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Chemical Characterization of Polysaccharide from the Slime Layer of the Cyanobacterium *Microcystis flos-aquae* C3-40

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Macromolecular material from the slime layer of the cyanobacterium *Microcystis flos-aquae* C3-40 was defined as material that adhered to cells during centrifugation in growth medium but was dislodged by washing with deionized water and retained within dialysis tubing with a molecular-weight cutoff of 3,500. At each step of this isolation procedure, the slime was observed microscopically. Cells in the centrifugal pellet were surrounded by large amounts of slime that excluded negative stain, whereas cells that had been washed with water lacked visible slime. Two independently isolated lots of slime contained no detectable protein (<1%, wt/wt) and consisted predominantly of anthrone-reacting polysaccharide. Sugars in a hydrolysate of slime polysaccharide were derivatized with trimethylsilylimidazole and examined by gas chromatography-mass spectrometry. The composition of the slime polysaccharide was 1.5% (wt/wt) galactose, 2.0% glucose, 3.0% xylose, 5.0% mannose, 5.5% rhamnose, and 83% galacturonic acid. This composition resembles that of the plant polysaccharide pectin, which was treated in parallel as a control. Consistent with earlier indications that *M. flos-aquae* slime preferentially binds certain cations, the ratio of Fe to Na in the dialyzed slime was 10^4 times that in the growth medium. The composition of the slime is discussed with respect to possible mechanisms of cation binding in comparison with other cyanobacterial exopolysaccharides and pectin.

The colonial cyanobacterium *Microcystis flos-aquae* (synonym, *M. aeruginosa* forma *flos-aquae*) is a prevalent planktonic organism in alkaline freshwater lakes worldwide. Because this organism often achieves large populations in natural waters, the voluminous slime layer that surrounds its colonies can substantially influence a number of chemical and ecological properties of freshwater ecosystems. For example, the slime from *M. flos-aquae* and related organisms has been shown to interact strongly with cations (7, 19, 21) and to be involved in oxidative precipitation of manganese nodules in certain lakes (25). Such adsorption of metal cations can influence the availability of several trace nutrients, sequester toxic metals, affect pH, and play a role in several biogeochemical cycles.

The potential of cation-binding microbes or their products for the preconcentration, speciation, separation, bioadsorption, and detection of metals is a topic of investigation (28, 30, 32, 33). The slime of *M. flos-aquae* may have utility for such techniques. Another practical application of the *M. flos-aquae* slime is as a gelling agent that coagulates in the presence of ions.

Here we describe the isolation and chemical composition of slime layer material from axenic cultures of *M. flos-aquae* C3-40. Comparison is made to the plant polysaccharide pectin, a commonly used food gelling agent (9–11, 15). Corresponding mechanistic interactions between cations and polysaccharides are discussed. Chemical composition is considered with respect to morphological differences between *M. flos-aquae* slime and those of other characterized cyanobacteria (1, 3, 5, 8, 13, 14, 17–19, 21, 23, 26, 27, 31, 34), including the related organism *M. aeruginosa* K3-A (19).

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MATERIALS AND METHODS

Cyanobacteria and culture conditions. *M. flos-aquae* C3-40 is a subclone of axenic culture C3-9 (21). Cultures were grown to a final density of 0.8×10^7 to 1.6×10^7 cells per ml in Jansen medium (5) at 30°C and with 30 microeinsteins^{-2} of irradiance s^{-1} from Westinghouse cool white fluorescent lamps.

Materials. All solutions and procedures utilized distilled deionized water, analytical grade (or better) reagents, and research quality gases (Linde). Metal standards (Mallinckrodt) and bovine serum albumin (BSA) standards (Armour Pharmaceuticals) were of certified concentrations.

Isolation of slime material. Lots 5, 7, and 9 were independently isolated from similarly grown cultures. Each culture was centrifuged at 23,000 × *g* for 10 min at 4°C. The pellet, which contained cells and adherent slime, was suspended in ≥16-MΩ deionized water to >31 times the pellet volume. (High-resistivity water was required to solubilize the slime.) The suspension was stored overnight at 4°C. The cells were removed by centrifugation as described above, followed by sequential filtration through Nuclepore 5.0-μm-pore-size and Gelman 0.45-μm-pore-size membrane filters 47 mm in diameter. The filtrate was concentrated to approximately 1/10 of its original volume by pervaporation (for lots 5 and 7) or rotary evaporation (for lot 9), dialyzed in washed tubing with a 3,500-molecular-weight cutoff (Spectrum 132725) for >48 h against at least five consecutive 1-liter changes of deionized water, and lyophilized to dryness. Before subsequent assay or hydrolysis, weighed portions of lyophilized slime were suspended in deionized water and dispersed by storage for >18 h at 4°C.

Negative staining and microscopy. During each slime isolation, samples were taken from (i) the initial culture, (ii) the pellet formed during initial centrifugation of that culture,

and (iii) the pellet formed during centrifugation of cells suspended in water. One drop of each sample was mixed with 4 drops of undiluted latex base drawing ink (3084-F; Koh-i-noor). Wet mounts were photographed under bright-field illumination with green light (Nikon GIF interference filter).

Assay of inorganic and organic components. All assays were performed on lot 9 slime and confirmed by assay of at least one other lot. The concentration of each metal ion was ascertained in a Perkin-Elmer 360 flame atomic absorption spectrophotometer-atomic emission spectrophotometer with an air-acetylene flame, and conditions were optimized for maximum sensitivity. Sample and standard metal ion solutions were matrix matched before measurement. Total carbohydrate, protein, and total organic-plus-inorganic phosphorus were assayed by the anthrone (12), Coomassie blue (29), and molybdenum blue (16) methods, respectively. Gas chromatographic assays of C, H, and N were performed by Galbraith Laboratories, Inc.

Gas chromatography-mass spectrometry. Ten-milligram portions of slime were hydrolyzed and neutralized (8), filtered through 0.45-μm-pore-size membrane filters (Millipore HA), rotary evaporated to dryness, reconstituted in 1.50 ml of anhydrous pyridine, and mixed with 0.5 ml of trimethylsilylimidazole. The resulting trimethylsilyl derivatives were characterized by gas chromatography-mass spectrometry. A Hewlett-Packard 5890 gas chromatograph and 5971A mass selective detector were operated with helium as the carrier gas (1.5 ml/min with a split ratio of 25 ml/min, 15 lb/in^2) on a polydimethylsiloxane column (30 m long, 0.25-μm inside diameter; Altech). Samples of 0.5 μl were injected. The column temperature was maintained for 2 min at 150°C and then increased to 250°C (8°C/min).

RESULTS

Effects of centrifugation and washing. Cells of *M. flos-aquae* C3-40 were surrounded by an extensive slime layer visible in negatively stained samples (Fig. 1A). Much of this slime still adhered to cells that had been centrifuged in growth medium (Fig. 1B) but was released by washing with deionized water (Fig. 1C). We therefore chose to recover slime material from deionized water washings of centrifuged cells. This procedure yielded 60 to 75 mg of dialyzed, lyophilized material per 10^{10} cells.

Elemental analysis. Table 1 lists the amounts of various elements in the dialyzed slime. The C, H, and N composition was consistent with that of organic material of low N content. Most of the other elements examined, especially sodium, the predominant metal cation in Jansen growth medium (6), were reduced to trace amounts by dialysis. The main exception was iron. The molar ratio of Fe to Na in the dialyzed slime was 10^4 times that in the growth medium. Furthermore, the dialyzed slime fraction contained 48% (wt/wt) of the iron found in the entire 1 liter of culture from which the slime was extracted. These observations suggest preferential retention of iron, compared with sodium, in the slime fraction.

Analysis of sugar composition. Gas chromatography-mass spectrometry of trimethylsilyl-derivatized sugars from hydrolyzed *M. flos-aquae* slime yielded baseline separation and 15 peaks corresponding to various conformations of six sugars. Parallel analysis of derivatized sugar standards was used to confirm the identifications and to measure detector response efficiencies, from which the percent composition (wt/wt) of each sugar in the slime polysaccharide was calculated (Table 2). These percentages correspond to molar ratios of galacturonic acid-rhamnose-mannose-xylose-glucose-galactose of approximately 43:3:3:2:1:1. Pectin, a plant polysaccharide that also contains galacturonic acid, was identically treated as a control. The sugar composition obtained for pectin (Table 2) resembled previously published values (4).

Assays of total carbohydrate. Initial anthrone assays of total carbohydrate in the slime material were based on both glucose and galacturonic acid standards (Table 3). In addition, a mixture of the six sugars in the same weight percentages found in *M. flos-aquae* slime was prepared and designated the sugar mixture. The sugar mixture reacted slightly more efficiently than galacturonic acid and much less efficiently than glucose in the anthrone assay. We therefore decided to use the sugar mixture as the standard for slime carbohydrate assays. Subsequent assays indicated the presence of 0.90 ± 0.08, 0.84 ± 0.02, and 0.72 ± 0.02 mg of sugar mixture-equivalent carbohydrate per mg of slime lots 5, 7, and 9, respectively (Table 3).

Assay of protein. BSA in the Coomassie blue microassay (29) reproducibly yielded a linear relationship of absorbance to protein amount between 0.5 and 10 μg, with a curve that passed through the origin. Repeated assays failed to detect protein in 50, 70, and 100-μg portions of slime from two different lots (Table 4). To determine the limits of detection and screen for possible interference from constituents of slime, known weights of BSA were assayed in the presence of known weights of slime (Table 4). In the presence of large amounts of slime, the detection limits for protein were higher than those for calibration standards in the absence of slime. Nonetheless, 0.48 μg of BSA was still easily detected in the presence of 70 μg of slime, as was 0.96 μg of BSA in the presence of 100 μg of slime (Table 4). We therefore conclude that the slime preparations contained less than 1% BSA-equivalent protein. This absence of detectable protein also indicates that the isolation procedure did not produce appreciable cell lysis. It is possible that some polypeptides large enough to be retained in dialysis tubing with a 3,500-molecular-weight cutoff might be too small to react strongly in the Coomassie blue protein assay.

DISCUSSION

Macromolecular material from the slime of *M. flos-aquae* C3-40 contains galacturonic acid, lesser amounts of five neutral sugars, and no detectable protein. Most other cyanobacterial slime layers or sheaths of known chemical composition also possess polysaccharide composed of at least one uronic acid and several neutral sugars, sometimes in combination with protein (1, 3, 5, 8, 13, 14, 17–19, 21, 23, 26, 27, 31, 34). It is noteworthy, however, that the *M. flos-aquae* slime material contains 83% uronic acid, much more than the approximately 20% found in most other cyanobacterial exopolysaccharides.

The slime material isolated from *M. aeruginosa* K-3A by Nakagawa et al. (19) differs chemically from that of *M. flos-aquae* C3-40 (Table 2). Protein, fucose, and arabinose are present in the former, whereas these three components were not detected in the latter. Galacturonic acid, rhamnose, and xylose each make up 20 to 28% of the total sugars in the *M. aeruginosa* polysaccharide. In contrast, the *M. flos-aquae* polysaccharide contains 83% galacturonic acid, with only 3% xylose and 5.5% rhamnose. The chemical differ-

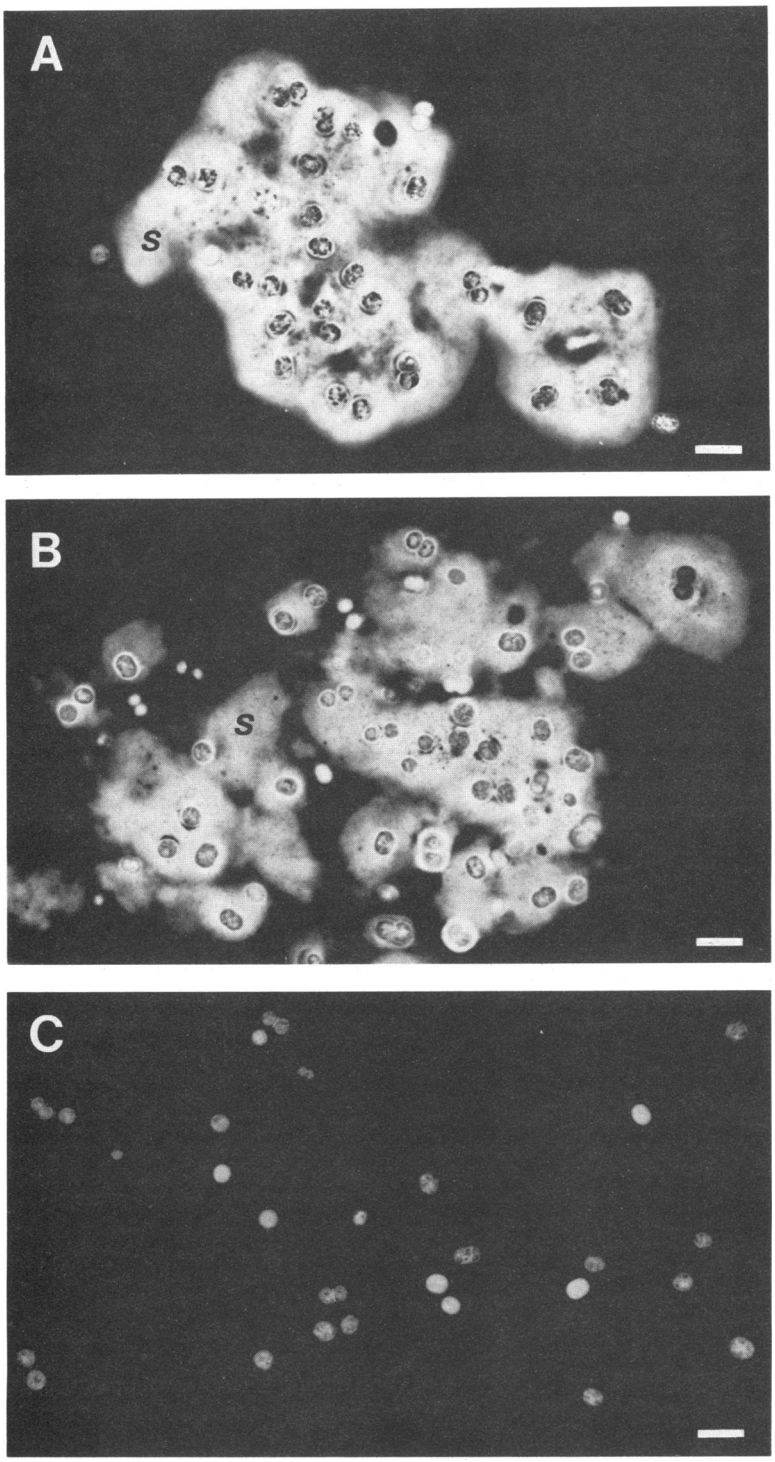

FIG. 1. Appearance of *M. flos-aquae* cells and slime at successive steps of the slime isolation procedure. (A) Culture immediately before centrifugation. (B) Pellet after centrifugation of the culture at 23,000 × *g* for 10 min. (C) Cells from which slime was removed by washing of the centrifugal pellet with 16-MΩ water. S, slime layer; black background, drawing ink negative stain; bar, 10 μm.

ences between the slimes of *M. aeruginosa* and *M. flos-aquae* are accompanied by differences in slime morphology. The tightly packed clusters of *M. aeruginosa* cells are characteristically surrounded by a ≥10-μm zone of relatively firm capsule with a distinct outer margin, whereas the more widely separated *M. flos-aquae* cells are embedded in a material that has a diffuse outer margin and is easier to shear by mechanical agitation (7, 19, 21). Furthermore, *M. flos-aquae* colonies ordinarily disperse and become unicells at cation concentrations that inhibit dispersal of *M. aeruginosa*

TABLE 1. Elements in *M. flos-aquae* C3-40 slime material

Element	Mean mg/g of slime ± SD
Na[a,b]	0.20 ± 0.04
K[a,b]	0.94 ± 0.02
Mg[a,b]	1.11 ± 0.01
Ca[a,b]	5.25 ± 0.01
Fe[a,b]	10.1 ± 0.1
P[a,c]	0.20 ± 0.01
C[d]	356.9
H[d]	60.3
N[d]	10.7

[a] Means of triplicate measurements of lot 9 are shown. Lot 7 gave similar results.
[b] Atomic absorption.
[c] Molybdenum blue assay.
[d] Gas chromatography of lot 9. Lot 7 gave similar results.

TABLE 3. Anthrone-reacting carbohydrate in *M. flos-aquae* C3-40 slime

Slime lot no.	No. of replicates[a]	Mean g of carbohydrate/g of slime ± SD[b]	
		Galacturonate equivalents	Sugar mixture equivalents
5[c]	5	0.94 ± 0.08	0.90 ± 0.08
7[c]	5	0.96 ± 0.03	0.84 ± 0.02
9[c]	5	0.84 ± 0.02	0.72 ± 0.02
9[d]	4	0.82 ± 0.04	0.72 ± 0.02

[a] Number of samples analyzed for each mean and standard deviation.
[b] Standards were galacturonic acid and a sugar mixture that simulated the composition of *M. flos-aquae* slime.
[c] Desiccated over Drierite at −10°C.
[d] Vacuum desiccated over phosphorus pentoxide at 4°C.

colonies (7, 21). These differences might arise because the polysaccharides differ or because the *M. aeruginosa* material contains protein, which is absent in *M. flos-aquae* slime. Cyanobacterial sheaths, which characteristically contain protein (1, 14, 23, 27, 31), are much more resistant to disruption procedures than are slime layers. In contrast, cyanobacterial slime layers usually lack protein (5, 8, 13, 17, 18, 20, 26, 34). Mutants in many *M. aeruginosa* cultures produce slime of decreased firmness (7). It should therefore be possible to test the hypothesis that decreased slime firmness correlates with decreased protein content, as opposed to altered carbohydrate composition.

The predominance of galacturonic acid in *M. flos-aquae* slime (Table 2) suggests that charge attraction to carboxyl groups contributes to cation binding. In addition, sugars with certain configurations of hydroxyl groups (axial, equatorial, and axial in hexoses) can complex with some metals (2). A well-studied comparison compound is the plant polysaccharide pectin, which resembles the *M. flos-aquae* slime in sugar composition (4). In pectin, not only carboxyl groups but also macromolecular conformation influence the specificity of cation binding and cation-dependent gelation (9, 10, 15, 24). Hanke and Northcote (11) report both weak nonspecific binding and strong, specific, sterically determined binding between pectin and the cationic dye ruthenium red, which also strongly stains the *M. flos-aquae* slime layer (7). Although pectin and the *M. flos-aquae* slime have strikingly similar sugar compositions (Table 2), it is not known whether their structural features, including linkages between sugars, are the same or different. A comparison of these two compounds could elucidate the relationship between polysaccharide structure and affinity for specific cations.

It is possible that siderophores of the hydroxamic acid, catechol, or phenolic class are immobilized within the slime and influence its ability to chelate Fe. Certain *M. flos-aquae* cultures apparently do produce siderophores, although usually under Fe starvation conditions that were not used here.

The pectinlike polysaccharide can be harvested from *M. flos-aquae* cells by washing with deionized water, a simpler procedure than that used to extract pectin from plant tissue (4, 22). Because genetic manipulation of procaryotes is relatively easy, the microbial system may also be a convenient source of altered pectins or enzymes that synthesize polygalacturonate.

TABLE 2. Sugar compositions of slime polysaccharide and pectin polysaccharide[a]

Sugar	% Composition (wt/wt)	
	Slime	Pectin
Arabinose	—[b]	2.5
Galactose	1.5	9.0
Glucose	2.0	1.5
Xylose	3.0	1.2
Mannose	5.0	1.4
Rhamnose	5.5	6.3
Galacturonic acid	83.0	78.0

[a] Determined by gas chromatography-mass spectrometry of trimethylsilyl derivatives.
[b] —, No arabinose was detected in the slime polysaccharide.

TABLE 4. Assay of protein in slime with and without added BSA

Slime added (μg)	BSA added (μg)	Mean A_{620}[a] ± SD	Mean protein found ± SD (μg of BSA equivalent)[b]
0.0	0.00	0.000 ± 0.002	0.00 ± 0.03
50.0	0.00	0.000 ± 0.002	0.00 ± 0.03
70.0	0.00	0.000 ± 0.002	0.00 ± 0.03
100.0	0.00	0.000[c]	NC[d]
50.0	0.48	0.027 ± 0.003	0.40 ± 0.03
70.0	0.48	0.020 ± 0.001	0.30 ± 0.01
100.0	0.48	0.000[c]	NC
0.0	0.96	0.064 ± 0.002	0.98 ± 0.02
50.0	0.96	0.063 ± 0.001	0.96 ± 0.01
70.0	0.96	0.063 ± 0.003	0.96 ± 0.03
100.0	0.96	0.061 ± 0.002	0.93 ± 0.02
0.0	1.92	0.124 ± 0.004	1.90 ± 0.05
50.0	1.92	0.124 ± 0.002	1.90 ± 0.02
70.0	1.92	0.124 ± 0.003	1.90 ± 0.03
100.0	1.92	0.119 ± 0.001	1.83 ± 0.01

[a] Means of triplicate Coomassie blue (29) assays of lot 9 are shown. Other assays of lots 9 and 7 gave similar results.
[b] Calculated from a linear standard curve for six different weights of BSA showing A_{620} readings between 0.000 ± 0.002 (for no BSA) and 0.310 ± 0.004 (for 4.80 μg of BSA).
[c] The A_{620} was less than that of the blank.
[d] NC, not calculated.

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