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Carotenoid-Containing Outer Membrane of *Synechocystis* sp. Strain PCC6714

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Outer membranes, free of cytoplasmic or thylakoid membranes and peptidoglycan components, were obtained from *Synechocystis* sp. strain PCC6714. Electron microscope studies revealed double-track outer membrane vesicles with a smooth-appearing exoplasmic surface, an exoplasmic fracture face covered by closely packed particles and a corresponding plasmic fracture face with regularly distributed holes. Lipopolysaccharide, proteins, lipids, and carotenoids were the constituents of the outer membrane of *Synechocystis* sp. PCC6714. Twelve polypeptides were found in outer membrane fractions, among them two dominant outer membrane proteins (M_rs, 67,000 and 61,000). Lipopolysaccharide-specific components were GlcN and an unidentified heptose. Outer membrane lipid extracts contained phosphatidylglycerol, sulfolipid, phosphatidylcholine, and unknown lipids. The carotenoids, myxoxanthophyll, related carotenoid-glycosides, zeaxanthin, echinenone, and β-carotene were found to be true constituents of the outer membrane of *Synechocystis* sp. PCC6714.

Cyanobacteria have three membrane systems: the thylakoid membrane, the cytoplasmic membrane, and the outer membrane. Until recently, purification of the different membrane types has not been achieved. In recent studies, the thylakoid and cytoplasmic membranes of *Anacystis nidulans* (25, 26), *Anabaena variabilis* (19, 20), and *Synechocystis* sp. strain PCC6714 (27) were separated by physical methods. Some biochemical data were published on cell envelopes of *A. nidulans* (9, 25), *Aphanothece halophytica* (13, 32), *Oscillatoria limnetica*, *Phormidium* sp. (32), *Synechococcus* sp. PCC6912 (31), and other cyanobacteria (for a review, see reference 7). Previously, cell walls, free of cytoplasmic and thylakoid membranes, were isolated from *Synechococcus leopoliensis* UTEX 625, *A. nidulans* R-2, *Synechococcus* sp. (28), and *Synechocystis* sp. PCC6714 (16a, 27; U. J. Jürgens, doctoral thesis, University of Freiburg, Freiburg, Federal Republic of Germany, 1984). Polypeptide patterns of cell wall fractions were dominated by two major proteins (16a, 27, 28). Recently, pore-forming activity was detected in outer membrane extracts of *A. variabilis* (1).

This paper describes the isolation of the outer membrane of *Synechocystis* sp. PCC6714, a unicellular spherical cyanobacterium that lacks a sheath and divides in two planes. A biochemical characterization revealing carotenoids to be constituents of the isolated outer membrane fractions in addition to lipopolysaccharide (LPS), proteins, and lipids is given.

MATERIALS AND METHODS

Cultivation of cyanobacteria. *Synechocystis* sp. PCC6714 was obtained from the Pasteur Culture Collection (PCC), Institut Pasteur (Paris, France). The strain was cultivated photoautotrophically in BG-11 medium (pH 7.5) at 25°C (29). Mass cultures were prepared in a 12-liter fermentor (Jungkeit; Göttingen, Federal Republic of Germany) and were gassed continuously by a stream of air (250 liters/h) and carbon dioxide (2.5 liters/h). Cells were harvested from the stationary phase of growth and washed once with 20 mM *N*-Tris hydrochloride (pH 8.0; Tris buffer, used throughout the isolation procedure) before use.

Preparation of outer membranes. The gradient-purified cell wall fraction and the Triton X-100-insoluble cell wall fraction of *Synechocystis* sp. PCC6714 were isolated as previously described (16a). Both fractions were digested with hen egg white lysozyme (53,000 U/mg of protein; Sigma Chemical Co., St. Louis, Mo.) in 20 mM ammonium acetate buffer (pH 6.5) at 37°C for 24 h (enzyme-substrate ratio, 1:25 [wt/wt]). The digested cell wall fractions were centrifuged (176,600 × *g*, 4°C, 2 h) and washed once with Tris buffer. Outer membranes were recovered from the final pellet and resuspended in a small volume of Tris buffer. The suspension was loaded on discontinuous sucrose gradients (10 ml of 60, 55, 50, 45, and 40% sucrose in Tris buffer) and run in an SW25.2 rotor (Beckman Instruments, Inc., Fullerton, Calif.) at 20,000 rpm for 12 h. Outer membranes were isolated from the band at 50% sucrose. Sucrose was removed by dialysis against Tris buffer. Outer membrane fractions, prepared from the gradient-purified cell wall fraction and from the Triton X-100-insoluble cell wall fraction of *Synechocystis* sp. PCC6714, were designated OM I and OM II, respectively.

Isolation of the cytoplasmic-thylakoid membrane fraction. The cell homogenate of *Synechocystis* sp. PCC6714 was prepared as described previously (14). After differential centrifugation (12,000 × *g*, 4°C, 30 min) and several washes with Tris buffer, the supernatants were combined and centrifuged (45,900 × *g*, 4°C, 30 min). The pellet was resuspended in Tris buffer and purified on discontinuous sucrose gradients (as described above). The cytoplasmic-thylakoid membrane fraction was collected from the band at 40% sucrose and dialyzed against Tris buffer.

Isolation of outer membrane constituents. The LPS fractions were prepared by hot phenol-water treatment of *Synechocystis* sp. PCC6714 cells (34) and purified by ultracentrifugation (105,000 × *g*, 4°C, 4 h). Lipids and carotenoids were extracted by trichloromethane-methanol extraction (2) of outer membrane fractions and separated by thin-layer chromatography as previously described (16a).

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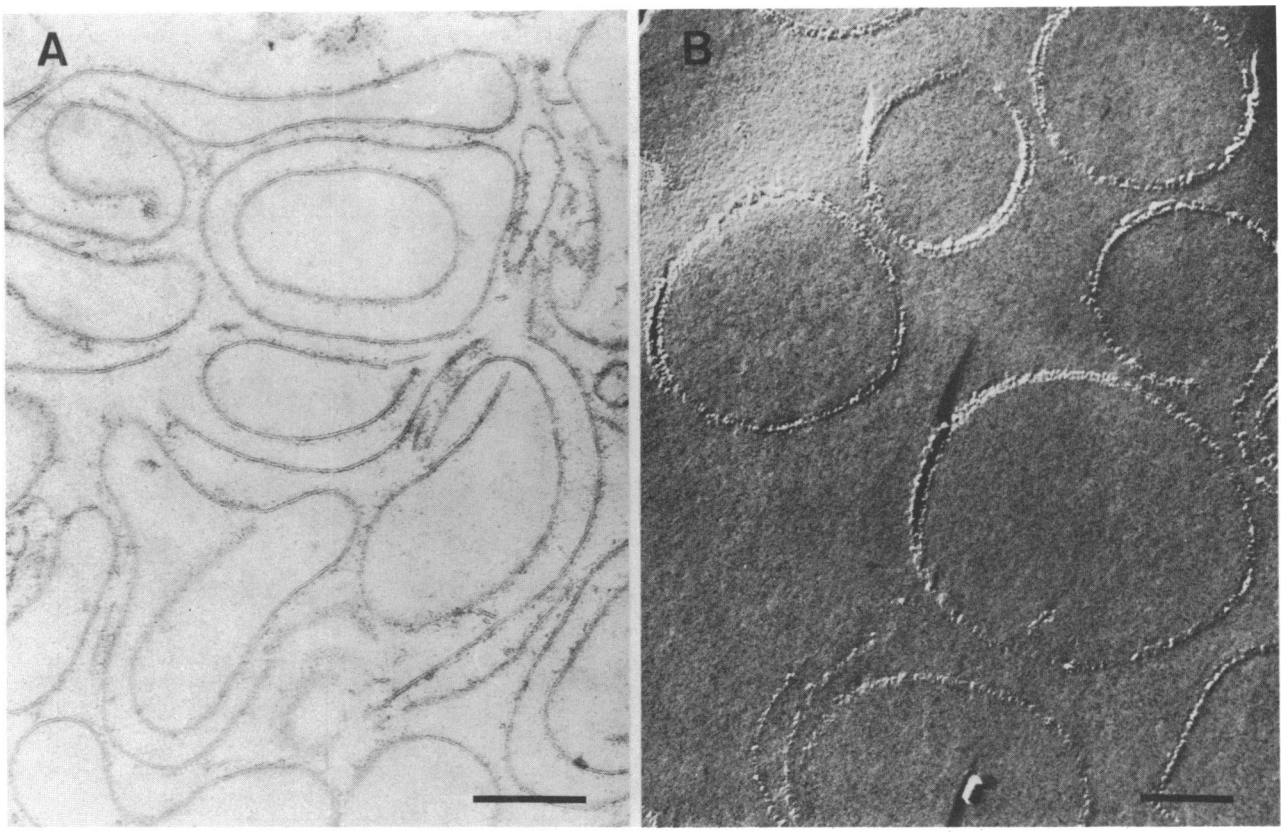

FIG. 1. Ultrathin sections (A) and freeze-etch preparations (B) of OM I prepared from the gradient-purified cell wall of *Synechocystis* sp. PCC6714. Note the maintained fine structure of the outer membrane (A) and the formation of membrane vesicles (B). Bar represents 0.2 μm.

Analytical procedures. Methods for the analysis of amino sugars, amino acids, neutral sugars, fatty acids, and organic phosphorus were described elsewhere (14). Protein was determined by amino acid analysis and by the Lowry method (21). Spectra of carotenoid extracts were recorded with a Perkin-Elmer spectrophotometer (model 330; Hitachi Ltd., Tokyo, Japan). Carotenoids were identified by their R_f-values and absorption spectra. The absorption coefficients, $E^{1\%}_{1\,cm}$, at the absorption maxima were 2,160 for myxoxanthophyll and related carotenoid-glycosides in acetone, 2,480 for zeaxanthin in ethanol, 2,340 in echinenone in acetone, 2,592 for β-carotene in *n*-hexane (4, 10, 25), and 820 for chlorophyll *a* in acetone (24).

PAGE. Membrane fractions (2 mg of protein per ml) were diluted with sample buffer (2% sodium dodecyl sulfate, 10% glycerol, and 10% mercaptoethanol in 62.5 mM Tris hydrochloride buffer [pH 6.8]) in a ratio of 1:1 (vol/vol) and solubilized at 100°C for 2 min. Up to 30 μg of membrane protein was applied to a slab gel, consisting of an upper stacking gel (1.5 cm by 10 cm by 1 mm; 3% acrylamide, 0.8% *N'*,*N*-methylenebisacrylamide) and a lower separating gradient gel (9 cm by 10 cm by 1 mm; 11 to 18% acrylamide, 0.8% *N'*,*N*-methylenebisacrylamide) and run at 20°C with a constant current of 10 mA. Bromophenol blue was used as a dye for the control of the electrophoresis. The slab gels were fixed, stained, and destained by the method of Laemmli (18). The apparent molecular weights of the membrane proteins were determined from M_r values of known marker proteins (phosphorylase *b* [M_r, 94,000], bovine albumin [M_r, 67,000], ovalbumin [M_r, 43,000], carbanhydrase [M_r, 30,000], soybean trypsin inhibitor [M_r, 20,100], and α-lactalbumin [M_r, 14,400]), which were separated by polyacrylamide gel electrophoresis (PAGE) in parallel with the membrane proteins (22).

Electron microscopy. The methods for embedding and ultrathin sectioning were as described elsewhere (14). For freeze-etching, the fixed membrane fractions were suspended in 30% glycerol and shock frozen in Freon (20). The membranes were freeze-etched by standard methods with a Balzer freeze-etching apparatus, model BA 360 M, at −100°C for 1 min (9). The specimens were examined in a Philips EM 400 electron microscope at 80 kV.

RESULTS

Morphology of outer membranes. Ultrathin sections revealed the double-track structured outer membrane (8 nm thick), resembling in appearance a unit membrane in fine structure (Fig. 1A). OM I and OM II, respectively, from the gradient-purified cell wall fraction (Fig. 1A) or from the Triton X-100-insoluble cell wall fraction (not shown) were retained and showed no differences in fine structure. Freeze-etch preparations showed that most of the outer membranes were closed to circles, demonstrating the formation of vesicles (Fig. 1B). The exoplasmic surface of the outer membrane appeared smooth with a fine granular pattern (Fig. 2A). The fracture plane through the inner hydrophobic layer of the outer membrane produced two fracture faces, the exoplasmic and the plasmic. The exoplasmic face of the outer membrane was shown to be covered with regular, closely packed particles of 7.5 to 8.0 nm in diameter (Fig. 2B). The plasmic face of the outer membrane contained

several holes, the distribution of which correlated with the regular arrangement of the particles of the corresponding exoplasmic face (Fig. 2C).

Chemical composition of outer membranes. The outer membrane of *Synechocystis* sp. PCC6714 was found to be composed of LPS, proteins, lipids, and carotenoids (Table 1). The sugars Glc (main sugar), Man, Gal, Fuc, GlcN, and an unidentified heptose, together with phosphorus and the β-hydroxy fatty acids, β-C_{14}OH and β-C_{16}OH, represented the LPS moiety of the outer membrane, which accounted for about 50% of the total outer membrane dry weight.

Outer membrane proteins were present in a total amount of about 30% fraction (dry weight) as revealed by amino acid analysis. Fatty acids other than the β-hydroxy fatty acids were typical components of membrane lipids. The fatty acid content (2% [dry weight]) of the outer membrane was relatively low. Carotenoids (about 0.2% fraction [dry weight]) were found in the isolated outer membrane of *Synechocystis* sp. PCC6714. Peptidoglycan components (MurN, m-A_2pm) and chlorophyll *a* were completely lacking. Furthermore, the purified outer membranes contained neither ManN nor GalN, components of the peptidoglycan-polysaccharide complex of the *Synechocystis* sp. PCC6714 cell wall (14, 15). The chemical composition of OM I and OM II, isolated from the gradient-purified and the Triton X-100-insoluble cell wall fractions, respectively, did not differ significantly except for the contents of lipids, which were decreased after the extraction with Triton X-100.

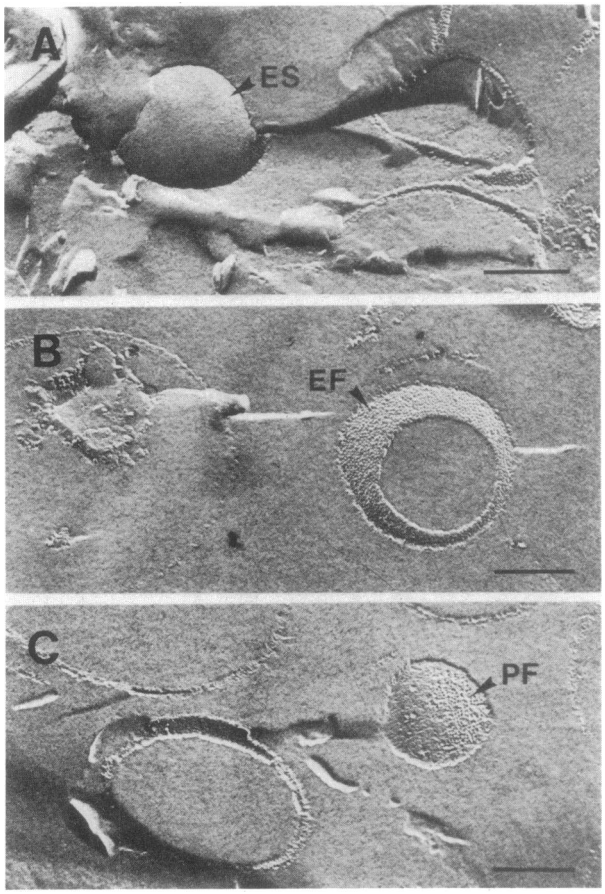

FIG. 2. Freeze-etch preparations of the exoplasmic surface (ES in panel A), the exoplasmic face (EF in panel B), and the plasmic face (PF in panel C) of OM I (Fig. 1) of *Synechocystis* sp. PCC6714. Bar represents 0.2 μm.

TABLE 1. Chemical composition of OM I and OM II prepared from the gradient-purified cell wall and the Triton X-100-insoluble cell wall, respectively and of the LPS from *Synechocystis* sp. PCC6714

Component	Amt of constituents in outer membrane and LPS fractions (μg/100 μg of fraction [dry wt])		
	OM I	OM II	LPS
Neutral sugars			
Fuc	0.9	1.3	1.5
Xyl	ND[a]	ND	1.7
Man	5.5	7.3	8.7
Gal	3.9	4.2	5.7
Glc	29.1	27.9	43.9
Hep	6.7	7.4	6.1
Amino sugars[b] GlcN	1.5	1.3	1.2
Amino acids[b]	30.2	31.2	—[c]
Fatty acids			
$C_{16:0}$	1.2	0.4	0.5
$C_{16:1}$	0.1	0.1	0.3
$C_{18:0}$	0.1	0.1	0.2
$C_{18:1}$	0.2	0.1	0.2
β-C_{14}OH	0.8	0.5	1.7
β-C_{16}OH	1.4	0.9	3.0
Unknown	0.2	0.3	0.6
Carotenoids	0.2	0.05	—
Phosphate	0.2	0.2	0.2

[a] ND, Not determined.
[b] Peptidoglycan components (MurN, m-A_2pm) were completely lacking.
[c] —, Absent.

LPS. The LPS fraction contained the neutral sugars, Glc (main sugar), Man, Gal, Xyl, Fuc, and an unidentified heptose, detected also in outer membrane fractions of *Synechocystis* sp. PCC6714 (Table 1). GlcN was the only amino sugar present in the LPS. The sugar spectrum was similar to that of respective outer membrane fractions. The predominant fatty acids were the β-hydroxy fatty acids, β-C_{14}OH and β-C_{16}OH. Small amounts of fatty acids other than the β-hydroxy fatty acids were also detected (Table 1). The phosphate content (0.2% [dry weight]) was relatively low.

Outer membrane proteins. Twelve polypeptides were detected in the outer membrane fractions of *Synechocystis* sp. PCC6714 by PAGE (Fig. 3, lanes b and c). The identical polypeptide patterns of both OM I and OM II were dominated by two major outer membrane proteins (M_rs, 67,000 and 61,000). These major proteins were found in purified cell wall fractions of *Synechocystis* sp. PCC6714 and shown to be peptidoglycan associated (16a). In addition, we found one polypeptide (M_r, 22,000) which could be visualized in polypeptide patterns of outer membrane fractions but not in cell wall fractions (Fig. 3). This polypeptide was also detected in rigid-layer (peptidoglycan plus covalently bound polymers) fractions of *Synechocystis* sp. PCC6714 (16; Jürgens, doctoral thesis). The polypeptide with an M_r of 16,000 is likely lysozyme used during the isolation procedure of outer membranes, although untreated cell wall fractions (Fig. 3, lane a)

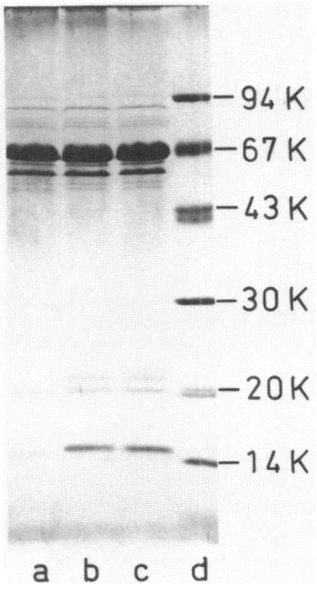

FIG. 3. Polypeptide patterns of OM I (lane b) and OM II (lane c) of *Synechocystis* sp. PCC6714 in comparison to that of the Triton X-100-insoluble cell wall fraction (lane a). Lane d: Marker polypeptides. Outer membrane and cell wall fractions were heated in PAGE sample buffer at 100°C for 2 min, and 30 μg of protein was applied to the gradient slab gel (11 to 18% acrylamide). Staining was with Coomassie brillant blue R-250.

of *Synechocystis* sp. PCC6714 contained small amounts of a protein (M_r, 16,000) which is different from lysozyme.

Lipids. In outer membrane fractions of *Synechocystis* sp. PCC6714, phosphatidylglycerol, phosphatidylcholine, sulfolipid, unknown lipids, and an unidentified, strong polar lipid were detected. These findings were in agreement with the previous description of cell wall lipids of *Synechocystis* sp. PCC6714 (16a). Interestingly, outer membrane lipid extracts contained the β-hydroxy fatty acids, β-C_{14}OH and β-C_{16}OH, in addition to the typical fatty acids ($C_{16:0}$, $C_{18:0}$) of membrane lipids (Table 2). However, the fatty acid spectrum of the respective cytoplasmic-thylakoid membrane fraction of *Synechocystis* sp. PCC6714 differed significantly from that of OM II (Table 2). Monogalactosyldiglyceride and digalactosyldiglyceride, typical galactolipids of the thylakoids, were lacking in outer membrane fractions of *Synechocystis* sp. PCC6714.

Carotenoids. The pigment extracts of the deep red-orange outer membranes and of the cytoplasmic-thylakoid membrane fractions of *Synechocystis* sp. PCC6714 were compared spectrophotometrically (Fig. 4). The absorption spectrum of outer membrane carotenoids showed maxima at 462, 485, and 513 nm, whereas typical absorption maxima of chlorophyll *a* (432 and 665 nm) were lacking. The absorption maxima of chlorophyll *a* were the predominant peaks in pigment extracts of the cytoplasmic-thylakoid membrane fraction in addition to carotenoids. In outer membrane fractions of *Synechocystis* sp. PCC6714, we detected echinenone, zeaxanthin, myxoxanthophyll, two related carotenoid-glycosides, β-carotene, and an unknown, polar carotenoid (R_f = 0; Fig. 5), which were also present in purified cell wall fractions of *Synechocystis* sp. PCC6714 (16a). The unknown, polar carotenoid was absent from respective cytoplasmic-thylakoid membrane fractions. Zeaxanthin, myxoxanthophyll, and the two related carotenoid-glycosides were the predominant carotenoids of the outer membrane and accounted for about 75% of the total outer membrane carotenoids (Table 3). OM I and OM II contained carotenoids in amounts of 1.9 and 0.5 μg/mg of protein, respectively. In respective cytoplasmic-thylakoid membrane fractions of *Synechocystis* sp. PCC6714, 18.3 μg of carotenoids per mg of protein was found in addition to 96.4 μg of chlorophyll *a* per mg of protein.

TABLE 2. Percent distribution of fatty acids in lipid extracts of OM II prepared from the Triton X-100-insoluble cell wall and the cytoplasmic-thylakoid membrane (CM/ICM) fraction of *Synechocystis* sp. PCC6714

Fatty acid	Distribution of fatty acids in lipid extracts (%) of[a]:	
	OM II	CM/ICM
$C_{12:1}$	—[b]	10.2
$C_{14:0}$	4.1	1.3
$C_{14:1}$	1.6	0.3
$C_{16:0}$	23.8	37.3
$C_{16:1}$	8.5	6.3
$C_{18:0}$	12.7	3.4
$C_{18:1}$	9.4	5.2
$C_{18:2}$	4.5	19.8
β-C_{14}OH	12.0	16.3
β-C_{16}OH	18.5	—
Unknown	3.5	—

[a] Values are percentages of total fatty acids.
[b] —, Absent.

DISCUSSION

The complete purification of outer membranes from *Synechocystis* sp. PCC6714 was achieved by applying lysozyme treatments of cell wall fractions followed by sucrose gradient centrifugations. Electron microscope studies of outer membrane fractions revealed that the fine structure of the outer membrane was retained, even in outer membrane fractions isolated from Triton X-100-insoluble cell walls of *Synechocystis* sp. PCC6714. Most of the outer membranes were found to be closed to circles, demonstrat-

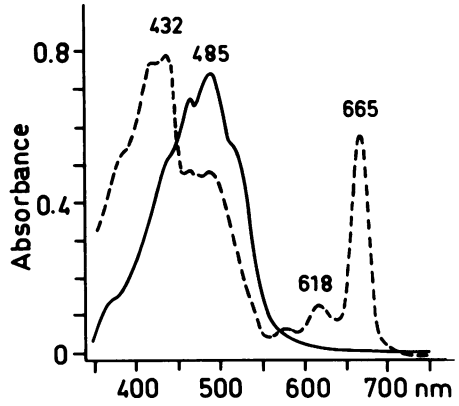

FIG. 4. Absorption spectra of pigment extracts of OM II (——) and the cytoplasmic-thylakoid membrane fraction (-----) from *Synechocystis* sp. PCC6714. The spectra were recorded with a Perkin-Elmer spectrophotometer at room temperature in trichloromethane.

ing the formation of vesicles which can occur only in the absence of peptidoglycan. The regularly arranged particles detected in freeze-etch preparations on the exoplasmic fracture face of the outer membrane may represent protein-LPS complexes which are postulated for those of the enterobacterial outer membrane (23). The isolated outer membrane fractions of *Synechocystis* sp. PCC6714 were free of cytoplasmic and thylakoid membranes as shown by PAGE, electron microscopy, and spectrophotometry. However, absorption spectra of outer membrane fractions revealed carotenoids to be true constituents of the *Synechocystis* sp. PCC6714 outer membrane. Nothing is known about their function. Probably they protect against high light intensities or have a structural function (17). The outer membrane as the site of carotenoid biosynthesis is also a possibility. The outer membrane of chloroplasts from green plants also contain carotenoids which are a suggested site of synthesis (3, 5, 6). The carotenoids may be associated with outer membrane proteins. Other carotenoid-protein complexes, loosely bound to the thylakoids, have been isolated from *Spirulina maxima*, *Aphanizomenon flos-aquae*, and *Microcystis aeruginosa* (11). The function of the small amounts of carotenoids and their complexes in the outer membrane of *Synechocystis* sp. PCC6714 cannot be identified at the moment. Carotenoids were also found in cell walls of other cyanobacteria (25, 27, 28), as well as in cell walls of the gram-positive *Micrococcus radiodurans* (35, 36) and the Knallgas bacterium, strain 12/60/x (8). In the outer membrane of the gram-negative *Flexibacter elegans*, flexirubins were assumed to have a structural function (12).

Other outer membrane constituents found in *Synechocystis* sp. PCC6714 were LPS, proteins, and lipids, similar to constituents of the outer membrane of gram-negative bacteria. However, differences from the typical gram-negative outer membrane are obvious. The major outer membrane proteins of *Synechocystis* sp. PCC6714, being peptidoglycan associated (16a), are found with M_rs of 67,000 and 61,000, whereas those of *Enterobacteriaceae* have apparent molecular weights of about 33,000 to 36,000 (23). It seems likely that the major outer membrane proteins of *Synechocystis* sp. PCC6714 are functioning as porins, but clear proof cannot be given yet. Recently, pore-forming activity was found in outer membrane extracts of *A. variabilis* (1). Major cell wall proteins were also detected in cell wall fractions of *S. leopoliensis* UTEX 625, *A. nidulans* R-2, and *Synechocystis* sp. (28) and *Synechocystis* sp. PCC6714 (16a, 27; Jürgens, doctoral thesis). In outer membrane fractions of *Synechocystis* sp. PCC6714, a protein (M_r, 22,000) was found which could not be detected in polypeptide patterns of respective cell wall fractions. However, it could be visualized by PAGE only after lysozyme digestion of cell wall fractions. The identical protein was also detected in rigid-layer fractions of *Synechocystis* sp. PCC6714 and could be released from the peptidoglycan with a muramidase (Jürgens, doctoral thesis). Thus, anchoring to the outer membrane and binding to the peptidoglycan of this polypeptide (M_r, 22,000) are assumed.

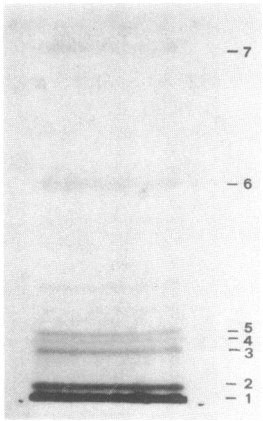

FIG. 5. Thin-layer chromatography of carotenoids from OM II on silica gel plates in the solvent system (10, 33): light petroleum benzene–isopropanol–water, 100:11:0.5 (vol/vol/vol). 1, Unknown polar carotenoid; 2, myxoxanthophyll; 3 and 4, two carotenoid-glycosides related to myxoxanthophyll; 5, zeaxanthin; 6, echinenone; 7, β-carotene.

TABLE 3. Percent distribution of carotenoids in OM I and OM II (Table 1) of *Synechocystis* sp. PCC6714

Carotenoid	Distribution in outer membrane fractions (%)[a]	
	OM I	OM II
β-Carotene	13.0	8.1
Echinenone	12.1	13.9
Zeaxanthin	14.3	15.2
Myxoxanthophyll	53.1	46.7
Unknown xanthophylls	7.5	16.1
Unknown polar carotenoid[b]	+[c]	+

[a] Values are percentages of total carotenoids.
[b] Polar carotenoid not further identified from the starting point of the silica gel plate (Fig. 5).
[c] +, Present.

It could be shown that phosphatidylglycerol, phosphatidylcholine, sulfolipid, and further unidentified lipids are constituents of the outer membrane bilayer of *Synechocystis* sp. PCC6714, but the major galactolipids, monogalactosyldiglyceride and digalactosyldiglyceride, of the cyanobacterial thylakoid membrane are not. Detailed analyses on the LPS of *Synechocystis* sp. PCC6714 were not performed. In cell wall and outer membrane fractions, an unknown heptose occurred which had not been described for the LPS of *Synechocystis* sp. PCC6714 (30). The unknown heptose could be found in the isolated LPS fraction of *Synechocystis* sp. PCC6714 (Table 1) and might be identical with the not-further-characterized heptoses of the LPSs of *Synechocystis* sp. PCC6308 and *Synechocystis* sp. PCC6807 (32).

Peptidoglycan components were absent from outer membrane fractions, showing the complete digestion of the peptidoglycan and removal from cell wall fractions. In addition, ManN and GalN, being specific components of the peptidoglycan-bound polysaccharide of *Synechocystis* sp. PCC6714 (14–16), were not detected in outer membrane fractions. Thus, the peptidoglycan-bound polysaccharide (16) is not anchored in the outer membrane of *Synechocystis* sp. PCC6714.

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