Characterization of Macromolecular Flocculants Produced by *Phormidium* sp. Strain J-1 and by *Anabaenopsis circularis* PCC 6720

Y. BAR-OR* AND M. SHILO

The Institute of Life Sciences, Division of Microbial and Molecular Ecology, The Hebrew University of Jerusalem, 91904 Jerusalem, Israel

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Several benthic cyanobacteria were found to produce significant amounts of extracellular flocculants. The macromolecular flocculants produced by *Phormidium* sp. strain J-1 and *Anabaenopsis circularis* PCC 6720 were characterized. The *Phormidium* flocculant is a sulfated heteropolysaccharide to which fatty acids and protein are bound. The polysaccharide backbone is composed of uronic acids, rhamnose, mannose, and galactose. The *A. circularis* flocculant is also an acidic polysaccharide containing keto acid residues and neutral sugars, but to which no fatty acids, proteins, or sulfates are linked. Both flocculants could be recovered from growth medium by precipitation with cetyltrimethylammonium bromide and were found to bind the cationic dye Alcian-blue in a linear proportion to their concentration in solution. The latter property was used to quantify flocculant concentrations in culture supernatants and natural water samples and to compute their anion densities.

The surface properties of bacterial cells determine their physical interaction with the environment. External cell wall components, such as lipoteichoic acid (2) or proteinaceous adhesins (1, 6, 13), mediate the adhesion of various bacteria to a diverse range of animate or inanimate substrate surfaces. Detachment and dispersal of such attached cells are in some cases facilitated by the excretion of amphiphilic molecules, such as emulsan (20) or emulcyan (7, 9), which accumulate on and coat the cell wall. While such modulations of the structure and properties of the cell surface modify the behavior of the cells toward their environment, there are also microbial modifications of the environment to suit specific requirements of the cells. One such example is the excretion of a potent macromolecular flocculant by the benthic cyanobacterium *Phormidium* sp. strain J-1 (8). This species was isolated from a drainage canal rich in suspended clay particles (7) which hamper light penetration through the water column to the photoautotrophic cyanobacterial mat. Excretion of the flocculant was found to bring about the flocculation and subsequent sedimentation of the clay particles, thus clarifying the water column. The aim of the present study was to characterize the biochemical nature of the *Phormidium* flocculant (designated F-J1) and to investigate the occurrence and nature of flocculant production in other cyanobacterial species.

**MATERIALS AND METHODS**

Cyanobacterial strains and culture conditions. The cyanobacterial strains used are described in Table 1. All cultures were axenic and were grown with shaking at 35°C in BG-11 medium (23), except for *Oscillatoria limnetica*, which was grown in BG-11 medium in Turks Island salt solution (29). The cultures were continuously illuminated with cool white fluorescent lamps (incident light intensity, $2.5 \times 10^{3}$ ergs/cm$^2$ per s).

Preparation of flocculants. Crude preparations of extracellular F-J1 were obtained by pronase digestion of concentrated supernatants followed by ethanol precipitation, as previously described (8). Further purification was achieved by treating aqueous solutions of the flocculant with hot phenol (28). Dissolution of the crude preparation was possible only after preliminary “wetting” with ethanol. The aqueous phase obtained from the phenol treatment step was dialyzed, lyophilized, and designated WF-J1. The material obtained was dissolved in 2 mM Tris-0.15 M NaCl buffer (pH 8.5) to a concentration of 2 mg/ml; 2 ml of this solution was loaded and run on a Sepharose CL-4B (Pharmacia, Inc., Piscataway, N.J.) column (45 by 2.5 cm) equilibrated with the same buffer. Fractions (3.5 ml) were collected and analyzed for flocculant activity. Active fractions were pooled, dialyzed, lyophilized, and analyzed biochemically.

The *Anabaenopsis circularis* flocculant (designated F-Ac) was obtained by addition of two volumes of cold ethanol to the filtered supernatant. The precipitate was dissolved in water, dialyzed, and lyophilized.

**Biochemical and chromatographic analyses.** Protein determination was done by the method of Lowry et al. (16). Neutral sugars were assessed with anthrone (26), using glucose as a standard unless otherwise specified. Uronic acids were evaluated by the carbazole method (5) modified by the addition of borate and sulfamate to prevent interference by neutral sugars (11). Keto acids were assayed with 2,4-dinitrophenylhydrazine by the method of Friedemann and Haugen (10) as modified by Katsuki et al. (15). Ester-linked sulfates were detected qualitatively in flocculant preparations by staining with aldehyde fuchsin followed by Alcian-blue (4). Quantitative analysis of sulfate was performed with benzidine (24). Estimation of fatty acid esters was done by hydrolysis with 0.5 M KOH (90 min, 50°C), extraction with petroleum ether, evaporation of the solvent, and weighing. Analysis of the carbohydrate components of F-J1 was done by thin-layer chromatography as follows. Purified flocculant preparations were hydrolyzed with 2 M trifluoroacetic acid at 120°C for 2 h and lyophilized. The hydrolysate, dissolved to a concentration of 10 μg/ml, was loaded onto a silica gel plate (Sigma Chemical Co., St. Louis, Mo.) along with marker carbohydrates (obtained from Sigma or BDH, Poole, England). The solvent system was a mixture of water, ethyl acetate, and n-pentanol (1:2:7,
TABLE 1. Production of flocculants by cyanobacterial cultures

<table>
<thead>
<tr>
<th>Cyanobacterium</th>
<th>Morphology</th>
<th>Ecological niche</th>
<th>Flocculant production</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Phormidium</em> sp. strain J-1; ATCC 39161</td>
<td>Filamentous</td>
<td>Benthic</td>
<td>+</td>
</tr>
<tr>
<td><em>Anabaenopsis circulalis</em> PCC 6720</td>
<td>Filamentous</td>
<td>Benthic</td>
<td>+</td>
</tr>
<tr>
<td><em>Calothrix desertica</em> PCC 7102</td>
<td>Filamentous</td>
<td>Benthic</td>
<td>+&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Oscillatoria limnetica</em>; isolated from Solar Lake, Israel, by Y. Cohen</td>
<td>Filamentous</td>
<td>Benthic</td>
<td>–</td>
</tr>
<tr>
<td><em>Plectonema boryanum</em> 594; Indiana University Culture Collection</td>
<td>Filamentous</td>
<td>Planktonic</td>
<td>–</td>
</tr>
<tr>
<td><em>Anacystis nidulans</em> PCC 6311</td>
<td>Unicellular</td>
<td>Planktonic</td>
<td>–</td>
</tr>
</tbody>
</table>

<sup>a</sup> Small quantities were found in late-stationary-phase cultures.

vol/vol). The chromatogram was developed by spraying it with a fresh solution of anisaldehyde-ethanol-concentrated H<sub>2</sub>SO<sub>4</sub>(0.5:9:0.5, vol/vol) (18). Spots were detected after the plate was heated to 100°C. Undeveloped spots, run in parallel, were scraped from the plate and analyzed by the anthrone method, using the corresponding carbohydrates as standards. The relative sugar proportions were thus revealed. This was necessary because the different sugars yield different specific colorimetric values by the anthrone method. Rhamnose was also determined independently, using the orcinol and the cysteine-H<sub>2</sub>SO<sub>4</sub> methods (12).

**Desulfation of flocculant preparations.** Solvolytic desulfation of flocculant preparations was done in dimethyl sulfoxide containing 10% methanol (17).

**Assay of flocculant activity.** The flocculating activity of the cyanobacterial culture supernatants was tested by a modification of the method previously described (8). Samples of the supernatant were added to fresh medium to make up a final volume of 4.5 ml; 0.5 ml of a standard bentonite suspension (6.0 mg/ml) was added, and the test tube was vortexed thoroughly. Turbidity was then measured continuously with a ratio turbidimeter (Hach, Loveland, Co.), and the time required to reach 50% of the initial turbidity was recorded. A 1-ml volume of supernatant causing a 50% decrease in 5 min was defined as containing 5 U of flocculant.

**Alcian-blue binding.** An indirect assay for calculation of flocculant concentrations in supernatants, and for comparison of the anion densities of different flocculants, was done by measuring the binding of Alcian-blue (MCB, Cincinnati, Ohio) by the method of Ramus (19), modified by using a 0.5-mg/ml dye solution and incubating the samples for 6 h. Anion density was expressed as micrograms of Alcian-blue bound per microgram of flocculant.

**RESULTS**

Production of flocculants by cyanobacteria. Several benthic, as well as planktonic, cyanobacteria were screened for production of extracellular flocculants (Table 1). Significant amounts were detected in the culture supernatants of *Phormidium* sp. strain J-1 (described previously [8]) and *A. circularis* PCC 6720 which, like *Phormidium* sp. strain J-1, is a benthic species possessing hydrophobic cell surface properties (7). Growth and extracellular flocculant production of *A. circularis* are shown in Fig. 1. Another benthic cyanobacterium, *Calothrix desertica*, produced small amounts of extracellular flocculant at the late lag phase of growth. Several other benthic and planktonic species did not produce significant amounts of flocculant.

**Comparative analysis of cyanobacterial flocculants.** Crude preparations of F-J1 contained considerable amounts of protein in addition to carbohydrates and lipids (8) (Table 2). Treatment with hot phenol removed some of the protein without affecting the flocculating properties of the material left in the aqueous phase (WF-J1) (Table 2). The protein recovered from the phenolic phase did not possess flocculating activity.

Qualitative demonstration of sulfate groups in WF-J1 was done by staining with aldehyde fuchsin followed by Alcian-blue. The violet fuchsin dye was retained by the polymer, indicating that it was sulfated.

Gel permeation chromatography of WF-J1 on Sepharose CL-4B yielded two broad UV absorption peaks (Fig. 2), the first beginning to elute at the void volume (corresponding to an estimated molecular weight of about $1.2 \times 10^6$) and containing 65% of the initial flocculating activity. A second peak eluted later, contained only protein, and lacked any activity.

Analysis of the material eluted in the first, active peak showed that it contained uronic acids, neutral sugars, proteins, fatty acids, and sulfates (Table 2). The carbohydrate components were analyzed by thin-layer chromatography.

![FIG. 1. Growth of (⊙) and flocculant production by (●) *A. circularis.*](https://example.com/fig1.png)
and found to be rhamnose, mannose, and galactose at an approximate ratio of 1:2:0.5, respectively, and an unidentified uronic acid with an $R_f$ value different from those of glucuronic, mannanuronic, and galacturonic acids. Rhamnose was also determined independently by the orcinol and cyanine-$H_2SO_4$ methods for deoxyhexoses (12) and found to account for 13% of WF-J1 on a weight basis.

An alternative way for recovery of F-J1 was by the addition of cetyltrimethylammonium bromide (CTAB) or other alkyl ammonium salts (such as cetlypyridinium chloride, benzalkonium chloride, or cetyldimethylammonium chloride), to the culture supernatant, yielding a highly gelatinous precipitate. Quantitative dissolution of the flocculant-quaternary ammonium complex proved to be difficult to accomplish. Incubation in 4 M NaCl, 2.5 M MgSO$_4$, or 4.2 M KCl was ineffective. Partial dissolution was achieved by incubating the complex in CaCl$_2$-saturated ethanol at 35°C overnight and then in water. Incubation in an alkaline solution dissolved the complex by destroying the cetyltrimethylammonium ion. However, much of the flocculating activity was lost as well. We finally found that incubation in 50 mM Na$_2$SO$_4$ (22) fully dissolved the complex. The biopolymer was then precipitated with cold ethanol, dissolved in water, and lyophilized. The biochemical composition of the CTAB-precipitated flocculant was similar to that of the WF-J1 preparation.

Analysis of F-Ac, obtained by ethanol precipitation, revealed that it was a polyanion containing neutral sugars and keto acid residues (Table 2). No proteins, uronic acids, or sulfates were detected. When chromatographed on Sepharose CL-4B, F-Ac eluted at the void volume, indicating that its molecular weight was above $1.2 \times 10^6$.

F-Ac could be recovered from spent medium of Anabaena sp. strain J-1 by addition of CTAB, forming a fine precipitate. The flocculant-CTAB complex dissolved readily upon incubation in dilute salt solutions. The critical electrolyte concentration required for dissolution was determined to be 0.13 M NaCl.

### TABLE 2. Biochemical characterization of cyanobacterial flocculants

<table>
<thead>
<tr>
<th>Prepn</th>
<th>Carbohydrates</th>
<th>Uronic acids</th>
<th>Keto acids</th>
<th>Proteins</th>
<th>Fatty acids</th>
<th>Sulfates</th>
<th>Anion density</th>
<th>Flocculating activity (U/μg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F-J1a</td>
<td>22</td>
<td>13.5</td>
<td>3.5</td>
<td>1.1</td>
<td>1.65</td>
<td>0.92</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WF-J1a</td>
<td>43</td>
<td>9.0</td>
<td>14.1</td>
<td></td>
<td></td>
<td></td>
<td>0.95</td>
<td>210</td>
</tr>
<tr>
<td>chr-WF-J1a</td>
<td>43</td>
<td>4.4</td>
<td>14.1</td>
<td></td>
<td>1.65</td>
<td>0.96</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F-Acb</td>
<td>31</td>
<td>0</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0.46</td>
<td>153</td>
<td></td>
</tr>
</tbody>
</table>

* From Phormidium sp. strain J-1. chr-WF-J1. Material eluted in the first, active peak.

* From A. circularis.

Assessment of flocculant preparations by binding of Alcian-blue. Determination of flocculant concentrations in culture supernatants or cell lysates has been routinely carried out by measuring the rate of flocculation and sedimentation of standard bentonite suspensions after addition of the tested liquid (8). This method has a number of disadvantages. (i) Linearity is maintained only within a narrow range of flocculant concentrations (0 to 25 μg/ml). (ii) Results are affected by variations in electrolyte concentrations and temperature. (iii) The method is time-consuming, requiring about 15 min for each sample. To avoid these drawbacks, we measured the correlation between flocculant concentrations and binding of Alcian-blue, a cationic dye with a high affinity for polyanions (19). Figure 3 shows that, under the experimental conditions used, a linear correlation existed between Alcian-blue binding and concentrations of WF-J1 or F-Ac up to 100 μg/ml. Binding of the dye was affected neither by temperatures up to 50°C nor by variations in salt concentrations (0 to 0.15 M NaCl). Dye binding also facilitated the computation of the anion densities in the two flocculants (Table 2). Solvolytic desulfation of WF-J1 resulted in the loss of ca. 80% of its flocculating activity and a decrease in its anion density from 1.4 to 0.30. Bacterial sulfatase (EC 3.1.6.1: 1.0 U/ml [Sigma]) was ineffective in removing sulfates from WF-J1 and did not reduce its anion density.

**DISCUSSION**

Screening of several cyanobacterial species for flocculant production revealed that this phenomenon is not unique to Phormidium sp. strain J-1 and is found also in A. circularis and C. desertica. The two cyanobacterial flocculants inves-
tigated in detail in this study differed markedly in their biochemical compositions, although both are polyanionic macromolecules. As determined by column chromatography, F-J1 is a polydisperse macromolecule, the anionic nature of which is due to uronic acids and sulfate groups. In F-Ac, the anionic nature is caused by keto acid residues. The carbohydrate-protein-lipid compositions are also dissimilar in these two flocculants.

To the best of our knowledge, F-J1 is the first described sulfated, extracellular polysaccharide of eubacterial origin. However, sulfated, cell wall polymers do occur in certain archaea bacteria (27). This means that sulfated exopolysaccharides are not unique to eucaryotes and may further establish the special status of cyanobacteria as an intermediate between procaryotes and photosynthetic eucaryotes. Production of sulfated exopolysaccharides, such as agar-agar or carrageenan, is common in higher algae, such as Gracilaria and Laminaria species (3).

Solvolytic desulfation of WF-J1 resulted in the loss of 80% of its flocculating activity and a similar decrease in Alcian-blue binding. Since these activities are, at least in part, due to the presence of acidic carboxyl groups (as is the case in F-Ac), desulfation by itself cannot explain these dramatic decreases in activity. Apparently, simultaneous decarboxylation and probably methylation of acidic residues take place (14).

Another unique feature of F-J1 is its tight binding of CTAB. In other acidic, or even sulfated, polysaccharides, it is a common practice to dissolve the CTAB complex in salt solutions or to exchange the alkyl ammonium ion with calcium (21). Such treatments were ineffective in dissolving the F-J1–CTAB complex.

In an earlier study, A. circularis was not found to produce significant amounts of flocculant (8). However, culture conditions in the present study were different; the temperature was elevated to 35°C, and incubation was done with shaking. We found that at 27°C without shaking, flocculant production was considerably reduced. In C. desertica, flocculant production is limited to the late lag phase of growth and therefore was overlooked previously (8).

Several negatively charged polysaccharides isolated from bacteria, yeast, and soil fractions are known to be capable of flocculating suspended clay particles (for a review, see reference 25). They act by adsorbing onto the surfaces of neighboring, negatively charged clay particles via cation bridges. The effectiveness of such polymers depends on the length of the molecule and the number of charged groups per unit length, i.e., the charge density. These factors determine the extent of interparticle bridging by the flocculant. We found that both cyanobacterial flocculants are large polyanion macromolecules. In F-J1, the anionic density is relatively high and is attributed to sulfate groups in addition to carboxyl uronic acid residues, while in F-Ac the ionic density is lower and is derived solely from carboxyl keto acid residues. These differences may explain the higher flocculating activity of F-J1 as compared with F-Ac.

There are many potential uses for flocculants of biological origin. Among such uses are clarification of solar ponds, reduction of suspended solid matter in water reservoirs intended for irrigation, soil conditioning for improvement of water-holding capacity, and stabilization of wastewater effluents, which are now being tested in our laboratory. Elucidation of the mechanisms of action of bioflocculants may also lead to the synthesis of potent chemical flocculants or to modification of natural polymers, improving their activity.

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LITERATURE CITED


