Effects of Light and Dark Incubation on the Lipid and Fatty Acid Composition of Marine Cyanobacteria

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The effect of light and dark incubation on the ultrastructure, pigments and lipids of five marine cyanobacteria was studied. All light-grown cyanobacteria contained in their lipid extracts the four major lipid classes characteristic of chloroplasts, namely monogalactosyldiacylglycerols, digalactosyldiacylglycerols, sulphoquinovosyldiacylglycerols, and phosphatidyl glycerols. However, the fatty acid patterns of the cyanobacterial total lipids and individual lipid classes were different from those of chloroplasts, and responded differently to dark incubation. In two cyanobacteria the fatty acid patterns of total lipids from light- and dark-incubated cultures were similar, but in the other three, dark incubation was associated with increased levels of oleic acid in the total lipids and individual lipid classes. The decrease of linolenic acid known to occur in lipids from photosynthetic eukaryotes in response to dark incubation was recorded only in *Spirulina subsalsa*. However, linolenic acid was not esterified in monogalactosyldiacylglycerols, as it is in chloroplasts. We also found the class of alcohol glycosides in three non-heterocystous cyanobacteria.

INTRODUCTION

Results of many studies have repeatedly confirmed that the major lipid classes in chloroplasts and cyanobacteria are quite similar (for a review see Quinn & Williams, 1983). However, this similarity does not involve the constituent fatty acids of the lipids. Lipids from photosynthetic eukaryotes contain particularly high concentrations of linolenic acid (Quinn & Williams, 1983). On the other hand cyanobacteria are unique among the oxygenic phototrophs in lacking polyenoic fatty acids in their lipids (Holton *et al.*, 1964; Parker *et al.*, 1967). Acyl moieties in cyanobacterial lipids usually consist of saturated and monoenoic C₁₆ and C₁₈ fatty acids (Holton *et al.*, 1968; Quinn & Williams, 1983; Sallal *et al.*, 1987). A few cyanobacteria, however, synthesize linolenic acid (Parker *et al.*, 1967; Nichols & Wood, 1968*a*; Sato & Murata, 1980, 1982), but the distribution of this trienoic fatty acid among the various lipid classes has not yet been extensively studied.

Chloroplasts, irrespective of their origin, exhibit reproducible responses to illumination in their fine structure, composition of their lipid classes and fatty acid patterns of their lipids. Parallel responses in cyanobacteria have not been systematically studied, and the major objective of the present work was to make this information available.

METHODS

Organisms and growth. Five marine cyanobacteria identified as *Anabaena constricta* (strain KCCA/C100), *Phormidium corium* (KCCA/C200), *Phormidium jenkelianum* (KCCA/C210), *Spirulina subsalsa* (KCCA/C300) and *Synechocystis* sp. (KCCA/C400) were isolated from a salt marsh in the Al-Khiran area, south of Kuwait. Water, mud and salt-crust samples were diluted and inoculated into medium MN (Waterbury & Stanier, 1978). After 2 weeks the cultures were microscopically examined for cyanobacteria. Individual filaments were picked up by micropipettes and plated on medium MN solidified with 1% (w/v) agar. The cyanobacteria were subcultured four

successive times on the same medium, incubating the cultures each time under continuous illumination (1500 lx) at a temperature of 38 °C to control contaminating diatoms and green algae (Allen & Stanier, 1968). Cyanobacteria free from diatoms and green algae were then passed four successive times in liquid medium MN supplemented with 150 U benzylpenicillin ml⁻¹, 100 U chloramphenicol ml⁻¹ and 1000 U streptomycin sulphate ml⁻¹ in an attempt to differentially inhibit growth of contaminating heterotrophic bacteria. Cyanobacterial cultures were considered axenic when they did not reveal any bacterial growth after 7 d incubation at 30 °C on peptone/sea water agar of the composition (l⁻¹): 5 g bacto-peptone, 0.1 g ferric phosphate, 10 g agar (dissolved in 75% v/v, sea water) pH 6.8. The organisms were identified by consulting Desikachary (1959) and Rippka *et al.* (1979). *A. constricta* does not produce heterocysts and could be identified as *Pseudanabaena constricta* according to Lauterborn (1915).

Growth measurement and pigment analysis. Cyanobacteria grown under continuous illumination for 7 d and then incubated for another 7 d in the dark, and cultures maintained under continuous illumination throughout the 14 d period, were studied. The organisms were grown at 30 °C in medium MN in 10-litre aspirators equipped with magnetic stirrers and sparged with sterile air, 30 ml min⁻¹. Light (3000 lx at the surfaces of the vessels) was provided by cool white fluorescent tubes. Culture samples (5 ml) were withdrawn daily, and after grinding the cyanobacteria mildly in a tissue grinder (Wheaton Scientific, Millville, USA) for 3 min, the percentage transmission was read at 678 nm using a spectrophotometer (Bausch & Lomb Spectronic 21). Pigments were extracted with 90% (v/v) acetone in water (Hansmann, 1973) and chlorophyll *a* and carotenoid concentrations determined using the equations of Parsons & Strickland (1965) with a Beckman model 25 spectrophotometer.

Growth in a nitrogen-free medium was examined by inoculating cyanobacteria into a medium of the following composition: 5 mM-MgSO₄, 0.3 mM-CaCl₂, 0.4 mM-KH₂PO₄, 1.5 µM-FeCl₃, 0.6 µM-CuSO₄, 1 µM-ZnSO₄, 0.6 µM-CoCl₂, 0.5 µM-NaMoO₄, 3.0% (w/v) NaCl; pH 8. Air, made free of nitrogen oxides and ammonia by passing it successively through NaOH, H₂SO₄ and distilled water, was bubbled into the cultures. All five cyanobacteria were able to grow in this medium when nitrate (5 mM-KNO₃) was present.

Electron microscopy. Cyanobacterial filaments were taken from actively growing cultures and fixed with 3% (v/v) glutaraldehyde in cacodylate buffer, pH 7.5, for 90 min at room temperature and embedded in 2% agar. Each agar block was divided into small cubes which were fixed with buffered 1% (w/v) OsO₄ for 90 min at 40 °C. The cubes were dehydrated by passing them through an ascending series of acetone and were then embedded in Epon. The cured resin blocks were sectioned using an LKB ultramicrotome equipped with a glass knife. The ultrathin sections were stained by uranyl acetate. Electron micrographs were recorded with a Jeol electron microscope.

Lipid extraction and analysis. As far as feasible, all steps were done in an atmosphere of oxygen-free nitrogen. Total lipids were extracted with boiling 2-propanol/chloroform (1:2, v/v) to deactivate lipases (Kates, 1972; Radwan, 1984) and purified following established procedures (Folch *et al.*, 1957). The composition of lipid classes was studied by thin-layer chromatography (TLC) using silica gel plates. Non-polar compounds were fractionated using hexane/diethyl ether/acetic acid (90:10:1, by vol.) (Mangold & Malins, 1960). Ionic and other polar lipids were resolved by two-dimensional TLC using chloroform/methanol/7 M-ammonium hydroxide (65:25:4, by vol.) in the first direction and chloroform/methanol/acetic acid/water (170:25:25:2, by vol.) in the second direction (Nichols, 1964). The lipid fractions were detected with iodine vapour or by charring after spraying the plates with 50% (v/v) H₂SO₄. Individual lipid classes were identified by comparing their chromatographic behaviour with that of authentic samples and by using specific spray reagents (Dittmer & Lester, 1964; Siakotos & Rouser, 1965; Vioque, 1984). To confirm the identity of a glycolipid tentatively identified as alcohol glycoside, samples of the pure compound were separated by preparative TLC and eluted from the adsorbent with chloroform/diethyl ether/ethanol (1:1:1, by vol.) (Kates, 1972) and subjected to acid hydrolysis (Gaver & Sweeley, 1965). Hydrolysis products soluble in hexane were analysed by TLC and sugar moieties remaining in the aqueous phase were identified by paper chromatography (Menzies & Seakins, 1976). Samples of total lipids and of individual lipid classes, separated by preparative TLC (Radwan, 1978), were subjected to methanolysis with 2% H₂SO₄ in absolute methanol at 90 °C for 90 min (Chalvardjian, 1964). The resulting methyl esters of fatty acids were recovered with hexane, purified by preparative TLC and analysed by GLC using a Pye-Unicam 204 gas chromatograph equipped with a glass column (1.83 m × 4 mm i.d.) packed with 15% (w/w) DEGS on Anakrom D (100–200 mesh) and operated isothermally at 170 °C with nitrogen as the carrier gas. Individual fatty acids were identified by comparing their retention times with those of standard samples as well as by using the 'equivalent chain length' method of Miwa *et al.* (1960).

RESULTS

Growth and pigments

The five axenic cultures of marine cyanobacteria grew well in medium MN under the conditions described above. Only *A. constricta* grew, although very weakly, in the nitrogen-free medium, indicating its poor ability to fix atmospheric nitrogen under aerobic conditions.

Table 1. *Effect of light on the lipid and pigment contents of marine cyanobacteria*

Values are the means of three determinations.

Cyanobacterium	Total lipids (% of dry wt)		Chlorophyll (mg l ⁻¹)		Carotene (mg l ⁻¹)		Carotene to chlorophyll ratio	
	Light	Dark	Light	Dark	Light	Dark	Light	Dark
<i>A. constricta</i>	8.2	1.6	29	13	38	22	1.3	1.7
<i>P. corium</i>	5.6	4.2	11	3	18	13	1.6	4.3
<i>P. jenkelianum</i>	4.5	1.4	11	9	36	34	3.3	3.8
<i>Spirulina subsalsa</i>	6.5	7.8	10	6	41	31	4.1	5.2
<i>Synechocystis</i> sp.	1.2	0.9	22	18	37	15	1.7	0.8

Growth in medium MN was exponential for 7 d, with population doubling times ranging between about 21 h for *Synechocystis* sp. and 13 h for *Spirulina subsalsa*. Light-incubated cultures apparently reached stationary phase, as they did not grow further after another 7 d period under continuous illumination or when transferred to the dark. However, in all cultures the chlorophyll *a* and carotene contents decreased in response to dark incubation (Table 1). The carotene to chlorophyll *a* ratio increased in the dark in *A. constricta*, *P. corium* and *Spirulina subsalsa*, remained unchanged in *P. jenkelianum* and decreased in *Synechocystis* sp. (Table 1).

Dark incubation was associated with morphological and visible colour changes. For example, cultures of *A. constricta* were deep blue-green in the light but yellowish green in the dark. The mean number of cells per trichome of *A. constricta* in the light was 80 ± 7 (mean $\pm$ SD, $n = 100$) and in the dark only 8 ± 2 . Cells in the light-incubated trichomes were more or less cylindrical and separated by clear constrictions, but in the dark they were spherical and commonly thicker (probably akinetes), giving the trichome a beaded appearance.

Ultrastructure

Electron micrographs showed that the photosynthetic lamellae of light-incubated *A. constricta* were compact and regularly appressed to each other (Fig. 1). In contrast, photosynthetic lamellae of dark-incubated cultures were much less compact and less regularly appressed to each other, and the intrathylakoidal spaces were dilated (Fig. 1). Light-incubated cells, particularly of *Spirulina subsalsa*, contained arrays of phycobilisomes in the interthylakoidal space, whereas dark-incubated cells contained few phycobilisomes.

Lipid composition

The total lipid content of *A. constricta* and *P. jenkelianum* decreased in the dark, whereas that of the three other cyanobacteria was relatively little affected (Table 1). The major lipid classes in extracts from light-incubated cyanobacteria (Fig. 2) were monogalactosyldiacylglycerols, digalactosyldiacylglycerols, sulphoquinovosyldiacylglycerols and phosphatidyl glycerols. Substantial proportions of triacylglycerols and fatty acids in addition to traces of several unidentified polar compounds were also present. Sterols and complex lipids containing sterols were not detected. Lipid extracts from *P. corium* and *Synechocystis* sp. and, to a lesser extent *A. constricta*, contained major proportions of alcohol glycosides, a class which was not detected in extracts of the two other cyanobacteria (Fig. 3). Acid hydrolysis of this fraction from *P. corium*, isolated by preparative TLC from total lipids, showed that the sugar moiety consisted of glucose bound to long-chain alcohols. These long-chain alcohols were identified by their thin-layer chromatographic behaviour and by their IR absorption band at 3500 cm^{-1} , characteristic of the (OH) group.

Dark incubation resulted in a decrease in the concentration of monogalactosyldiacylglycerols, digalactosyldiacylglycerols and sulphoquinovosyldiacylglycerols in extracts from *P. corium* and *A. constricta* but not from *P. jenkelianum*, *Spirulina subsalsa* and *Synechocystis* sp. (Fig. 3). Alcohol glycosides in lipid extracts from *P. corium*, *Synechocystis* sp. and *A. constricta* decreased in the dark. The fatty acid patterns of total lipids from marine cyanobacteria are summarized in

Fig. 1. Electron micrographs of (a) light- and (b) dark-incubated cells of *A. constricta*. PL, photosynthetic lamellae; SF, sheath fibre; PM, plasma membrane; NP, nucleoplasmic region; LD, lipid droplets; PP, polyphosphate. Bars, 1 μ m.

Fig. 2. Typical two-dimensional thin-layer chromatogram showing the composition of lipid classes of *P. corium* incubated under continuous illumination. TLC was done using silica gel G plates and the following solvent systems: chloroform/methanol/7 M-ammonium hydroxide (65:25:4, by vol.) in the first direction, and chloroform/methanol/acetic acid/water (170:25:25:2, by vol.) in the second direction. The lipid fractions were visualized by charring after spraying with 50% H_2SO_4 . O, origin; NL, neutral lipids + pigments; MGDG, monogalactosyldiacylglycerols; PG, phosphatidylglycerols; AG, alcohol glycosides; DGDG, digalactosyldiacylglycerols; SQDG, sulphoquinovosyldiacylglycerols. Several unidentified polar lipids are present in small amounts.

Fig. 3. Typical TLC plate showing the effect of light on the major glycolipids of cyanobacteria. TLC was done using silica gel G and chloroform/methanol/acetic acid/water (85:12:12:1, by vol.) as solvent. The glycolipid fractions were visualized by spraying with α -naphthol and H_2SO_4 and heating at 100 °C. MGDG, monogalactosyldiacylglycerols; P, pigments; AG, alcohol glycosides; DGDG, digalactosyldiacylglycerols; SQDG, sulphoquinovosyldiacylglycerols; X, unknown glycolipid. *P. corium* light (lane 1), dark (lane 2); *P. jenkalianum* light (lane 3), dark (lane 4); *Spirulina subsalsa* light (lane 5), dark (lane 6); *A. constricta* light (lane 7) dark (lane 8); *Synechocystis* sp. light (lane 9), dark (lane 10).

Table 2. Effect of light on the constituent fatty acids of total lipids from marine cyanobacteria

Values are expressed as percentages and are means of two determinations. tr., Trace.

Fatty acid	<i>A. constricta</i>		<i>P. corium</i>		<i>P. jenkalianum</i>		<i>Spirulina subsalsa</i>		<i>Synechocystis</i> sp.	
	Light	Dark	Light	Dark	Light	Dark	Light	Dark	Light	Dark
16:0	32.1	26.0	34.8	38.0	28.6	28.5	15.0	21.8	23.8	23.5
16:1	14.1	11.3	13.2	10.4	18.6	18.4	16.7	20.4	4.8	4.2
16:2	1.3	1.0	14.2	2.0	tr.	tr.	19.2	5.6	3.0	5.0
18:0	1.8	5.2	tr.	tr.	tr.	tr.	tr.	tr.	0.9	0.9
18:1	17.6	37.7	30.4	39.6	35.7	35.9	27.6	44.4	18.8	17.1
18:2	29.3	18.2	7.4	10.0	11.4	11.5	11.4	7.8	23.4	26.9
18:3	3.8	0.5	tr.	tr.	5.7	5.7	10.1	tr.	25.2	22.1

Table 2. Saturated and monoenoic C_{16} fatty acids and monoenoic and dienoic C_{18} fatty acids predominated. Considerable proportions of $C_{16:2}$ were present in lipids from *P. corium* and *Spirulina subsalsa* and of $C_{18:3}$ in lipids from *Synechocystis* sp. and *Spirulina subsalsa*. In *P. jenkalianum* and *Synechocystis* sp. the fatty acid patterns of total lipids were similar in the light and dark. In lipids from the other three cyanobacteria, dark incubation was associated with increases in the concentration of oleic acid ($C_{18:1}$). Simultaneously the levels of $C_{16:2}$ in lipids from *P. corium* and *Spirulina subsalsa* and of $C_{18:3}$ in lipids from *Spirulina subsalsa* decreased.

In the major lipid classes of *A. constricta*, *P. corium* and *Spirulina subsalsa*, oleic acid ($C_{18:1}$) increased considerably in the dark (Table 3). In these three organisms the highest concentration

Table 3. *Effect of light on the patterns of constituent fatty acids of major lipid classes from marine cyanobacteria*

Values are expressed as percentages and are means of two determinations. tr., Trace.

Fatty acid	<i>A. constricta</i>		<i>P. corium</i>		<i>Spirulina subsalsa</i>	
	Light	Dark	Light	Dark	Light	Dark
Monogalactosyl-diacylglycerols						
16:0	31.8	25.3	26.8	33.4	34.0	23.7
16:1	15.9	13.7	16.5	10.7	47.8	22.2
16:2	tr.	tr.	31.1	11.7	tr.	tr.
18:0	tr.	tr.	tr.	tr.	tr.	tr.
18:1	18.2	36.4	22.0	44.1	18.1	25.2
18:2	34.1	24.3	3.4	tr.	tr.	28.9
18:3	tr.	tr.	tr.	tr.	tr.	tr.
Digalactosyl-diacylglycerols						
16:0	32.7	27.1	8.5	37.6	10.6	33.9
16:1	20.8	13.3	21.6	6.3	tr.	32.7
16:2	6.7	1.4	66.7	11.0	10.6	tr.
18:0	tr.	4.2	tr.	tr.	tr.	4.9
18:1	23.4	33.3	3.2	45.1	20.9	25.7
18:2	16.5	20.6	tr.	tr.	21.3	tr.
18:3	tr.	tr.	tr.	tr.	36.6	2.7
Sulphoquinovosyl-diacylglycerols						
16:0	31.0	18.3	49.3	50.1	10.0	tr.
16:1	23.1	2.1	9.9	9.7	tr.	tr.
16:2	tr.	tr.	19.7	5.8	46.8	tr.
18:0	tr.	4.1	tr.	tr.	tr.	tr.
18:1	8.7	59.6	21.1	39.2	15.8	80.3
18:2	37.2	15.9	tr.	tr.	14.5	19.7
18:3	tr.	tr.	tr.	tr.	13.0	tr.
Phosphatidyl-glycerols						
16:0	50.5	31.9	64.8	45.3	60.1	45.3
16:1	3.8	2.9	11.1	9.6	6.9	11.6
16:2	tr.	tr.	tr.	tr.	tr.	tr.
18:0	tr.	4.4	tr.	tr.	tr.	tr.
18:1	26.1	56.4	23.8	45.1	33.0	31.3
18:2	19.6	4.4	tr.	tr.	tr.	5.2
18:3	tr.	tr.	tr.	tr.	tr.	6.4

of palmitic acid ($C_{16:0}$) was detected in phosphatidylglycerols and the concentration of this fatty acid decreased in the dark. Most of the $C_{16:1}$ was esterified in monogalactosyldiacylglycerols and digalactosyldiacylglycerols of the three cyanobacteria, although relatively high concentrations of this fatty acid were also present in sulphoquinovosyldiacylglycerols of *A. constricta* and to a lesser extent of *P. corium*. Most of the $C_{16:2}$ was esterified in digalactosyldiacylglycerols of the three organisms; in *P. corium* it was also found in monogalactosyldiacylglycerols, and in *Spirulina subsalsa* in sulphoquinovosyldiacylglycerols. Linolenic acid ($C_{18:3}$) occurred in *Spirulina subsalsa* only, in digalactosyldiacylglycerols and sulphoquinovosyldiacylglycerols, and its concentration in these glycolipids decreased in the dark.

DISCUSSION

The five marine cyanobacteria studied did not respond to dark incubation in the same way. Response variations involved the pigmentation, the total lipid contents and the fatty acid patterns of total lipids and of individual lipid classes. These variations reflect the heterogeneity of cyanobacteria as a taxonomic group.

All the cyanobacteria studied showed an ultrastructural change in response to the dark incubation similar to that described for other cyanobacteria (Fogg *et al.*, 1973; Lang & Whitton, 1973; Stulp, 1982) and chloroplasts (Luning & Schmitz, 1988). In this context it should be noted that most of the cyanobacterial lipids occur in the photosynthetic membranes with minor proportions in the plasma membranes. None of the five cyanobacteria showed responses similar to those known for chloroplasts, especially as far as lipids are concerned. The total lipid content of chloroplasts and photosynthetic eukaryotes decreases in the dark (Radwan & Mangold, 1980), but in our study this was true for *A. constricta* and *P. jenkelianum* only. Although, confirming earlier reports (Quinn & Williams, 1983; Sallal *et al.*, 1987), the composition of lipid classes in the five cyanobacteria incubated in the light was similar to that of illuminated chloroplasts, incubating the cyanobacteria in the dark brought about important differences. The major lipid classes of chloroplasts are known to decrease in the dark (Quinn & Williams, 1983). This was found true for *A. constricta* and *P. corium* only. Total chloroplast lipids are rich in linolenic acid, which significantly decreases in the dark (Nichols, 1970). Of the five cyanobacteria studied, only *Synechocystis* sp. and *Spirulina subsalsa* contained significant amounts of linolenic acid in their total lipids, and only in the latter organism did this fatty acid decrease in the dark. However, linolenic acid in *Spirulina subsalsa* was not esterified in monogalactosyldiacylglycerols, as is always the case in chloroplasts, but occurred in digalactosyldiacylglycerols and sulphoquinovosyldiacylglycerols. Also $C_{16:1}$, which occurs almost exclusively in phosphatidyl glycerols in chloroplasts, is distributed among the various major classes of cyanobacteria. In none of the five cyanobacteria we studied do the individual lipid classes reveal fatty acid patterns known for comparable classes in chloroplasts, nor are the light-associated differences in the fatty acid patterns of the comparable lipid classes similar.

In conclusion, the light-associated variations in the lipids of cyanobacteria, especially in the constituent fatty acids of these lipids, are distinctly different from those known for photosynthetic eukaryotes. Another important finding is the detection of alcohol glycosides in the total lipids from three cyanobacteria. Alcohol glycosides have never been found in chloroplasts, and their presence in cyanobacteria has only been observed in heterocysts (Nichols & Wood, 1968*b*; Walsby & Nichols, 1969; Winkenbach *et al.*, 1972). The detection of alcohol glycosides in cyanobacteria devoid of heterocysts suggests that this lipid class may be more frequent among cyanobacteria than previously assumed, and that it is not restricted to the specialized sites of aerobic nitrogen fixation.

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