

BIOCHEMICAL CHARACTERIZATION OF CYANOBACTERIAL EXTRACELLULAR POLYMERS (EPS) FROM MODERN MARINE STROMATOLITES (BAHAMAS)

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ABSTRACT

A range of biochemical characterizations were used to examine the extracellular polymeric secretions (EPS) of two cyanobacteria, *Synechocystis* sp. and *Oscillatoria* sp., isolated from marine stromatolites, Bahamas. Two-dimensional gel electrophoresis was successfully used to fractionate proteins in EPS. The results suggest that cyanobacterial EPS is composed of a network of macromolecules having different biochemical properties, which may contribute to extracellular functions.

INTRODUCTION

The matrix of extracellular polymeric secretions (EPS) is a key structuring component of microbial biofilms. EPS secreted by cyanobacteria often contain carbohydrates, proteins, glycoproteins, and lipids.^{1,2} The biochemical properties of EPS may be instrumental in regulating a range of functions to their associated microbial communities.^{1,3}

A major role of EPS is in enhancing the mechanical stability and adhesive capacity of microbial cells to surfaces.^{4,5} This has been attributed to the proteinaceous components in EPS.⁶ Lipids may help bacterial cells overcome the strong surface tension of the surrounding water, thus facilitating growth on surfaces.

The protein component of EPS may be potentially important in the cohesive properties and long-term stability of the biofilm.⁸ Despite the potential significance, few biochemical studies have focused on the proteinaceous moieties of EPS. In cyanobacteria, studies of EPS have been largely limited to analyzing the total protein content and/or amino acid composition.²

In the present study, two cyanobacterial species (*Synechocystis* sp., and *Oscillatoria* sp.) were isolated from modern marine stromatolites at Highborne Cay in the Bahamas, and maintained in culture in our laboratory. EPS was later extracted and used for the further biochemical analyses. For the first time, the proteinaceous components in cyanobacterial EPS were characterized using two-dimensional polyacrylamide gel electrophoresis (2D-PAGE).

EXPERIMENTAL

Cyanobacteria Culture and EPS Isolation

Two cyanobacteria (*Synechocystis* sp., and *Oscillatoria* sp.) were isolated from the marine stromatolite at Highborne Cay, Bahamas. Each species was grown in CHU-10 medium (0.004M Na₂SiO₃, 9H₂O, 0.006M Ca(NO₃)₂, 4H₂O, 0.014M K₂HPO₄, 0.025M MgSO₄, 7H₂O, 0.05M Na₂CO₃, 0.012 M Fe-EDTA, 3.7 x 10⁻⁸ M B₁₂, 4 x 10⁻⁷ M biotin and 5.9 x 10⁻⁷ M thiamin) in seawater (32 ppt salinity) and a light:dark cycle (12: 12 hr; approx. 100 μEinstein). Sterilized Bahamas sediment, consisting of well-sorted calcium carbonate ooids (mean grain size 250-500 μm), cleaned in sodium hypochlorite and rinsed thoroughly in distilled H₂O, were added to culture flasks to a depth approximately 0.5 cm as a substratum for growth.

Culture medium was discarded, then each of the cultures was suspended, stirred, and gently heated (40 °C) for at least 30 minutes to strip EPS from the cyanobacteria. The suspension was centrifuged (12000 rpm; 15 minutes) in order to shear any remaining EPS from the cell. Supernatant, containing EPS, was drawn off from the resulting cell pellets and then lyophilized completely.

Lyophilized supernatant was rehydrated with de-ionized water, and combined with cold ethanol (final concn. 75%; 6 - 8 h) to precipitate the EPS. The suspension was centrifuged (3000 rpm; 5 min) and the supernatant removed, leaving a white pelletized mass of EPS. This procedure was repeated.

Afterwards, the solution was dialyzed (MW cutoff 14,000 Da.) against deionized water (48 h). Dialyzed EPS was lyophilized to dryness and stored at -20 °C.

Carbohydrate Monomer Analyses

Samples of purified EPS were hydrolyzed using freshly prepared 1M methanolic-HCl for 16h at 80°C. Released sugars were derivatized with Tri-Sil and the sample was analyzed by gas chromatography using a Supelco column. Myoinositol was added (40 µg) as an internal standard. Monomeric analyses were conducted by the CCRC (Complex Carbohydrate Research Center, University of Georgia).

Determination of Uronic acid, Glycosaminoglycans (GAGs), and Protein in EPS

Uronic acid content was measured using carbazole in 80% sulfuric acid with borate ions added⁹. GAG content was measured using dimethyl methylene blue¹⁰. Protein content was measured according to Bradford's method using BioRad Protein Assay Kit.¹¹ All samples were measured in triplicate.

2D-PAGE

Two-dimensional polyacrylamide gel electrophoresis was carried out on EPS from two cyanobacteria using BioRad Mini-PROTEAN II 2-D Cell according to the method of O'Farrell.¹² One mg of EPS was treated at room temperature with 200 µL of first-dimension sample buffer (9.5M urea, 2.0% Triton X-100, 5% β-mercaptoethanol, 1.6% Bio-Lyte 5/7 ampholyte and 0.4% Bio-Lyte 3/10 ampholyte). The first-dimension tube gel was run at 750 V for 4 hours. For the second-dimensional separation, 10 % SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was run at 40mA for 45 minutes.¹³ Proteins were visualized using a BioRad silver stain kit.¹⁴

Lectin Blot Assay

Seven different biotinylated lectin probes were used to profile carbohydrate moieties in the EPS. Following separation of EPS by SDS-PAGE, carbohydrate and protein were transferred to nitrocellulose membrane.¹⁵ After blotting, the nitrocellulose membrane was incubated in blocking buffer (3% non-fat dry milk in Tris-buffer saline (TBS)) for 30 minutes, and briefly washed with TBS.

Table 1
Concentrations of Protein, Glycosaminoglycans (GAGs), and Uronic Acids in EPS From *Synechocystis* sp. and *Oscillatoria* sp.

	Protein ($\mu\text{g}/\text{mg}$ EPS \pm SD)	GAGs ($\mu\text{g}/\text{mg}$ EPS \pm SD)	Uronic Acids ($\mu\text{g}/\text{mg}$ EPS \pm SD)
<i>Synechocystis</i> sp.	3.53 ± 0.35	4.40 ± 0.17	4.63 ± 1.36
<i>Oscillatoria</i> sp.	6.33 ± 1.53	11.00 ± 1.00	39.00 ± 2.65

The nitrocellulose membranes were transferred to a Bio-Rad Mini-PROTEAN II Multi-Screen Apparatus. Biotinylated lectins (600 μL) in 1 % non-fat dry milk in TBS (dilution: 1:1000), were applied to each well, and incubated at 4 °C overnight. The membranes were washed three times in 0.1% Tween 20 in TBS, incubated in Streptavidin-Alkaline Phosphatase conjugates in 1% non-dry milk in TBS (dilution; 1:1000) for 1 hour. Then, membranes were washed three times in 0.1% Tween 20 in TBS and developed in the color development buffer. The following lectin probes were used: *Dolichos biflorus* agglutinin (DBA), *Ulex europaeus* agglutinin 1(UEA 1), Peanut agglutinin (PNA), Concanavalin A (Con A), Wheat germ agglutinin (WGA), Soybean agglutinin (SBA) and *Ricinus communis* agglutinin 1 (RCA₁₂₀). All lectins were purchased from Vector Laboratories, (Burlingame, CA).

RESULTS AND DISCUSSION

The EPS matrix of stromatolite biofilms serves to physically stabilize the uppermost layers of cells against the high-energy wave environments in which these structures commonly occur¹. EPS may additionally provide a chemically protective microenvironment for cellular and extracellular activities¹. Reactive ligands on EPS serve to bind and concentrate Ca²⁺ and Mg²⁺ ions from the surrounding seawater³. These are present as a range of proteins, carbohydrates, amino-sugars, and other molecules which are typically present in EPS.

Determination of Protein, Uronic Acids, and GAGs

Protein contents of EPS from *Synechocystis* sp. and *Oscillatoria* sp. were 0.35% and 0.63%, respectively (Table 1). GAGs contents of *Oscillatoria* sp. EPS (1.1%) were approximately three times higher than those of *Synechocystis* sp. EPS (0.44%). Also, *Oscillatoria* sp. EPS (3.9%) contains higher amounts of uronic acids than EPS from *Synechocystis* sp. (0.46%) (Table 1).

Table 2

**Carbohydrate Composition (mole %) of EPS from
Synechocystis sp. and *Oscillatoria* sp.**

	<i>Synechocystis</i> sp.	<i>Oscillatoria</i> sp.
Glucose	58.3	26.7
Galactose	19.0	37.1
Xylose	7.3	13.4
Arabinose	7.4	1.8
Rhamnose	5.4	7.5
Fucose	2.6	13.5

Although relatively small amounts of uronic acids and GAGs were found in EPS from *Synechocystis* sp. and *Oscillatoria* sp., the presence of these moieties contributes to the anionic nature of EPS.² These groups may play important roles in binding Ca²⁺ and Mg²⁺ ions, and in stabilizing the microbial extracellular environment. The ability of the immediate extracellular environment to bind and concentrate ions may enhance the utilization of bicarbonate for photosynthesis.¹⁶ Also, the effective chelation of excess dissolved Ca²⁺ ions may prevent precipitation of CaCO₃ (Kawaguchi & Decho, in prepn.).

Observed protein contents of cyanobacterial EPS have been typically small; however, protein has been found in almost every cyanobacterial EPS investigated.^{2,17,18} In our study, weight-specific protein contents from both *Synechocystis* sp. and *Oscillatoria* sp. EPS were low, 0.35% and 0.63%, respectively.

The protein moieties of EPS may serve a range of functions to cyanobacteria and other microorganisms. The hydrophobicity and adhesive capacity of EPS have been attributed to protein in EPS.^{19,20} Also, a range of extracellular enzymes are known to be secreted by photosynthetic microorganisms and serve important functions.¹⁶ These enzymes are closely associated with the EPS fraction, and the activities of the enzymes may be stabilized by the protective microenvironment afforded by EPS,²¹ and even facilitate H⁺ cycling to enhance bicarbonate uptake.¹⁶ Such enzymes would be extracted as part of the EPS fraction using most separation techniques.²² Decho and Lopez²³ proposed that certain EPS proteins may form a structural backbone

Table 3

Lectin Binding Assay of EPS from *Synechocystis* sp. and *Oscillatoria* sp.

Lectin*	<i>Synechocystis</i> sp.	<i>Oscillatoria</i> sp.
DBA		
UEA 1		
PNA		
Con A	++	++
WGA	+++	+++
SBA	+++	+++
RCA ₁₂₀	++	++

++ = Reaction; +++ = Strong reaction. * DBA = Dolichos Biflorus Agglutinin, UEA 1 = Ulex Europaeus Agglutinin 1, PNA = Peanut Agglutinin, Con A = Concanavalin A, WGA = Wheat Germ Agglutinin, SBA = Soybean Agglutinin, RCA₁₂₀ = Ricinus Communis Agglutinin 1.

for the EPS matrix. Subsequent studies by Higgins and Novak⁸ have shown that lectin-like proteins contribute to the formation of three-dimensional networks of the biofilm matrix by cross-linking polysaccharides, either directly or indirectly, through multivalent interactions.

Carbohydrate Content and Composition

Carbohydrate content of both types of EPS were approx. 50% (Table 2). Compositional analyses of carbohydrate monomers showed the presence of hexoses (glucose, galactose, mannose), pentoses (ribose, xylose, arabinose), deoxy-sugars (rhamnose and fucose), and acidic sugars (glucuronate and galacturonate), however, the relative molar ratios of these monomers differed in the two cyanobacteria. EPS from *Oscillatoria* sp. was enriched in galactose (37%), xylose (13%), fucose (13%) and uronic acids (4.6%), while *Synechocystis* sp. EPS had high mole-percents of glucose (58%), and galactose (19%).

Ten different monosaccharides have been found in cyanobacterial EPS. These include the hexoses: glucose, galactose and mannose; the pentoses: ribose, xylose and arabinose; and the acidic hexoses: glucuronate and galacturonate (see De Phillippis & Vincenzini² for review). Zhou *et al.*²⁴ emphasized the potential importance of deoxy-sugars (*e.g.* rhamnose and fucose) and sulfate half-esters in the cohesive properties of EPS polysaccharides. The *Oscillatoria* sp. EPS in our study had a 13% (wt/wt) content of the deoxy-sugar fucose (Table 2), which may have contributed to the cohesive properties of this polymer.

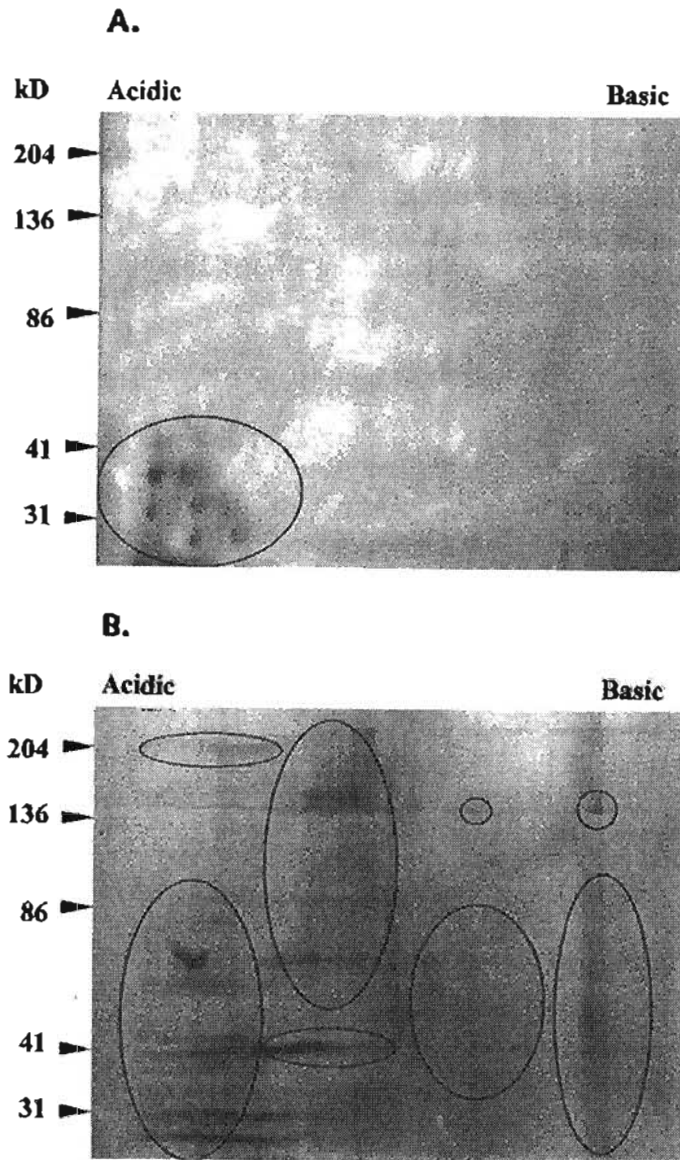


Figure 1. 2-D PAGE profiles of EPS isolated from *Oscillatoria* sp. (A) and *Synechocystis* sp. (B).

Lectin Binding Assays

The presence of amino sugars in cyanobacterial EPS has been considered to be rare.²⁵⁻²⁸ Our lectin binding assays, however, indicated that EPS from both species contained relatively high amounts of *N*-acetylglucosamine and α - or β -linked *N*-acetylgalactosamine. This was evidenced semi-quantitatively by the

binding of WGA and SBA lectins to EPS components after SDS-PAGE (Table 3). Con-A and RCA₁₂₀ showed a relatively weak binding showing the presence of α -mannose and oligosaccharides ending in galactose, respectively (Table 3). The specificity of DBA is for α -linked N-acetylgalactosamine, UEA 1 for glycoproteins and glycolipids containing α -linked fucose residues, PNA for galactosyl (β -1, 3) N-acetylgalactosamine, Con A for α -linked mannose, WGA for N-acetylglucosamine, SBA for oligosaccharide structures with terminal α -linked or β -linked N-acetylgalactosamine, RCA₁₂₀ for oligosaccharides ending in galactose.

2-D PAGE

For the first time, two-dimensional gel electrophoresis was successfully used to fractionate proteinaceous components in cyanobacterial EPS. While total protein contents of EPS in both cyanobacteria (*Oscillatoria* sp. = 0.63%, *Synechocystis* sp. = 0.35%) were low, two-dimension gel electrophoresis indicated that the extracellular proteins represented a range of different molecular sizes and isoelectric points (Fig. 1). The nature of these proteins are under investigations. Our studies using 2D-PAGE have revealed that EPS from both *Synechocystis* sp. and *Oscillatoria* sp. contained a range of proteins having different molecular weights (MW) and pI values. To our knowledge, this is the first report demonstrating the separation of proteins in cyanobacterial EPS using 2D-PAGE. In conclusion, the 2D-PAGE together with other biochemical analyses provide important information about proteins in EPS for better understanding the biochemical characteristics of EPS.

ACKNOWLEDGMENTS

We gratefully acknowledge Dr. Lee Prufert-Bebout (NASA-Ames Laboratory) for help in identifications of isolated strains of cyanobacteria. We also acknowledge the scientists of RIBS (Research Initiative on Bahamian Stromatolites) and CoBOP (Coastal Ocean Benthic Optical Properties-ONR) for input during this work. This work was supported by grants from the National Science Foundation (OCE 96-17738) and the Office of Naval Research (N 0001497-10024), and represents RIBS Contribution #8.

REFERENCES

1. A. W. Decho, *Oceanogr. Marine Biol. Ann. Rev.*, **28**, 73-153 (1990).
2. R. De Philippis, M. Vincenzini, *FEMS Microbiol. Rev.*, **22**, 151-175 (1998).
3. I. W. Sutherland, **Biotechnology of Microbial Exopolysaccharides**,

4. T. R. Neu, K. C. Marshal, *Biofouling*, **3**, 101-112 (1991).
5. C. Mayer, R. Moritz, C. Kirschner, W. Borchard, R. Maibaum, J. Wingender, H.-C. Flemming, *Int. J. Biol. Macromol.* **26**, 3-16 (1999).
6. M. Ganter, P. Rowell, W. W. Kerby, I. W. Sutherland, *Biol. Fertil. Soils*, **19**, 41-48 (1995).
7. T. R. Neu, *Microbiol. Rev.*, **60**, 151-166 (1996).
8. M. J. Higgins, J. T. Novak, *J. Environ. Engineer*, **123**, 479-485 (1997).
9. K., A. Taylor, J. G. Buchanan-Smith, *Anal. Biochem.*, **201**, 190-196 (1992).
10. S. Chandrasekhar, M. A. Esterman, H. A. Hoffman, *Anal. Biochem.*, **161**, 103-108 (1987).
11. M. Bradford, *Anal. Biochem.*, **72**, 248-254 (1976).
12. P. H. O'Farrell, *J. Biol. Chem.* **250**, 4007-4021 (1975).
13. U. K. Laemmli, *Nature*, **227**, 680-685 (1970).
14. C. R. Merrill, D. Goldman, S. A. Sedman, M. H. Ebert, *Science*, **211**, 1437 (1981).
15. H. Towbin, T. Staehelin, T. Gordon, *Proc. Natl. Acad. Sci. USA*, **76**, 4350-4354 (1979).
16. T. A. McConnaughey, J. F. Whelan, *Earth Sci. Rev.*, **42**, 95-117 (1997).
17. A. Flaibani, Y. Olsen, T. J. Painter, *Carbohydr. Res.*, **190**, 238-248 (1989).
18. M. Marra, A. Palmeri, A. Ballio, A. Segre, M. E. Slodki, *Carbohydr. Res.*, **197**, 338-344 (1990).
19. Y. Bar-Or, M. Shilo, *FEMS Microbiol. Ecol.*, **53**, 169-174 (1988).
20. M.-F. Dignac, V. Urbain, D. Rybacki, A. Bruchet, D. Snidaro, P. Scirbe, *Water Sci. Technol.*, **38**, 45-53 (1998).
21. M. Hoffman, A. W. Decho, "Extracellular Enzymes Within Microbial Biofilms and the Role of the Extracellular Polymer Matrix," in **Microbial Extracellular Polymeric Substances-Characterization, Structure and Function**, J Wingender, T. R. Neu, H.-C. Flemming, eds., Springer-Verlag, Berlin, 1999, pp. 217-230.

22. P. H. Nielson, A. Jahn, "Extraction of EPS," in: **Microbial Extracellular Polymeric Substances**, J. Wingender, T. R. Neu, H.-C. Flemming, eds., Springer-Verlag, Berlin, pp. 49--72.
23. A. W. Decho, G. R. Lopez, *Limnol. Oceanogr.*, **38**, 1633-1645 (1990).
24. J. Zhou, K. Mopper, U. Passow, *Limnol. Oceanogr.*, **43**, 1860-1871 (1998).
25. J. Bender, S. Rodriguez-Eaton, U. M. Ekanemesang, P. Phillips, *Appl. Environ. Microbiol.*, **60**, 2311-2315 (1994).
26. D. Fischer, U. G. Schlosser, P. Pohl, *J. Appl. Phycol.*, **9**, 205-213 (1997).
27. I. W. Sutherland "Biofilm Specific Polysaccharides - Do They Exist?" in **The Life and Death of a Biofilm**, J. W. T. Wimpenny, P. Handley P. Gilbert H. M. Lapin-Scott, eds., Bioline Publ., Cardiff, 1995, pp 103-106.
28. I. W. Sutherland, *FEMS Microbiol. Revs.*, **16**, 323-347 (1995).

Received February 8, 2000
Accepted March 22, 2000

Author's Revisions May 7, 2000
Manuscript 7084