Comparative Composition of the Sheath of the Cyanobacterium *Gloeotheca* ATCC 27152 Cultured with and without Combined Nitrogen

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The extracellular sheath was isolated from cultures of a unicellular diazotrophic cyanobacterium, *Gloeotheca* ATCC 27152. Chemical analysis of lyophilized cell-free preparations indicated that the sheath was composed of an acidic heteropolysaccharide containing the neutral sugars rhamnose, 2-*O*-methylxylose, xylose, mannose, galactose and glucose, and the uronic acids mannuronic, glucuronic and galacturonic acid. Protein, pyruvic acid and *O*-acetyl and sulphate groups were also detected. Quantitative differences in the carbohydrate and protein content of the sheath were observed between material collected from cultures grown with and without a source of combined nitrogen. The extent to which the external layers bound cadmium ions was affected by these differences.

INTRODUCTION

The majority of cyanobacteria produce an external layer of polysaccharide, reminiscent of the glycocalyx and slime layers of bacteria. These extracellular investments occupy a strategic position at the outermost region of the bacterial cell envelope and thereby play a fundamental role in the interaction of cells with their environment (Cheng & Costerton, 1975). Once in contact with a substrate, external layers help to anchor the cells and thereby permit further colonization by additional organisms (Daniels, 1980). Furthermore, bacterial external layers provide protection against phagocytic predation, antibody recognition, desiccation and lysis by bacteria and viruses (Dudman, 1977).

Bacterial external layers are composed of acidic polysaccharides containing an array of uronic acid and neutral sugar residues, frequently substituted with pyruvic acid and *O*-acetyl groups (Sandford, 1979; Troy, 1979; Sutherland, 1985). Sulphated and *O*-methyl sugars, common among eukaryotic extracellular polysaccharides (Percival, 1979; McCandles, 1981; Painter, 1983), have yet to be reported (Sutherland, 1985). Chemical analyses of the slime layers of certain cyanobacteria have revealed an array of uronic acid and neutral sugar residues similar to those found in bacterial external layers (Hough *et al.*, 1952; Bishop *et al.*, 1954; Moore & Tischer, 1965; Dunn & Wolk, 1970; Kokyrsta & Chekoi, 1972; Sangar & Dugan, 1972; Wang & Tischer, 1973; Mehta & Vaidya, 1978; Painter, 1983). The more structurally resilient sheaths of *Chlorogloeopsis* PCC 6912 (Schrader *et al.*, 1982), *Gloeotheca* PCC 6501 (Jurgens & Weckesser, 1985) and *Chroococcus minutus* SAG B.41.79 (Adhikary *et al.*, 1986) have been shown to contain *O*-methyl sugars and a protein component, in addition to the typical sugar residues detected in previously studied external layers.

The production of both bacterial and cyanobacterial external layers is affected by changes in culture conditions (Duguid & Wilkinson, 1953; Wilkinson *et al.*, 1954; Moore & Tischer, 1964; Findley *et al.*, 1970; Tischer & Davis, 1971; Sangar & Dugan, 1972; Fletcher & Floodgate, 1973; Williams & Wimpenny, 1978; Mehta & Vaidya, 1978; Konopka & Schnur, 1980). Increased production of extracellular polysaccharides appears to be directly proportional to the C:N ratio. Changes in light intensity, temperature, culture age, and the concentrations of sulphur,

phosphorus and potassium can also affect this production. Variations have been observed in the proportion of uronic acid residues in alginic acid (Haug *et al.*, 1974) and in the extracellular polysaccharide of *Pseudomonas atlantica* (Uhlinger & White, 1983). However, additional work is needed to determine the significance of such changes and the frequency with which they occur among external layers in general.

This paper describes the chemical characterization of the sheath of *Gloeotheca* ATCC 27152 and the extent to which changes in the mode of nitrogen assimilation affect its composition.

METHODS

Growth conditions. *Gloeotheca* ATCC 27152 (obtained from the American Type Culture Collection) is a member of section I of the generic classification scheme of Rippka *et al.* (1979). Cultures were grown photoautotrophically on medium BG-11 (Allen & Stanier, 1968) with and without NaNO_3 , at 26 °C, in 15 litre glass carboys aerated with prefiltered compressed air. Illumination was continuously provided by two cool-white fluorescent lights (3.4 W m^{-2}). Cultures were harvested after 1 month incubation, while in the exponential growth phase.

Sheath removal and isolation. Carboy cultures were allowed to settle to a density of 10^7 cells ml^{-1} . From a total cell concentrate of 1–2 litres per carboy, 30 ml portions were transferred to a 2 litre porcelain jar containing 250 glass beads (3 mm in diameter) and revolved for 30 min at 4 °C. This was repeated until all of the cell concentrate was milled. Sequential centrifugation for 5–10 min at 250, 1000 and 38000 g separated liberated sheath from intact aggregates, sheathless cells and debris.

Lysozyme and SDS treatment. Since differential centrifugation was not totally effective in isolating pure sheath material, remaining cells were removed by treating the crude preparation with a 1% (w/v) solution of lysozyme (Sigma) for 20 h at 4 °C. After 10 rinses with deionized-distilled water, each followed by centrifugation at 38000 g for 10 min, the sheath preparation was treated with a 3% (w/v) aqueous solution of sodium dodecyl sulphate (SDS) for 1 h at room temperature. The resulting frothy suspension was centrifuged at 38000 g for 10 min and washed with deionized-distilled water 10 times. This material was transparent, colourless and virtually cell-free. Sheath material, before and after treatment with lysozyme and SDS, was lyophilized and stored at –20 °C.

Analytical procedures. The water content of the final sheath preparation was determined by gravimetric analysis before and after dehydration at 95 °C for 1 h. The water loss observed was an indication of the amount of water absorbed by this material.

Phosphorus was determined after acid digestion of lyophilized sheath material in concentrated sulphuric and nitric acids, by the molybdenum blue assay according to Ma & Rittner (1979).

Total lipid-soluble material was determined by extracting lyophilized sheath material for 3 h at room temperature in chloroform/methanol (2:1, v/v), and weighing the dried extract. Sulphur was determined by combusting sheath material in a Schonizer oxygen flask, followed by the standard barium titration in ethanol, using thiorin as the indicator reagent (Ma & Rittner, 1979). Sulphate was determined by the turbidimetric-barium chloride procedure (American Public Health Association, 1980). Samples were hydrolysed in 0.5 M- and 1.0 M-HCl for 1 and 28 h at 100 °C.

A modified ninhydrin procedure was followed to determine the protein content of the sheath material (Rosen, 1957). Samples were hydrolysed in 6 M-HCl at 100 °C from 15 min to 24 h. Amino acid analysis was performed on a model 121 Beckman amino acid analyser. Samples (5 mg) were hydrolysed in 6 M-HCl for 20 h at 110 °C. After evaporation over NaOH *in vacuo*, 1 ml 0.2 M-sodium citrate buffer (pH 2.2) was added. The amino acids were chromatographed at 55 °C in a three-buffer system (pH 3.49, 4.20 and 6.40) using type W-1 ion-exchange resin supported in a 56 cm $\times$ 9 mm glass column. Colorimetric detection using ninhydrin was performed at 670 nm (except for proline, 440 nm).

SDS-PAGE was performed according to Laemmli (1970) in the presence of 0.1% SDS using a discontinuous buffer system. Samples containing 5 mg freeze-dried sheath preparation were suspended in 3% (w/v) SDS, 0.5% Tris/HCl buffer pH 6.8, 10% (v/v) glycerol and 5% (w/v) 2-mercaptoethanol and heated for 3 min in a boiling water bath. Gel slabs (0.75 mm thick), containing 3% (v/v) (stacking gel) and 10% (v/v) (running gel) acrylamide were placed in a Hoeffer SE-600 vertical slab gel unit that received a constant current of 15 mA and kept at 10–15 °C.

Total carbohydrate, uronic acid, pyruvic acid and *O*-acetyl groups were determined by the anthrone (Dreywood, 1946), *m*-phenylphenol (Blumenkrantz & Asboe-Hansen, 1973), 2,4-dinitrophenylhydrazine (Lessie & Whiteley, 1969) and hydroxylamine (Hestrin, 1949) colorimetric assays, respectively. The presence of hexosamines was measured by the Morgan–Elson procedure (Gerhardt, 1981). Neutral (Swardeker, 1965) and uronic acid (Lehrfeld, 1985) sugars were identified by gas-liquid chromatography of their alditol acetate and *N*-propylamide acetate derivatives, respectively. A Perkin–Elmer model 900 gas chromatograph with dual ionization detectors was used in a dual column compensation mode. Stainless steel columns (168 cm $\times$ 3 mm) were packed with 80–100 mesh Supelcoport (Supelco, Inc.), coated with 3% (w/w) OV-225. The temperature was programmed from 140 to 240 °C

at 5 °C min⁻¹ and finally kept isothermal at 240 °C until all components eluted. Nitrogen was used as the carrier gas at a rate of 55 ml min⁻¹. Acid hydrolysis of lyophilized sheath was performed with 2 M-trifluoroacetic acid (TFA) and 0.5 M-H₂SO₄ at 100 °C from 15 min to 20 h. (It should be noted that TFA interfered with *N*-propylamide acetate derivatization of uronic acid residues.) H₂SO₄ was neutralized with BaCO₃, and TFA was evaporated over NaOH *in vacuo*. A 0.03% (w/v) aqueous solution of arabinose was used as an internal standard. Gas chromatography-mass spectrometry was used to assist identification of neutral sugars. Electron impact and chemical ionization using isobutane was performed on a model HP 5985 Hewlett-Packard GC-mass spectrometer coupled by an open split interface.

Cadmium-binding studies. Lyophilized sheath preparations (2.0 mg) were exposed to a range of cadmium (as cadmium nitrate) concentrations (25, 50, 75, 100, 150 and 200 µg Cd²⁺ ml⁻¹). After 30 min the sheath/metal suspension was allowed to settle for 2 h. Cadmium concentrations were determined on 1:25 dilutions of the supernatant and on standard solutions, using a Perkin-Elmer ICP (inductively-coupled plasma) model 6500 atomic emission spectrophotometer.

RESULTS

Cultivation and sheath removal

Differences in the growth rate of *Gloeotheca* ATCC 27152 grown with N₂ or with NaNO₃ as nitrogen source were negligible. Average generation times for the cultures were approximately 4 d. Accelerating the growth rate by providing additional inorganic carbon resulted in a diminished sheath/cell volume ratio. Sheath removal was best performed on aggregates containing 8–16 cells (Fig. 1).

Removal procedures were directed at the outermost sheath investment. Exposure of concentrated cell suspensions to ball milling for short periods fractured the external sheath, resulting in the dispersion of the enclosed cell clusters. While this may not have been the most productive method to obtain sheath material, it proved to be the best for minimizing cell breakage.

The outer sheath investment maintained a sack-like conformation (Fig. 2) throughout the removal procedures. Purification of this material involved separating the extended sacks of sheath from cell clusters, sheathless cells and debris (fragments of broken cells). Lysozyme and SDS were used to break down the remaining cells and debris not removed by centrifugation. Isolated sheath material was considered to be homogeneous based upon the absence of whole cells and upon its low phosphorus (0.06%) and lipid (0.3–0.5%) content, suggesting negligible membrane contamination.

Approximately 150 mg lyophilized sheath material could be collected from an exponentially growing carboy culture (15 litres).

Chemical analyses

In general, the sheath of *Gloeotheca* ATCC 27152 contained the neutral sugars rhamnose, 2-*O*-methylxylose, xylose, mannose, galactose and glucose, and the uronic acids mannuronic, glucuronic and galacturonic acid (Fig. 3). Pyruvic acid, *O*-acetyl and sulphate groups were also detected; this is believed to be the first report of these groups in extracellular polysaccharide from cyanobacteria. While pyruvate and *O*-acetyl groups are common in bacterial polysaccharides, the occurrence of sulphates and methylated sugars is unique.

The results from the Morgen-Elson test for hexosamines were negative. This, however, should not be considered absolute. The limited supply of sheath material necessitated using small amounts for a test that depends upon larger quantities for precision. Further research regarding the presence of amino sugars in the sheath of *Gloeotheca* ATCC 27152 is required.

Amino acid analysis revealed a wide variety of residues, suggesting the presence of complex proteins. The high proportion of aspartic acid and glycine detected (Table 1) was similar to results observed with the sheath of *Chlorogloeopsis* PCC 6912 (Schradler *et al.*, 1982). SDS-PAGE revealed two sharp major bands of *M_r* 56 000 and 51 000, a broad minor band of *M_r* 18 000 and a broad major band of *M_r* 14 000 (Fig. 4).

Fig. 1. Photograph of a cell aggregate of *Gloeotheca* ATCC 27152 showing characteristic multi-laminated sheath investments. Bar, 10 μ m.

Fig. 2. Photograph of the sheath material after removal procedures. Bar, 10 μ m.

Quantitative differences were observed in the composition of the sheath material collected from cultures grown with NaNO_3 and from those fixing N_2 (Table 2). When viewed under the light microscope, material from cultures grown with N_2 was more refractile and voluminous than that from cultures grown with NaNO_3 , but it contained 26% and 42% less total carbohydrate and protein, respectively, than the latter material. The water content (prior to lyophilization) of sheath from cultures grown with N_2 was 98.8%, compared to 99.5% for sheath

Fig. 3. FID (flame ionization detector) trace of the *N*-propylamide and alditol acetate derivatives of the uronic acid and neutral sugar residues from the sheath of *Gloeotheca* ATCC 27152 grown (a) with NaNO_3 and (b) with N_2 as nitrogen source, analysed by gas chromatography. Peak numbers 1 to 10 refer to rhamnose, 2-*O*-methylxylose, arabinose, xylose, mannose, galactose, glucose, mannuronic acid, glucuronic acid and galacturonic acid, respectively.

Fig. 4

Fig. 4. Line drawing of an SDS-PAGE gel showing the protein standards (Stds) and bands from two samples of sheath from cells grown with N_2 . A, 4.98 mg material treated with lysozyme and SDS; B, 5.00 mg material treated with SDS only. Both wells received 30 μl sample extract.

Fig. 5

Fig. 5. Adsorption of cadmium ions to the sheath of *Gloeotheca* ATCC 27152 collected from cultures grown with NaNO_3 (●) and with N_2 (■) as nitrogen source. Points represent single determinations.

Table 1. *Amino acid composition of the protein component in the sheath preparation from nitrate-grown Gloeotheca ATCC 27152 after lysozyme/SDS treatment*

Values represent a single analysis and are based upon a percentage of alanine.

Amino acid	Content (mol %)	Amino acid	Content (mol %)
Asp	110	Met	9
Thr	56	Ile	50
Ser	83	Leu	78
Glu	79	Tyr	31
Pro	8	Phe	59
Cys	8	His	14
Gly	102	Lys	50
Ala	100	Arg	70
Val	62		

Table 2. *Chemical composition (% dry wt) of the sheath of Gloeotheca ATCC 27152 collected from cultures assimilating nitrate or fixing N₂*

Parameter	N ₂ as nitrogen source	NO ₃ ⁻ as nitrogen source
Total carbohydrate	41.0	55.4
Total uronic acids	7.6	6.0
Protein		
3 h hydrolysis	4.3	7.3
8 h hydrolysis*	6.3	10.9
Water		
Gel preparation	99.8	99.5
Sheath preparation	4.3	4.3
Total sulphur	5.3	5.2
Sulphate		
1 h hydrolysis	13.2	8.7
28 h hydrolysis	15.4	17.5
O-Acetyl	2.2	3.1
Pyruvate†	2.3	ND

ND, Not determined.

* Maximum protein value determined from a hydrolysis series.

† Maximum 8 h pyruvate value determined from a hydrolysis series.

Table 3. *Peak area and proportions (relative to xylose), determined by gas chromatography (Fig. 3), of the N-propylamide and alditol acetate derivatives of the sugar residues from samples of sheath material from cultures assimilating nitrate or fixing N₂*Samples were hydrolysed for 10 h at 100 °C in 0.5 M-H₂SO₄. Arabinose served as internal standard (1.5 µg per 0.5 µl injection).

Peak no.	Component	N ₂ as nitrogen source*		NO ₃ ⁻ as nitrogen source†	
		Peak area (cm ²)	Ratio	Peak area (cm ²)	Ratio
1	Rhamnose	1.11	3.50	0.96	3.00
2	2-O-methylxylose	0.46	1.50	0.51	2.00
3	Arabinose	0.96	—	1.01	—
4	Xylose	0.32	1.00	0.28	1.00
5	Mannose	1.91	6.00	1.83	6.50
6	Galactose	2.75	9.00	2.78	10.00
7	Glucose	2.55	8.00	2.31	8.00
8	Mannuronic acid	0.02	0.06	0.04	0.14
9	Glucuronic acid	0.03	0.09	0.03	0.11
10	Galacturonic acid	0.16	0.50	0.11	0.40

* 2.61 mg sample.

† 2.58 mg sample.

from cultures grown with NaNO_3 . While the proportion of neutral sugars was virtually the same in material from the two cultures, the uronic acid of sheath from N_2 -fixing cultures contained much less mannuronic acid (Table 3).

Cadmium-binding ability

Alterations in the chemical composition of the sheath of *Gloeotheca* ATCC 27152 affected its capacity to bind cadmium ions: sheath material from cells grown with NaNO_3 bound considerably more than that from N_2 -fixing cultures (Fig. 5).

DISCUSSION

The sheath of *Gloeotheca* ATCC 27152 was primarily composed of an acidic heteropolysaccharide, substituted with several functional groups, representative of both eukaryotic and prokaryotic extracellular polysaccharides. The presence of protein, uronic acids, pyruvic acid and *O*-methyl, *O*-acetyl and sulphate groups indicates a significant degree of complexity. The presence of a variety of components in the sheath of *Gloeotheca* ATCC 27152 may convey resistance to bacterial decomposition, since several enzymes may be needed to achieve complete breakdown of the heteropolysaccharide.

A possible function of external layers may be the concentration of essential trace elements, present at submarginal concentrations in oligotrophic environments (Lange, 1976; Geesey, 1982; Uhlinger & White, 1983). The occurrence of several functional groups (carbonyl, carboxyl, hydroxyl and sulphate) would provide various attachment sites for cations (Eichorn, 1975), and thereby facilitate nutrient uptake into the cell. By concentrating certain nutrients at the cell surface, an organism could proliferate in an environment otherwise uninhabitable due to an insufficient nutrient supply. While cadmium is not an essential nutrient, its substantial adsorption to the sheath of *Gloeotheca* ATCC 27152 indicates the appreciable metal-binding capacity of this extracellular investment. The variations in composition observed in the sheath material collected from cultures grown with and without a combined nitrogen source resulted in differences in metal-binding capacity. Future research should be directed at determining the extent to which changes in the metal-binding affinity of external layers are directed by the cells. It would be interesting to determine if a binding preference results during periods of rapid assimilation and/or limited nutrient supply (e.g. adsorption of iron and molybdenum during N_2 fixation).

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