ni	2	9	7
Co	2	2	0.2
Ca	14000	347	420
K	10000	103	< 25
Na	92000	207	< 25

* 50 mg lyophilized sheath corresponds roughly to the amount isolated from 1 l of culture.

† Medium I, low phosphate, high iron; medium II, high phosphate, low iron (see Methods for details).

0.48 (relative to inositol, as alditol acetate derivatives) on gas-liquid chromatography showed primary fragments at m/e 333 and 118 and secondary fragments at m/e 259, 171 and 139, indicating it to be a 2-*O*-methylhexose (Jansson *et al.*, 1976). The other *O*-methyl sugar (relative retention time 0.53) was present in only small amounts. Among other fragments, it showed one of m/e 118, characteristic of a 2-*O*-methyl sugar. It was not further characterized. In addition to some galacturonic acid, small amounts of glucosamine, muramic acid and diaminopimelic acid were found. The latter three compounds were probably due to contamination by peptidoglycan. The fatty acid and phosphorus contents were very low (Table 1). About 10% of the amino acid residues could not be removed by the detergent treatment. Essentially comparable data were obtained with the sheath of strain D550 (data not shown). The absolute amount of metals in the sheath was determined for strain D550 (Table 2). Heavy metals were bound up to above 0.7% of sheath dry weight with the effectiveness (measured as absolute quantities) $\text{Fe} > \text{Zn} > \text{Cu} > \text{Ni} > \text{Mn} > \text{Mo} > \text{Co}$. A high enrichment of Ni, Cu, Zn and Fe, relative to their respective concentrations in the culture medium, was observed, while Mn, Ca, Mo, K and Na were only slightly enriched.

A similar sheath composition was obtained when a different isolation procedure was applied, in which the cell homogenate was treated with hot phenol/water (Table 1). With both strains, the sheath was found entirely in the phenol/water interphase (yield 25% of cell dry weight). Further purification by lysozyme treatment and a subsequent SDS treatment (as above) did not change the composition (Table 1). Accordingly, an additional hot phenol/water treatment of the sheath fraction, obtained by SDS extraction, again extracted the sheath into the phenol/water interphase. The chemical composition remained unchanged (examined with strain D550; data not shown).

There were no significant differences in yield and chemical composition of the sheath fractions isolated from cells grown in media with different phosphate and iron contents (medium I or medium II).

Several attempts were made with the sheath of *C. parietina* D550 to cleave off the polysaccharide moiety from the protein portion. They included treatment of the sheath at 60 °C in 0.5 M-NaOH for 12 h or in 2 M-NaOH for 48 h. In both cases some material was not solubilized, and no clear-cut separation of the polysaccharide from the protein moiety was achieved upon centrifugation (48000 *g*, 4 °C, 20 min). The sheath could not be dissolved in

Schweitzer reagent. However, after treatment with HF (48%, v/v, 0 °C, 48 h) and centrifugation (176000 g, 4 °C, 60 min) about 70% of the sheath dry weight was obtained in the supernatant. The latter was highly enriched in neutral sugars, while the sediment contained nearly all of the amino acids (data not shown).

Sheath of *C. scopulorum*

C. scopulorum had a somewhat thinner sheath layer than the two *C. parietina* strains, as judged by light microscopy. The yield of isolated sheath in the phenol/water interphase of hot phenol/water extracts of cell homogenates was 20% of the cell dry weight. The chemical composition and metal-binding capacity of the *C. scopulorum* sheath were essentially identical to that of the two *C. parietina* strains (data not shown).

DISCUSSION

The hot phenol/water procedure proved to be a suitable method for the isolation of the sheath of the two *Calothrix* species. It is technically easy and gave highly enriched sheath fractions in good yields in the phenol/water interphase with all three strains examined. The partition into this phase is caused by the high insolubility of the *Calothrix* sheath, and thus this method may also be applicable to the insoluble sheath fractions from other cyanobacteria such as *Chlorogloeopsis* (Schrader *et al.*, 1982a). In contrast, the more soluble sheath of the unicellular *Gloeotheca* sp. was extracted into the water phase by a comparable extraction method (Weckesser *et al.*, 1987). There may be a correlation between insolubility and the staining density of the osmiophilic fine structure: both were high with *Calothrix* spp. and *Chlorogloeopsis* sp., whereas the sheaths of *Gloeotheca* sp. have a less osmiophilic fine structure (Weckesser *et al.*, 1987).

The chemical composition of the sheath remained essentially unchanged on application of the various drastic extraction conditions such as hot phenol/water or boiling SDS treatment. This might indicate high purity of the sheath fraction, but it might also be explained by the presence of insoluble material other than sheath. The sheath fraction certainly contains peptidoglycan, as indicated by the presence of muramic and diaminopimelic acids. Thus, the possibility cannot be excluded that some of the sugars found are part of a peptidoglycan-polysaccharide complex, as in the unicellular *Synechocystis* sp. PCC 6714 (Jürgens & Weckesser, 1986). However, such a contribution would be small, because of the low relative content (about 0.1%) of peptidoglycan-specific compounds in the fraction. With *Chlorogloeopsis* sp. PCC 6912 a polysaccharide different from that of the sheath was found and suggested to be a cell wall constituent (Schrader *et al.*, 1982b). No comparable polysaccharide was detected in the water phase of the phenol/water extracts of cell homogenates of the *Calothrix* strains.

Like the sheaths of other cyanobacteria (Drews & Weckesser, 1982; Adhikary *et al.*, 1986; Weckesser *et al.*, 1987), the complex sugar spectrum with pentoses, hexoses, and *O*-methyl sugars indicates high specificity of the sheath in *Calothrix* spp. *O*-Methyl sugars are characteristic constituents of cell wall polysaccharides from phototrophic prokaryotes (Drews & Weckesser, 1982; Weckesser *et al.*, 1979). The sugars are not due to contamination by lipopolysaccharide, since β -hydroxy fatty acids, which are characteristic constituents of lipopolysaccharides of Gram-negative bacteria and cyanobacteria (Weckesser *et al.*, 1979), were lacking in all the sheath fractions isolated in this study. Interestingly, a protein content of at least 5% (of sheath dry weight) was not removed by any of the drastic extraction conditions applied. A possible binding between polysaccharide and protein would be worthy of study.

Only about 60% of the sheath fraction dry weight was identified. This may be due to the observed partial insolubility of the sheath under the hydrolytic conditions used and by a possible degradation of identified compounds during hydrolysis. Insolubility, together with mechanical stability and resistance to enzymic treatment, will render sheath layers from cyanobacteria relatively stable against destruction in their natural environment. Thus, the sheaths of cyanobacteria can play a major part in sedimenting detritus material in lakes and in geological sediments (Aizenshat *et al.*, 1984; Krumbein, 1983).

The sheaths of both *Calothrix* spp. bound heavy metals, which were enriched in the order

Ni > Cu > Zn > Fe > Co > Mo, relative to their concentrations in the medium. However, as is clear from the data presented in Table 2, some contribution has to be ascribed to additional, contaminating metals (in non-defined amounts) collected by the sheath during cell cultivation and the isolation of the sheath. This holds true certainly at least for the excess of Fe, Cu, Ni and Zn determined in the sheath relative to their concentration in the culture medium. The concentration factor for Mn, needed in photosystem II, was low. Ca, K and Na also showed low enrichment factors. A comparable concentration of metals has been described for the sheath of *Microcystis* from a natural habitat (Amemiya & Nakayama, 1984), except that a higher relative concentration factor for Mn was measured in that study and the relative Ca, K and Na content of the sheath of the *Calothrix* spp. was much lower than described for that of *Microcystis*. The sheath of *Calothrix* spp. seems to bind only heavy metals, which in excess amounts are toxic to the cell, and it might therefore have a protective function.

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