Lipop polysaccharide containing L-Acofriose in the Filamentous Blue-Green Alga *Anabaena variabilis*

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For the first time, an O-antigenic lipopolysaccharide (LPS) has been isolated from a filamentous blue-green alga (*Anabaena variabilis*). It was extractable with phenol-water, resulting in extraction of the bulk of the LPS into the phenol phase. The polysaccharide moiety of this LPS consists of L-rhamnose, its 3-O-methyl ether L-acofriose, D-mannose, D-glucose, and D-galactose. L-Glycerol-D-mannoheptose and 2-keto-3-deoxyoctonate, the two characteristic sugar components of enteric LPS, and phosphate groups are absent from the *A. variabilis* O antigen. The only amino sugar present is D-glucosamine. Three hydroxy fatty acids were identified, namely, \(\beta\)-hydroxymyristic, \(\beta\)-hydroxypalmitic and \(\beta\)-hydroxysearic acids, in addition to palmitic and unidentified fatty acid. The LPS of *A. variabilis* is localized in the outermost cell wall layer and behaves like a bacterial O antigen in serological tests. The passive hemagglutination yielded high titers with isolated LPS (pretreated by heat or by alkali) and rabbit antiserum prepared against living or heat-killed cells. The position of the precipitation arcs after immunoelectrophoresis of the O antigen indicates the lack of charged groups. The water phase of the phenol-water extract contains, in high yield, a glucose polymer. It is serologically inactive as shown by the passive hemagglutination test and by agar-gel precipitation.

It has been shown previously (for review see reference 4) that cell walls of *Cyanophyta* (blue-green algae) are more closely related to bacterial cell walls than had been assumed previously (24). There are significant similarities in the chemical composition and morphological organization of the cell walls of these different organisms. Frank et al. (6) demonstrated the presence of murein in isolated cell walls of *Cyanophyta*, a component that formerly was thought to be restricted to bacterial cell walls. This finding, verified by several other investigators (4), explains the susceptibility of some *Cyanophyta* towards penicillin and lysozyme.

Like cell walls of gram-negative bacteria, the cell wall of blue-green algae consists of multiple layers. Recent observations on ultrathin sections of different Oscillatoriaceae and Chroococcaceae (2, 6, 14) and on freeze-etchings of *Anabaena variabilis* (9) show a far reaching similarity between their cell walls and the complex cell walls of gram-negative bacteria. Finally, Weise et al. (40) were able to extract from isolated cell walls of *Anacystis nidulans* high-molecular-weight material which, as judged by its extraction properties and chemical composition, could be considered to be a counterpart of the O antigens (lipopolysaccharides [LPS]) of gram-negative bacteria. This O antigen of *A. nidulans*, a unicellular blue-green alga, has been, to date, the only reported LPS isolated from *Cyanophyta*. Whether other species, especially filamentous strains, also possess similar LPS is of considerable interest from a taxonomic and a phylogenetic point of view.

The present paper describes the isolation and the chemical and serological characterization of the O antigen of the filamentous blue-green alga *A. variabilis* (*Hormogonales*). It will be shown that this LPS carries O-specific groups similar to bacterial O antigens, but that it differs considerably in its chemical composition and extraction properties from the well-known enterobacterial O antigens.

**MATERIALS AND METHODS**

**Organism.** The strain used was obtained from N. G. Carr, Liverpool, and was derived from the *A. variabilis* (Kützing) strain described by Kratz and Myers (17). It was cultivated in a modified Jüttnner medium (15) containing: NaNO₃, 1.6 g; K₂HPO₄, 80 mg; MgSO₄·7 H₂O, 80 mg; CaCl₂·2 H₂O, 60 mg; ethylenediaminetetraacetic acid-iron (III)-sodium salt (Fluka A.G. Buchs, Switzerland), 6 mg; K₂CO₃, 10 mg; H₂BO₃, 0.6 mg; MnCl₂·4H₂O, 0.6 mg;

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ZnSO₄·7H₂O, 0.01 mg; Na₂MoO₄·2H₂O, 0.02 mg; CuSO₄·5H₂O, 0.02 mg; Co(NO₃)₂·6H₂O, 0.01 mg; and glass-distilled water, 1,000 ml. The pH was adjusted to 7.5. Mass cultures were prepared in a 10-liter Microferm MF-114 (New Brunswick Sci. Corp. Inc.). A light unit of fluorescent tubes was set up in front of the culture vessel (ca. 5,000 lx on the surface of the Microferm vessel). A mixture of air and CO₂ (98:2) was bubbled continuously through the culture. The temperature was adjusted to 32°C. The strain does not fix nitrogen, and grows in turbulent, stirred cultures as very short filaments (four to eight cells). In agar cultures, it forms long filaments of 30 to 50 cells. Heterocysts were not observed.

**Isolation of LPS.** Lyophilized mass cultures were extracted by the hot phenol-water method of Westphal et al. (41). The water and phenol phases were freed from phenol by extensive dialysis against tap water and finally against distilled water. After removal of insoluble material by centrifugation (2,500 x g, 30 min), the extracts were freed from low-molecular-weight material by ultracentrifugation (three times at 105,000 x g for each run). Extraction of *A. variabilis* with liquid phenol-chloroform-petroleum ether was performed as described by Galanos et al. (7).

**Sugar analysis.** Neutral sugars were liberated by hydrolysis with 1 N sulfuric acid at 100°C for 4 h. The hydrolysates were neutralized with a saturated aqueous solution of Ba(OH)₂. Sugars were separated by descending paper chromatography (Whatman no. 1) with the solvent system pyridine-butan-1-ol-water (4:6:3, vol/vol/vol) and were stained with silver nitrate-NaOH (34) or with anilinium hydroxide phthalate (30).

For gas-liquid chromatography, the LPS was hydrolyzed in 0.1 N HCl at 100°C for 48 h. The aldoses of the neutralized hydrolysates (ion-exchanger IRA 410, HCO₃⁻) were converted into alditol acetates by the method of Sawardeker et al. (31). A Varian aerograph (model 1520 B) fitted with a glass column (0.32 by 152 cm), filled with ECNSS-M (3% on Gas-Chrom Q, 100 to 120 mesh) at a column temperature of 165°C, and at a nitrogen flow rate of 28 ml/min was used. Mass spectra of the isolated O-methyl sugar were determined, at 70 eV, in a Perkin-Elmer mass spectrometer (model 270B) fitted with a Honeywell Visicorder (model 3508).

Conditions employed in determining the presence or absence of 2-keto-3-deoxyoctonate (KDO) were hydrolysis of the LPS with 0.1 N sulfuric acid at 100°C for 20 min and reaction with thiobarbituric acid reagent by the method of Heath and Ghalambor (10). Amino sugars were liberated by hydrolysis in 6 N HCl at 100°C for 16 h. After removal of HCl by repeated evaporation in vacuo in the presence of KOH in a desiccator, amino sugars were identified by high-voltage paper electrophoresis in the vertical chamber system described by Kickhöfen and Warth (16), using a pyridine-acetic acid-water (10:4:86, vol/vol/vol) buffer (pH 5.3). An amino acid analyzer (Beckman, Type 120 B) packed with Custom Research Resin (type AA-15) was used for the quantitation of glucosamine.

**Amino acids and phosphorus.** Amino acids, liberated by hydrolysis in 6 N HCl at 100°C for 16 h, were freed from HCl and identified in an amino acid analyzer as described above. Phosphorus was determined by the method of Lowry et al. (19).

**Fatty acid analysis.** Fatty acids were liberated by hydrolysis in 4 N HCl at 100°C for 6 h, extracted from the hydrolysate with petroleum ether (40 to 60°C), weighed, and esterified with 2 N methanolic HCl at 100°C for 2 h. For identification, a Perkin-Elmer gas chromatograph (model F20) fitted with a Castor wax (2.5% on Chromosorb G, 80 to 100 mesh) stainless-steel column (0.32 by 152 cm) at a column temperature of 170°C and a nitrogen flow of 25 ml/min, or alternatively an EGSS-X (15% on Gaschrom P, 100 to 120 mesh) stainless-steel column (0.32 by 150 cm) at a column temperature of 165°C and a nitrogen flow of 30 ml/min, was used. Mass spectrometry was carried out as described above, using a coupled gas chromatography-mass spectrometry system.

**Hydroxylaminolysis.** Ester-bound fatty acids were transformed into ethanol-soluble hydroxamic acids by the method of Snyder and Stephens (33). The LPS residue with its presumable amide-bound fatty acids became insoluble in ethanol and was precipitated by centrifugation (1,450 x g, 30 min). After the supernatant fluid was decanted, hydroxamic acids were hydrolyzed by addition of 6 N HCl to the supernatant fluid (pH 2.0) and heating at 100°C for 2 h. The amide-bound fatty acids in the sediment were split off from glucosamine in alkali (4 N NaOH, 100°C, 5 h). The identification of amide- and ester-bound fatty acids was achieved by gas chromatographic analysis of the fatty acid methyl esters (see above).

**Serological tests.** Antisera were prepared by intravenously immunizing albino rabbits, three times at 4-day intervals, with increasing amounts (0.25, 0.5, and 1.0 ml) of *A. variabilis* cell suspensions containing 10⁶ cells/ml in 0.9% saline. The immunization was carried out either with living or with heat-killed cells (100°C, 2.5 h). The rabbits were bled 5 days after the last injection. For passive hemagglutination tests, human erythrocytes (blood group A) were sensitized with untreated, heat-treated (100°C, 60 min), or alkali-treated (0.25 N NaOH, 56°C, 60 min [27]) LPS samples. Serial dilutions of antisera were prepared with phosphate-buffered saline (pH 7.2) by using the Metrimpex microtitrator system (Metrimpex type OX-603). Fifty-microliter amounts of the serum dilutions were mixed with equal amounts of LPS-sensitized erythrocyte suspensions (25 μlitters of packed cells, suspended in 5 ml of saline). The titers were read after a 60-min incubation of the plates at 37°C. The reciprocal serum dilution of the last dilution cups showing complete agglutination were recorded as hemagglutination titers. Agar-gel immunoelectrophoresis was carried out, by the method of Scheidegger (32), using an electrophoresis chamber from the Gelman Instrument Company (model 51170-1) and a diethyl-barbituric acid–sodium Veronal–calcium lactate buffer (12) at 10 V/cm for 1 h. Agar-gel precipitation was performed as described by Ouchterlony (29). Concanavalin A was obtained from Miles Yeda Ltd., Rehovoth, Israel.
Incubation with α- and β-amylase. Incubation of the water-phase material with α-amylase from bacteria or with β-amylase from barley (both from Fluka A.G., Buchs, Switzerland) was carried out as described by Mayer et al. (23). The incubation mixture was examined directly by paper chromatography to detect any release of maltose (staining with AgNO₃-NaOH [34]). Bacterial glycogen from Escherichia coli B was used as a positive control.

RESULTS

High-molecular-weight material (LPS) from the phenol phase of phenol-water extracts: (i) isolation. The hot phenol-water procedure is generally adopted for isolation of O antigens of gram-negative bacteria. By this method, a high-molecular-weight material was extracted from A. variabilis cells into the phenol phase. The yield was about 1% of the cell dry weight. To remove possible contamination by phospholipids and other lipophilic materials that would also be extracted into the phenol phase, the high-molecular-weight fraction was washed extensively with chloroform-methanol (2:1, vol/vol). In spite of its lipophilic character, we were not able to extract this material with phenol-chloroform-petroleum ether, a method recently found to be highly suitable for extracting lipophilic glycolipids (R-LPS) of enterobacterial R-mutant strains (7). The high-molecular-weight material isolated from A. variabilis represents a LPS by chemical criteria and an O antigen by its serological activity (see below).

(ii) Analyses for neutral sugars. The neutral sugar fraction obtained by acid hydrolysis (1 N H₂SO₄, 100°C, 4 h) of the LPS was analyzed by paper chromatography and by gas-liquid chromatography after the sugars were converted into their respective alditol acetates. Rhamnose and glucose were present in large amounts; mannose and galactose, however, were present in lower percentages (Table 1). The optical rotation values of the isolated sugars, separated by preparative paper chromatography, revealed a D-configuration for mannose, glucose, and galactose, and an L-configuration for rhamnose. It is noteworthy that L-glycero-D-mannoheptose, an ubiquitous constituent of enterobacterial O antigens, is absent from the LPS of A. variabilis.

An additional neutral sugar, characterized by its high Rf value in pyridine–butan-1-ol–water (Rf Rhamnose = 1.24), amounted to 18.4% of the LPS dry weight. It was recognized by its intensive brown staining with anilinium hydrogen phthalate and by its weak reactivity with AgNO₃-NaOH. The sugar could easily be obtained in a pure state due to its high chromatographic mobility. For mass spectrometry, its deuterium-labeled alditol acetate was prepared by using NaBH₄ in deuterium oxide for the reduction. Fragmentation at 70 eV gave the following main fragments: m/e 43, 88, 101, 143, 190, and 203, and a fragment with lower intensity (about 1% of the base peak at m/e 43) at m/e 290. These fragments are typical for the fragmentation of 3-O-methyl-ethers of 6-deoxyhexoses (38). The parental 6-deoxy-hexose, obtained by demethylation of the O-methyl sugar under investigation, was identified as rhamnose by paper and gas-liquid chromatography. Authentic 3-O-methyl-L-rhamnose showed the same properties in mass spectrometry and in chromatography. It gave a single peak in gas-liquid chromatography after its alditol acetate was mixed with that of the unknown sugar on an ECNSS column. The optical rotation of L-acofriose was reported by Muhr et al. (26) to be [α]D + 39.1°, whereas that of D-acofriose was described by Morrison et al. (25) to be [α]D -27°. The definitive positive optical rotation of the sugar under investigation proved its L-configuration. The O-methyl sugar of the LPS of A. variabilis was, therefore, identified as the 3-O-methyl-ether of L-rhamnose (L-acofriose).

(iii) Analyses for amino sugars and KDO. Separation of acid hydrolysates (6 N HCl, 100°C, 16 h) of the A. variabilis LPS on an amino acid analyzer gave only a glucosamine peak, and no further amino sugars could be present.
traced. The same result was obtained when the hydrolysate was separated by high-voltage paper electrophoresis at pH 5.3.

The assay for KDO in a mild hydrolysate (0.1 N H₂SO₄, 100 C, 20 min) of the LPS was negative: no color was obtained with the thiobarbituric acid reagent, indicating that KDO was absent from the LPS of A. variabilis. Preliminary observations concerning the isolation of the lipid A moiety of A. variabilis by mild acid hydrolysis showed that it could not be split off from the LPS by hydrolytic conditions, usually applied for the isolation of lipid A of gram-negative bacteria (21). This is very probably due to the lack of KDO in the A. variabilis O antigen, which forms the acid-labile linkage between lipid A and the polysaccharide moiety of enterobacterial O antigens.

(iv) Analyses for fatty acids. The material for fatty acid analysis, obtained from acid hydrolysates (4 N HCl, 100 C, 6 h) by extraction with petroleum ether, corresponded to 10.7% (dry weight) of the LPS. Gas-liquid chromatography and mass spectrometry of the fatty acids converted into their methyl ester derivatives allowed identification of three hydroxy fatty acids: \( \beta \)-hydroxy myristic acid (the main fatty acid), \( \beta \)-hydroxy palmitic acid, and \( \beta \)-hydroxy stearic acid. Also detected were palmitic acid and an unidentified fatty acid with \( T_n = 5.6 \) (palmitic acid = 1) on an EGSS-X column. The type of bonding by which individual fatty acids are bound to glucosamine was estimated by hydroxymalolysis. The three different \( \beta \)-hydroxy fatty acids are bound to the amino group of the glucosamine, whereas palmitic acid is ester bound.

(v) Analyses for phosphorus and amino acids. The LPS of A. variabilis is almost completely free from phosphorus (0.03% [dry weight] of the LPS) (Table 1). The total amount of amino acids did not exceed 8.4%, and analysis on an amino acid analyzer showed none to be predominate. No diaminopimelic acid was detected. These results indicate that the protein contained in the LPS sample represents a contaminant in the phenol-soluble LPS, rather than a specific peptide moiety.

(vi) Serological studies. Antisera against heat-killed or living A. variabilis cells reacted equally strongly in the passive hemagglutination test with erythrocytes coated with the isolated A. variabilis LPS (Table 2). It was necessary, however, to pretreat the O antigen either by alkali (0.25 N NaOH, 56 C, 60 min) or by heat (100 C, 60 min) to obtain an effective coating of human erythrocytes. Both methods of sensitization gave essentially the same results.

(It should be mentioned that most of the enteric O antigens show properties identical to those mentioned above.) Agar-gel precipitation studies showed that no differences exist between antisera obtained with heat-killed and living cells, indicating the heat stability of this O antigen. In immunoelectrophoresis with the alkali-treated LPS (pH 8.6, 10 V/cm, 60 min), the precipitation arcs were formed around the antigen well, demonstrating that charged groups are virtually absent in the A. variabilis O antigen.

L-Acofriose, one of the major sugar constituents of the A. variabilis O antigen, was detected earlier in the LPS of the photosynthetic bacterium *Rhodopseudomonas capsulata* 37b4 (38). The O antigens of both organisms were tested for serological cross-reactivity, since it is known that lipophilic sugars, such as dideoxyhexoses and O-methyl sugars, very often occupy the nonreducing terminal positions in O antigens and, therefore, are of special importance to the serological specificity of the respective O antigen. No cross-reactions were observed, however, in the passive hemagglutination test, neither by exposing erythrocytes coated with A. variabilis LPS to *R. capsulata* antisemum, nor vice versa.

**Macromolecular material (glycogen) from the water phase of phenol-water extracts.** Macromolecular material different from that of the phenol phase could be isolated from the water phase after extraction of the cells with phenol-water. It amounted to about 6% of the cell dry mass. Qualitative and quantitative gas-liquid chromatography of acid hydrolysates and optical rotation measurements showed that the polymer consists exclusively of d-glucose (about 100% [dry weight] of the material). Fatty

![Table 2](http://jb.asm.org/)

<table>
<thead>
<tr>
<th>Serum no.</th>
<th>LPS, untreated</th>
<th>LPS, heat-treated (100 C, 60 min)</th>
<th>LPS, alkali-treated (0.25 N NaOH, 56 C, 60 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>119*</td>
<td>&lt;10</td>
<td>2,560</td>
<td>2,560</td>
</tr>
<tr>
<td>120b</td>
<td>&lt;10</td>
<td>5,120</td>
<td>2,560</td>
</tr>
<tr>
<td>737*</td>
<td>&lt;40</td>
<td>1,280</td>
<td>2,560</td>
</tr>
<tr>
<td>784*</td>
<td>&lt;80</td>
<td>2,560</td>
<td>2,560</td>
</tr>
</tbody>
</table>

* Serum prepared with heat-killed cells.
* Serum prepared with living cells.
acids were present only in very small amounts (<0.3%). The polymer was inactive in passive hemagglutination and in agar-gel precipitation tests against antisera obtained with heat-killed and living A. variabilis cells. A very strong reaction was observed, however, in agar-gel precipitation with concanavalin A (22), a lectin that is known to react with polysaccharides carrying α-glycoside-linked terminal units of glucose, mannose, or N-acetylgalcosamine (8). With immunoelectrophoresis with concanavalin A, no migration of the water-phase material was observed, indicating the absence of charged groups in the glucan. Treatment of the polymer with α- or β-amylase resulted in a high yield of maltose in addition to a number of oligosaccharides. The same result was obtained with bacterial glycogen from E. coli B.

**DISCUSSION**

The present paper described the detection of a LPS with O antigenic activity in the filamentous blue-green alga A. variabilis. In spite of several striking similarities to enterobacterial LPS (i.e., extractability with phenol-water, presence of amide-linked β-hydroxy fatty acids), the sugar and fatty acid composition deviates remarkably from that of enterobacterial O antigens. L-Rhamnose, its 3-O-methyl ether (L-acofriose), D-mannose, D-glucose, and D-galactose are the neutral sugars of the A. variabilis O antigen, and D-glucosamin its only amino sugar.

It is interesting that the two sugars L-glycero-D-mannoheptose and KDO, both components of the enterobacterial R core region, are absent in the A. variabilis LPS. Either this LPS is devoid of an R core or it is built up by other constituents. It would be particularly interesting to know which sugar forms the linkage between the lipid moiety and the polysaccharide part, since KDO is absent here. The only hitherto described LPS of Cyanophyta, originating from A. nidulans, is also free from heptose, but it does still contain KDO (40). The same is true for the O antigens of Xanthomonas campestris (11, 35), Rhodopseudomonas capsulata 37b4 (36) and R. viridis F (39). In contrast the LPS of Neisseria catarrhalis (1), Bacteroides fragilis and B. melaninogenicus (13) are free from both heptose and KDO. O-methyl sugars, like L-acofriose, are rare constituents in O antigens of gram-negative bacteria. In photosynthetic bacteria of the family Rhodospirillaceae, O-methyl sugars were however encountered frequently (37–39). It is noteworthy, that A. variabilis represents another photosynthetic prokaryote in which the O antigen contains an O-methyl sugar. Since this sugar represents 18.4% of the LPS dry weight, it seems to be present in every repeating unit of the polysaccharide. Other LPS usually possess O-methyl sugar constituents in only minor amounts. R. capsulata 37b4, for example, contains only 1% L-acofriose and 15% L-rhamnose, indicating that not all repeating units of the O-specific chain contain the O-methyl sugar (36). Similar observations were made by Björndal et al. (3) with the O antigen of Klebsiella K73:010. A thorough investigation of this LPS by a methylation study showed that L-acofriose is restricted to the terminal repeating unit.

Phosphate is not present in the LPS of A. variabilis, indicating that the lipid A moiety of A. variabilis differs considerably from enteric lipid A. Phosphate, as an integral part of the enteric LPS provides a bridging between two glucosamine-disaccharide units in the lipid A (20). β-Hydroxyymristic acid, the main fatty acid of enteric O antigens, also represents the main fatty acid of the A. variabilis LPS, but the other fatty acids do not correspond to those usually found in lipid A.

The occurrence of three amide-linked β-hydroxy fatty acids in a procaryotic O antigen is unusual. In addition, this composition is of special interest, since the fatty acid analyses of blue-green algae that are presently available were usually carried out with whole cells, and they predominantly demonstrate the presence of saturated, mono-, di- or tri-unsaturated long-chain fatty acids, but not the presence of hydroxy fatty acids (for summary see reference 28).

All of these data demonstrate that the O antigens of Cyanaphyta, as well as of some gram-negative bacteria, do not strictly follow the general structural concept of enterobacterial O antigens.

The LPS of A. variabilis carries O specificity. It behaves like a bacterial O antigen in passive hemagglutination and agar-gel-precipitation, but does not migrate upon immunoelectrophoresis, substantiating the absence of phosphate and KDO observed by chemical analysis. The LPS seems to be localized in the outermost layer of the multiply layered cell wall, and it is obviously not covered by any capsular or slime antigens, since similar titers were observed when using antisera against either heat-killed or living cells.

Like other blue-green algae, the investigated strain of A. variabilis is gram positive. However, the fine structure and the chemical composition
of the cell walls of blue-green algae so far investigated clearly demonstrate the gram-negative character of these cell walls.

The high-molecular-weight material from the water phase of the phenol-water extracts is closely related to bacterial glycogen. This material has been described as a typical reserve component in some Cyanophyta (cells for summary see reference 5). The polymer is serologically inactive and does not seem to be part of the cell wall.

The present paper shows that LPS comparable to the O antigens of gram-negative bacteria exist not only in unicellular species (A. nidulans), but also in the filamentous Cyanophyta. It should be pointed out, however, that up to now investigations have been restricted to only one representative of each group, so that the results cannot be generalized. It would, therefore, be highly desirable to obtain more precise knowledge on the O antigens and other cell wall constituents from other strains of these organisms.

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LITERATURE CITED