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LIPID AND FATTY ACID COMPOSITION OF MEMBRANES FROM CYANOBACTERIA - IMPLICATIONS FOR EVOLUTION

RODULIO CAUDALES[1], ROBERT A. MOREAU[2], JOHN M. WELLS[2] and ALAN D. ANTOINE[1]

[1]Department of Biochemistry and Microbiology, Rutgers-The State University, New Brunswick, NJ, 08903-0231,USA; [2]U.S.Department of Agriculture, ARS, 600 East Mermaid Lane, Philadelphia, PA, 19118, USA

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INTRODUCTION

Cyanobacteria are morphologically diverse, ranging from simple unicellular to complex filamentous organisms. They are advanced prokaryotic organisms having one of the older fossil records (nearly 3.5 X 10^9 years), and were the earliest of the oxygenic photosynthetic organisms (14, 20).

Vegetative cells of cyanobacteria of the order *Nostocales*, have the same gross membrane organization as Gram-negative prokaryotic eubacteria. In addition they have thylakoids, analogous to the chloroplasts in higher plants (6, 9). The lipid composition of the genera *Anabaena* and *Nostoc* resembles that of plants, and includes the components monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), phosphatidylglycerol (PG), sulfoquinovosyldiacylglycerol (SQDG), (4, 11, 19, 21) phosphatidylethanolamine (PE), phosophatidylcholine (PC) (4, 21), lanosterol (21), bacteriohopanetetrol (4, 18), cardiolipin and several other unknown lipid components (4).

The cellular fatty acid composition of these genera include 16-and 18-carbon straight and polyunsaturated chains as major components (2, 13, 19, 23). In addition, other groups of fatty acids such as saturated odd-carbon, hydroxy-substituted, branch-chained, cyclopropane and unsaturated branched chains have been reported in cyanobacteria (2, 3, 4). In contrast, the fatty acid composition of eubacteria generally contains low percentages of straight-chained carbons, and high concentrations of *iso* and *anteiso* branch- chained acids, hydroxy-substituted and cyclopropane acids (12). The lipid and fatty acid composition of cyanobacteria thus make them an interesting group of organisms to study the evolutionary link between eubacteria and plant chloroplasts. In this paper we describe the lipid and fatty acid composition of cell membranes and of thylakoids of *Nostoc*, and the implications it may have in the evolution of bacteria and chloroplasts.

MATERIALS AND METHODS

Growth conditions

Vegetative cells of the cyanobacterium *Nostoc foliaceum* UTEX 1624 (University of Texas, Austin, TX) were grown in 2.8 l Fernbach flasks containing 1 l of BG-11 medium (17) under continuous cool-white fluorescent light at 50 $\mu mol.m^{-2}.s^{-1}$ for 12 days at temperature of 25 $\pm$ 1 °C (2).

Preparation of Membranes

Cyanobacteria were harvested by centrifugation, homogenized in a buffer containing 10 mM HEPES pH 7.0 to release part of the mucilaginous material, and centrifuged 2x for 10 min at 1000 g. Cells were resuspended in the same buffer by sonication with 5 bursts of 20 sec each followed by 30 sec cooling in ice. Unbroken cells were sedimented by centrifugation at 3000 g for 10 min, and the supernatant then centrifuged at 150,000 g for 2 hours. Pellets were resuspended in 0.5 M sucrose in the same buffer, then loaded into a sucrose density gradient (0.5, 1.0, 1.5, 2.0 and 2.5 M) and centrifuged at 140,000 g for 12 hours. After centrifugation each band was collected for lipid and fatty acid analysis. All centrifugations were at 4 °C

Lipid analysis

Each sucrose density gradient band was extracted with chloroform-methanol as described by Bligh and Dyer, (1), dried under a stream of N_2, and redissolved in chloroform-methanol (85/15, v:v). Lipid separation and quantification was performed by a high pressure liquid chromatography (HPLC) method described by Moreau et al. (15) involving separation of lipids on a silica column eluted with an isooctane-isopropanol-water gradient, and quantification by flame ionization detector (FID) (15). Most peaks were identified by comparison of retention times with known lipid standards.

Fatty acid analysis

Bands were saponified by mixing in 1 ml of 1.2 N NaOH in 50% aqueous methanol, and heating for 30 min in a boiling water bath. Samples were neutralized by adding 0.5 ml 6 N HCl and esterified by adding 1 ml 12% BCl_3-methanol, and heating for 5 min at 85 °C, based on the method of Moss (16). Methyl esters were then extracted with 1 ml hexane: diethylether (1:1), washed with 3 ml of 0.3 N NaOH, and concentrated to approximately 10 ul. Two to 5 ul of sample was injected into a Varian 3700 Gas Chromatograph with a flame ionization detector (Varian Associates, Sunnyvale, Ca, USA) and a 15 m x O.25 mm capillary glass column coated with SPB-1 (Supelco Inc., State College, PA, USA) as a non-polar stationary phase. Using operating conditions previously described (2), fatty acids between 8 and 20 carbons in length were initially identified by co-chromatography with available reference standards (Supeco Inc. State College, PA). Major fatty acids (each constituting at least 0.90% the total) were confirmed by mass spectrometer (Finnegan 8230 HR) and by chemical tests as previously described (2, 3). Equivalent carbon chain lengths (ECL) were calculated for each peak, and used for further confirmation of identities by reference to ECL's of published reports (2, 3, 7).

The major fatty acids in cell membrane were the saturated 16:0 (4.05% of the total); the unsaturated straight-chain 18:1 *cis*-9 (2.02%), and 16;1 *cis*-9 (1.99%), 18:1 *trans*-9 (3.01%) and a partly-characterized 16-carbon (16:?) with an ECL of 15.5 (1.52%); the hydroxy-substituted *iso* 3OH-17:0 (13.40%), 3 OH-17:0 (3.18%), 2 OH-12:0 (2.20%) and 2 OH-10:0 (0.92%); branched-chain *iso*-19:0 (4.93%) and *iso*-11:0 (0.94%); the unsaturated branch-chained *iso*-17:1 (33.30%), *iso*-15:1 (3.83%), and *iso*-18:1 (1.25%); and one provisionally characterized 17:1 alcohol (13.60%) (Table 2).

Cell membranes also contained minor quantities (less than 0.9% of the total) of unsaturated 14:1 *cis*-7 (0.62%) and 20:2 *cis*-11 (0.82%); hydroxy-substituted 3 OH-10:0 (0.53%), *iso* 3OH-11:0 (0.87%), and 3OH-12:0 (0.88%); and the branched chain *iso* 14:0 (0.84%). No cyclopropane acids were detected.

The major fatty acids in the thylakoids were the saturated even-carbon straight chain 16:0 (14.78%); the unsaturated straight chain 18:3 *cis*-9 (20.40%), 18:2 *cis*-9 (12.58%), 16:1 *cis*-9 (17.40%), 18:1 *cis*-9 (2.72%), 14:1 *cis*-7 (1.57%), 16:? (unsaturation sites undetermined) (1.36%), 16:1 *trans*-9 (1.26%) and 18:3 *cis*-6 (1.09%); the hydroxy- substituted *iso* 3 OH-15:0 (1.43%), 2 OH-17:0 (1.87%) and 3 OH-17:0 (2.66%); the branch-chain acid *iso*-16:0 (1.00%); the unsaturated branch chained *iso*-15:1 (2.76%), anteiso-17:1 (3.77%), and *iso*-17:1 (1.46%); and the provisionaly- identified 17:1 alcohol (4.19%). Minor components were the saturated 18:0 (0.88%), 14:0 (0.24%) and 12:0 (0.13%); the unsaturated 16:4 (0.58%) and 18:4 *cis*-6 (0.25%); cyclopropane 17:0 (0.29%), and branched *iso* 18:1 (0.46%).

Table 2: Percentage (of the total) of fatty acids in cell membranes, thylakoids and whole cells of *Nostoc foliaceum* UTEX 1624.

Fatty acids Chemical class	ECL[2]	Cell Fraction[1] Membranes	Thylakoids	Whole Cells
A. Saturated, even-carbon straight chains:				
12:0	12.0	0.00	0.13	0.06
14:0	14.0	0.01	0.24	0.18
16:0	16.0	4.05	14.78	23.97
18:0	18.0	0.53	0.88	0.96
20:0	20.0	0.00	0.01	0.04
Class A total		4.59	16.04	25.21
B. Saturated, odd-carbon straigt chains:				
11:0	11.0	0.00	0.00	0.02
13:0	13.0	0.00	0.00	0.04
15:0	15.0	0.00	0.00	0.74
17:0	17.0	0.00	0.01	0.46
19:0	19.0	0.00	0.00	0.11
Class B total		0.00	0.01	1.37

Table 2, Continued: Percentage (of the total) of fatty acids in cell membranes, thylakoids and whole cells of *Nostoc foliaceum* UTEX 1624.

Fatty acids Chemical class	ECL[2]	Cell Fraction[1] Membranes	Thylakoids	Whole cells
C. Unsaturated, straight chain acids:				
12:1	11.8	0.00	0.00	0.18
14:1 *cis*-7	13.8	0.62	1.51	0.62
14:1 *cis*-9	13.9	0.00	0.00	5.33
16:4	15.2	0.00	0.58	0.10
16:?	15.5	1.52	1.36	1.31
16:4 *cis*-4	15.55	0.00	0.00	1.00
16:3 *cis*-6	16.6	0.00	0.00	2.21
16:1 *cis*-9	15.8	1.99	17.40	13.96
16:1 *trans*-9	15.85	0.00	1.26	2.53
18:3 *cis*-6	17.2	0.00	1.09	0.30
18:4 *cis*-6	17.5	0.00	0.25	0.13
18:2 *cis*-9	17.7	0.00	12.58	9.83
18:3 *cis*-9	17.75	0.00	20.40	20.76
18:1 *cis*-9	17.8	2.02	2.72	3.01
18:1 *trans*-9	17.85	3.01	0.00	2.03
20:2 *cis*-11	19.6	0.82	0.01	0.65
Class C total		9.98	59.16	63.95
D. Hydroxy-substituted:				
2OH-10:0	11.15	0.92	0.00	0.00
3OH-10:0	11.4	0.53	0.00	0.03
iso 3OH-11:0	12.45	0.87	0.00	0.02
20H-12:0	13.2	2.20	0.00	0.00
30H-12:0	13.5	0.88	0.00	0.03
3OH-13:0	14.5	0.00	0.00	0.09
iso 3OH-15:0	16.1	0.00	1.43	0.00
iso 3OH-17:0	18.1	13.25	0.00	0.15
2OH-17:0	18.2	0.00	1.87	0.00
3OH-17:0	18.4	3.18	2.66	0.27
Class D total		21.83	5.96	0.59

Table 2, Continued: Percentage (of the total) of fatty acids in cell membranes, thylakoids and whole cells of *Nostoc foliaceum* UTEX 1624.

Fatty acids Chemical class	ECL[2]	Cell Fraction[1] Membranes	Thylakoids	Whole cells
E. Branched chain acids:				
iso-11:0	10.6	0.94	0.00	0.02
iso-14:0	13.6	0.84	0.10	0.03
iso-15:0	14.6	0.00	0.63	0.13
iso-16:0	15.6	0.00	1.00	1.52
iso-17:0	16.6	0.00	0.26	0.40
anteiso-17:0	16.7	0.00	0.00	0.84
iso-19:0	18.6	4.93	0.00	0.19
Class E total		6.52	1.99	3.13
F. Cyclopropane acids:				
cyclo-17:0	16.9	0.00	0.29	0.16
cyclo-19:0	18.9	0.00	0.00	0.06
Class F total		0.00	0.29	0.22
G. Unsaturated branch-chained fatty acids:				
iso-15:1	14.35	3.83	2.76	2.33
iso-17:1	16.4	33.30	1.46	0.26
anteiso-17:1	16.43	0.00	3.77	0.18
iso-18:1	18.4	1.25	0.46	0.34
Class G total		38.38	8.45	3.11
H. Alcohols				
17:1 alcohol[3]	16.33	13.60	4.19	0.26
Unidentified components		5.10	3.91	2.17

[1]. Mean percentage of triplicated tests.
[2]. ECL = equivalent (carbon) chain length
[3]. Tentatively identified

The fatty acid composition of whole cells of *N. foliaceum* UTEX 1624 was similar to that previously published for the genus *Nostoc* (2). The unsaturated straight chain 14:1 *cis*-9, present in whole cells, was found only in the non-membrane carotenoid fraction. The fatty acids 16:4 *cis*-4 and 16:3 *cis*-6, also present in whole cells, were not present in any membrane fraction, suggesting it may be a major component of heterocysts.

DISCUSSION

Giovannoni et al. (8), studying 16S rRNAs from selected cyanobacteria and the cyanelle of *Cyanophora paradoxa*, concluded that chloroplasts belong to the same evolutionary group as cyanobacteria. Woese (24) found that analysis of oligonucleotide catalog data did not entirely resolve the question of origins of chloroplasts, and suggested independent testing of the hypothesis by different methodologies. Guckert et al. (10) found that phenotypic relationships among bacteria, based on fatty acid analysis, have compared favorably with phylogenetic associations based on 16S rRNA data. Since lipid and fatty acid composition is highly conserved in these organisms, they continue to be useful as phylogenetic tools to investigate genetic diversity.

The lipid composition in cell membrane and thylakoids were qualitatively similar. Both contained the lipids ASG, MGDG, DGDG, PE, SQDG, PS and major quantities of an unknown lipid (retention time= 27) referred to as lipid 4 in *Azolla* cyanobionts (4). Differences between the two fractions were mainly quantitative. Lipid 4, DGDG and PE were in higher concentration in the cell membranes, and ASG, MGDG were higher in thylakoids. PG and PC were present only in thylakoids, which also lacked an unidentified lipid (retention time = 16) that was found in the cell membranes.

Lipid composition in plant thylakoid membranes, was reported to consist of MGDG, 53%; DGDG, 27%; SQDG, 7%; PG, 7; and phosphatidylinositol (PI), 2% (24). No PI was found in *Nostoc*. PE (and PS) is found in high concentration in bacteria, suggesting similarity with membrane lipids. PE for example is major lipid in *Escherichia coli*. (R. Moreau, unpublished data). In plants such as potato, corn, spinach and tobacco, PE and PS are minor components (15).

PS has not been reported previously in cyanobacteria. Another lipid of interest was ASG. ASG is a lipid comprised of three components, a sterol covalently linked to monosaccharide and a fatty acid. Another sterol, lanosterol, of non-photosynthetic origin has been reported in *Chlorogloepsis fritschii* (21). It is possible that other sterols of photosynthetic lineage will be found in cyanobacteria as analytical techniques improve, and further information developed that will assist in evolutionary studies.

Differences in fatty acid composition between the membrane and thylakoid fractions were clear. Thylakoids contained mainly polyunsaturated acids--the 18:3 *cis*-9 and 18:2 *cis*-9--comprising almost 33% of the total. Mono- unsaturated acids were also present in high proportion, totaling 23.05%, as well as the saturated 16:0 (14.78%) similar to what has been found in higher plants (5). The combined totals for other classes of fatty acids, the hydroxy-substituted, branched chains, and cyclopropane, were relatively low (16.71%).

Cell membranes, on the other hand, contained low percentages of the saturated 16:0 (4.05%), and the total for unsaturated straight-chain fatty acids was less than 10%. The only poly-unsaturated acid in the membranes was the 20:2 *cis*-11, present at low concentrations (less than 1% of the total). Mono-unsaturated acids were also low--consisting mainly of the 18:1 *cis*- and *trans*-9, 16:1 *cis*-9, an unsaturated 16:(?), and 14:1 *cis*-7. The unsaturated branch- chains were the most important membrane component, and included the *iso*-17:1 (33.03%), *iso*-15:1 (3.83%), and *iso*- 18:1 (1.25%).

Other classes of fatty acids in membranes were also high compared to the thylakoids--27.87% for the hydroxy- substituted acids, and 6.72% for branched-chain acids. The presence of these classes of fatty acids in relatively high concentrations is also characteristic of the prokaryotes (12).

At this point it cannot be implied that a particular group of prokaryotes gave origin to cyanobacterial membranes, but specific lipid or fatty acid markers could be used in the future to construct possible origins of cyanobacteria and of chloroplasts. The presence of characteristic groups of fatty acids in cell membranes and in thylakoids, as discussed above, may be a reflection of the two evolutionary lineages-- thylakoids related to chloroplasts, and cell membranes reflecting an origin in the eubacteria.

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Reprint requests to Dr. Rodulio Caudales