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Identification and Analysis of a Lipopolysaccharide in Cell Walls of the Blue-Green Alga *Anacystis nidulans*

G. Weise and G. Drews

Institut für Biologie II der Universität Freiburg i. Br.
Lehrstuhl für Mikrobiologie

B. Jann and K. Jann

Max-Planck-Institut für Immunbiologie, Freiburg i. Br.

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Summary. A lipopolysaccharide was isolated from cell walls of *Anacystis nidulans* by extraction with 45% aqueous phenol at 65°, and further purified by repeated high speed centrifugation. It contains 30—40% of lipid and about 60% of carbohydrate components. The carbohydrate moiety contains predominantly mannose and smaller amounts of galactose, glucose, fucose, rhamnose, 2-keto-3-deoxyoctonate, glucosamine and a second aminosugar. The latter was identified as a 2-amino-2-deoxyheptose with the gluco-configuration from C3 to C7. The lipid moiety contains glucosamine and fatty acids (C22:0, C18:2, C16:0, C12:0 and C14:βOH). The lipopolysaccharide has a very low phosphate content and does not contain heptose. It shows low pyrogenicity in rabbits and it is not toxic in mice.

The type of cell organization which is represented by blue-green algae *(Cyanophyceae)* and bacteria is known as the procaryotic cell (Stanier and van Niel, 1962). Both groups of organisms have many structural and chemical features in common. In the eucaryotic cell, however, the nuclear region, the photosynthetic apparatus, and the membranes responsible for respiration are not separated from the cytoplasm by membranes. The cell walls of nearly all bacteria consist of macromolecular components such as murein, lipopolysaccharide and teichoic acid, which have never been found in cell walls of eucaryotic cells. Recent investigations have revealed that murein (glycopeptide) is a structural component, also of the blue-green algal cell wall (Frank *et al.*, 1962; Drews and Meyer, 1964; Höcht *et al.*, 1965).

In the outer layers of *Anacystis* carbohydrates lipids and peptides were found. (Höcht *et al.*, 1965; Drews and Gollwitzer, 1965.) However,

Abbreviations: KDO = 2-Keto-3-deoxy-octonate; LPS = Lipopolysaccharide.

the composition and macromolecular organization of these compounds is unknown. In the present investigation we were able to demonstrate a lipopolysaccharide in the cell wall of *Anacystis*. In spite of some similarities to the lipopolysaccharides of enteric bacteria there are marked differences in composition and physiological features.

Materials and Methods

Cultivation of the Organism. Stock cultures of *Anacystis nidulans (Lauterbornia nidulans)*, which were obtained from the Algensammlung der Universität Göttingen, were maintained on agar slants. The organism was grown in the medium of Kratz and Myers (1955). Mass cultures were prepared in an 10 L-Microferm MF-114 of New Brunswick Sci. Corp. Inc. fitted out with fluorescent tubes. A second light unit was set up in front of the culture vessel. The cells were cultivated at 35°. The light intensity on the surface of the fermenter vessel was 5000 Lux. A mixture of air and CO_2 (98:2) was bubbled continously through the culture. The vessel was inoculated with 500 ml of an Erlenmeyer flask preculture.

Preparation of Cell Walls. The cells were harvested by centrifugation at the end of the logarithmic growth phase. The suspension of washed cells was cooled to 0° and shaken for periods of 15 sec with ballotini beads (diameter 0.1 mm) in a Braun-Homogenizer (B. Braun, Melsungen, Germany). After removal of the beads by filtration, the homogenized cell material was centrifuged 10 min at 1200×g. The supernatant was discarded and the loosely packed layer above the pellet was isolated. This represents the cell walls. The still intact cells, which were located in the pellet, were disintegrated again. The cell walls were washed and freeze dried.

Isolation and Purification of the Lipopolysaccharide. Cell wall material or whole cells were extracted for 10 min with 45% aqueous phenol at 65—68° C according to Westphal *et al.* (1965). The aqueous phase was dialysed against distilled water, concentrated in vacuo and centrifuged at 3000 RPM for 15 min. The supernatant was freeze dried. An aqueous solution (1—2%) of the crude extract was further purified by repeated centrifugation at 105,000×g for 2 hrs. The final sediment represents the purified LPS.

Analytic Procedures. For the qualitative analyses of sugar constituents the following methods were used.

For the detection of hexoses and deoxyhexoses the LPS (20 mg) was hydrolyzed in 2 ml of N sulfuric acid for 4 hrs at 100° C. The hydrolysates were neutralized with bariumhydroxyde, centrifuged and evaporated *in vacuo*. The liberated sugars were identified on paper chromatograms by comparison with authentic reference substances. Generally, butanol/pyridine/water (6:4:3) was used as solvent. For the differentiation of 6-deoxyhexoses the chromatographic procedure described by Kraus *et al.* (1960) was applied. Sugar spots were detected by spraying the chromatograms with anilinephthalate (Partridge, 1949).

Aminosugars were liberated by hydrolysis of the LPS with 6 N HCl for 15 hrs at 100° C. HCl was removed by repeated evaporation under reduced pressure. For identification, paper chromatography in pyridine/butanol/water (6:4:3) and high voltage electrophoresis (40 volts/cm; pyridine acetate, pH 5.3) was used. The aminosugars were stained with ninhydrin, the Elson-Morgan reagent (Partridge, 1948) and silvernitrate (Trevelyan *et al.*, 1950). Further identification was achieved by ninhydrin degradation (Stoffyn and Jeanloz 1954) and paper chromatography of the neutral sugars formed. Preparative separation of the aminosugars was achieved by chromatography on columns of Dowex 50 H^+.

Detection of 2-keto-3-deoxy octonic acid (KDO) was carried out by paper electrophoresis of neutralized hydrolysates (0.1 N H_2SO_4, 10 min/100° C) of the LPS. The electropherograms were sprayed with periodate/thiobarbiturate reagent (Warren, 1960).

Fatty acid esters were hydrolyzed with 3 N HCl for 12 hrs at 100° C. The fatty acids were extracted from the hydrolysates with ether and analyzed by paper chromatography (Novotny *et al.*, 1958) and gas liquid chromatography.

Quantitative determination of total neutral sugars was carried out with the anthron method (Shields *et al.*, 1960). Glucose was determined with glucose oxidase (Boehringer & Soehne, Mannheim/Germany), galactose with galactose oxidase (Worthington, Freehold, N.Y./U.S.A.) in the modification of Fischer and Zapf (1964) and mannose was determined by comparative paper chromatography according to Wilson (1959). 6-Deoxyhexoses were determined by the cysteine sulfuric acid method and 2-keto-3-deoxy octonate according to Ghalambor *et al.*, (1966). Determination of aminosugars was carried out using the method of Strominger *et al.* (1959). Specific determinations of D-glucosamine were carried out enzymatically with the aid of yeast acetylase as described by Lüderitz *et al.* (1964). Phosphorus was determined by the method of Lowry *et al.* (1954).

Results

The Fine Structure of the Cell Wall. The cell wall of *Anacystis nidulans* is a multilayered structure about 24 nm in thickness (Fig. 1). Immediately adjacent to the cytoplasmic membrane (*CM*; Fig.1) is an electron-transparent space which is the L_I layer in the terminology of Jost (1965), (Allen, 1968). The basal layer of the cell wall (L_{II}, Allen, 1968) is a relatively thick and electron-opaque layer (Fig.1, *M*). Chemical and electron

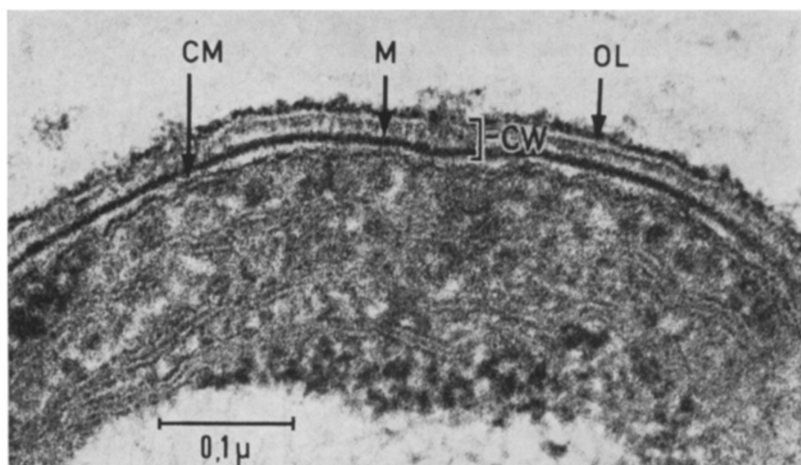

Fig. 1. Ultrastructure of *Anacystis nidulans*. [Fixation: Kellenberger *et al.*: J. biophys. biochem. Cytol. 4, 671 (1958); staining: uranylacetate, lead hydroxide.] *CM* cytoplasmic membrane; *M* murein, rigid layer (glycopeptide); *OL* over layer; *CW* cell wall. ×180,000. Preparation and micrograph: Rita Ladwig

microscopical investigations on gram-negative bacteria and blue-green algae have revealed that this layer is composed of murein (Frank *et al.*, 1962; Murray *et al.*, 1965; Frank and Dekegel, 1965; de Petris, 1967; Guysen, 1968; Lamont, 1969). Even in the basal rigid layer of *Anacystis nidulans* the typical components of murein were found (Drews and Meyer, 1964). The murein sacculus is covered with various layers of more electron-transparent material. These layers are not sharply limited. Micrographs of other blue-green algae show an unit membrane-like layer in this position (Frank *et al.*, 1962; Lamont, 1969). On the outside of the cell envelope is a thin irregular electron-dense overlayer which is probably mucilage (Fig.1). An extensive mucilaginous sheath is not seen in Fig. 1 (see Allen, 1968; Lamont, 1969).

Isolation

The LPS was obtained by extraction of either isolated cell walls or whole cells with 45% aqueous phenol. The material, which was obtained from the aqueous phase after dialysis and lyophilization, was subjected to high speed centrifugation ($105{,}000 \times g$). The LPS, which was obtained as an opalescent pellet, was further purified by two additional ultracentrifugations. The final sediment (LPS) was taken up in a minimum amount of water and lyophilized. The yield of LPS which was obtained from isolated cell walls was much lower (0.7—0.8%) than that after extraction of whole cells (2—3%). This can easily be explained by extensive loss of surface material in the course of the preparation of cell walls. There was no difference in chemical composition between LPS preparations which had been isolated from cell walls and from whole cells, respectively. Therefore whole cells were used for most extractions.

Analysis

Treatment of the LPS with mild acid (1% acetic acid/100°/60 min) results in the precipitation of a material which is insoluble in water, but soluble in pyridine and chloroform. Further hydrolysis of this material (6N HCl 100°/18 hrs) liberates glucosamine (6.5%) and fatty acids. For identification the latter were subjected to paper chromatography according to Novotny *et al.* (1958) and, after esterification with diazomethane, to gas liquid chromatography. The following fatty acids were found: behenic acid (C 22); stearic acid (C 18); oleic acid (C 18 =); palmitic acid (C 16); β-hydroxymyristic acid (C 14 OH); lauric acid (C 12).

From these results it is evident that the water insoluble material represents the lipid component of the LPS. Its phosphorus content is very low ($< 1\%$).

After acid hydrolysis of the LPS (1 N sulfuric acid/100°/3 hrs) the neutralized hydrolysate was subjected to thin layer chromatography in butanol/pyridine/water (4 : 6 : 3). Thus, the component sugars mannose, galactose, fucose, rhamnose, and glucosamine were detected.

When the neutralized hydrolyzate was subjected to paper electrophoresis (in pyridine acetate of pH 5.4/40 volts/cm) a component was detected, in addition to glucosamine, which could be stained with alcaline silvernitrate, ninhydrin and with the Elson-Morgan spray reagent. From the staining properties it can be concluded that this is a 2-amino-2-deoxysugar. The substance has an electrophoretic mobility of $M = 0.85$ (relative to glucosamine). Glucosamine and the unknown aminosugar were separated on columns (1.2 cm × 20 cm) of Dowex 50 H^+. Neutral sugars were eluted by percolation with water and the aminosugars then separated by elution with 0.5 N HCl. For further purification the aminosugar was subjected to preparative paper electrophoresis. The isolated aminosugar was then oxidatively degraded with ninhydrin (Stoffyn and Jeanloz, 1954). The reaction product, which was homogeneous on paper chromatography in pyridine/butanol/water (4 : 6 : 3) had a mobility identical to that of glucose. Identity with D-glucose was confirmed by a positive reaction with D-glucose oxidase. These results show that the aminosugar is a 2-amino-2-deoxy heptose which has the D-gluco configuration from C3—C7.

When the LPS was hydrolysed under mild conditions (0.1 N H_2SO_4, 100°/30 min) 2-keto-3-deoxy-octonic acid (KDO) could be identified on paper electropherograms, after staining with periodate/thiobarbiturate (Warren, 1960).

Results of the quantitative analyses are given in the Table.

Fucose and rhamnose were not determined separately; both were assayed together with cystein-sulfuric acid. Since there is no specific assay

Table. *Quantitative sugar composition of the lipopolysaccharide from Anacystis nidulans*

Sugar constituent	Amount % of total weight
Galactose	5.3
Glucose	3.4
Mannose	33.0
Fucose + Rhamnose	2.7
Glucosamine	6.4
2-Amino-2-deoxyheptose	0.7
2-Keto-3-deoxyoctonate	1.5

[a] Represents a minimal value.

for 2-amino-heptose, this sugar constituent was estimated by subtracting the value found for glucosamine in the enzymatic assay (Lüderitz *et al.*, 1964) from the total aminosugar content (Strominger *et al.*, 1959). The value thus obtained is rather low, which is probably due to a low color yield of the amino heptose in the aminosugar assay.

Studies on the Lipopolysaccharide

Aqueous solutions of the LPS are opalescent. During centrifugation the LPS sediments within a short time at 40,000–60,000 $\times$g. This is indicative of either aggregation or of a very high molecular weight. Aggregates of amphipathic substances and those composed of subunits (Stellwagen and Schachmann, 1962) may be dissociated with the aid of detergents. The LPS of *Anacystis nidulans* gave clear solutions in Tris buffer (0.1 M, pH 8), which was 1% with respect to sodium dodecylsulfate (SDS). When such a solution was passed through Sephadex G75 in the presence of SDS one sharp peak of carbohydrate containing material was obtained. This was eluted (Tris buffer pH 8.1; 1% SDS) within the exclusion volume of the column. This indicated a rather low molecular weight of the LPS in sodiumdodecylsulfate containing solutions. After removal of the sodiumdodecylsulfate by dialysis against Tris buffer (0.1 M, pH 8) the LPS was excluded by Sephadex G75 but could be eluted from Sephadex G100. Its solution in Tris buffer

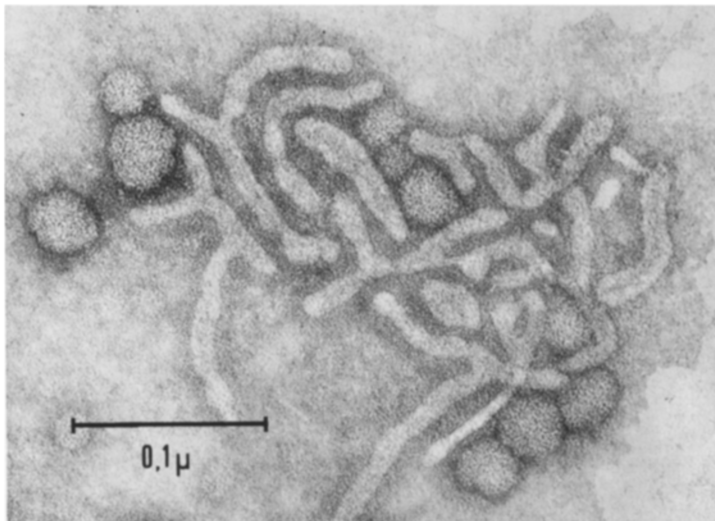

Fig. 2. Lipopolysaccharide of *Anacystis nidulans* (Staining: uranylacetate). $\times$250,000. Micrograph: Elisabeth Freund-Mölbert

(0.1 M; pH 8) sedimented slowly in the ultracentrifuge as a distinct peak at 261,000 × g. After dialysis against distilled water and treatment with aqueous phenol the LPS showed the same physical properties as did the untreated one. These observations show that the LPS of *Anacystis nidulans*, when isolated with 45% aqueous phenol, forms large aggregates (see Fig. 2). This phenomenon which was first described by Oroszlaw and Mora (1963), and extensively investigated by Ribi *et al.* (1966), is typical for LPS.

Biological Studies

The lipid moiety of the *Anacystis nidulans* LPS differed in some properties from the one encountered in enteric bacteria. It has a rather low glucosamine content and very little phosphate. Therefore, the biological properties of the *Anacystis nidulans* LPS were studied in comparison with the LPS (endotoxin) of *E. coli.*

Pyrogenicity. This study was carried out on rabbits as described by Eichenberger *et al.* (1955). The *Anacystis nidulans* LPS exerted a pyrogenic property which was tenfold less than that of the *E. coli* lipopolysaccharide. The biphasic character of the fever curve was not very pronounced.

Toxicity. Groups of 6 mice were injected with 4 different concentrations of the *Anacystis nidulans* LPS (50—200 μg/animal). Control injections were performed—as with the pyrogenicity tests—with saline. For comparison the same type of experiment was carried out using the *E. coli* LPS. In contrast to the known toxic effect of the *E. coli* LPS, the LPS from *Anacystis nidulans* did not elicit any toxic effect. Even with the highest concentrations, which in case of the *E. coli* LPS proved to be lethal within 2 days, no toxic effect could be observed on any animal within one week.

Discussion

From *Anacystis nidulans* a lipopolysaccharide was obtained, which could be extracted not only from whole cells but also from isolated cell walls. This indicates that the LPS is located on the cell surface. It is composed of 30—40% of lipid and about 60% of carbohydrate material. The lipid moiety contains a number of long chain fatty acids, which include β-hydroxy-myristic acid. No exhaustive chemical analysis of the lipid material was performed, so that further components may be detected on more detailed investigations. The carbohydrate moiety consists of mannose, glucose, galactose, fucose, rhamnose, 2-keto-3-deoxy octonic acid, glucosamine and a second aminosugar, which was tentatively identified as 2-amino-2-deoxy-heptose (with D-gluco configuration on

C3—C7). The LPS dissolved in water to give opalescent, colloidal dispersions. These can be clarified by detergents with concomittant disaggregation of LPS micelles. This characteristic is also found with LPS from enteric bacteria. The *Anacystis nidulans* LPS has further similarities to the LPS of enteric bacteria such as the components KDO and β-hydroxymyristic acid. On the other hand there are also marked differences in composition; in contrast to the enteric LPS the material described here is devoid of heptose, contains very little phosphate and has a rather low glucosamine content in its lipid moiety. For this reason the comparison between the LPS from enteric bacteria (*E. coli* as representative) and *Anacystis nidulans* were extended to studies of biological properties. It was found that whereas the LPS from *E. coli* was both pyrogenic (in rabbits) and toxic (in mice) the one from *Anacystis nidulans* showed neither of these properties. Since the term "lipopolysaccharide" is defined merely on chemical and physical grounds, it is obvious that the surface material from *Anacystis nidulans* has to be classified as such. However, it has to be pointed out that, mainly on biological grounds but also with respect to typical components, the *Anacystis nidulans* LPS differs greatly from the LPS which were isolated from enteric bacteria and which have been termed endotoxins. The occurrence of this class of substances on algal surface is of interest from biological and taxonomic view points. More definite considerations would be speculative at this time and have to await further investigations.

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Prof. Dr. Gerhart Drews
Lehrstuhl für Mikrobiologie
D-7800 Freiburg i. Br., Schaenzlestr. 9—11

Drs. Klaus u. Barbara Jann
Max-Planck-Institut für Immunbiologie
D-7800 Freiburg-Zähringen, Stübeweg 51