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MODIFICATION OF FATTY ACID COMPOSITION IN HALOPHILIC ANTARCTIC MICROALGAE

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Key Word Index—*Dunaliella*; Chlorophyceae; fatty acids; salinity; sorbitol; osmotic pressure; microalga; Antarctica.

Abstract—The major fatty acids in two *Dunaliella* isolates originating from Antarctic hypersaline lakes were 18:3ω3, 16:0, 16:4ω3. 18:1ω9, 18:2ω6, and 16:1ω7. The relative amount of individual fatty acids was modified by partially or fully replacing ionic osmotic pressure (Na^+, K^+ and Cl^-) with molecular osmotic pressure (sorbitol) in the growth medium. The effects of sorbitol on the extent of cellular fatty acid unsaturation differed in the two isolates. The results suggest that the appropriate environment necessary for the growth of these "halophilic" species is a certain level of osmotic pressure in the medium, but that this can be provided by either ionic or molecular osmotic pressure, or a mixture of both.

INTRODUCTION

The main functions of fatty acids and lipids in plants are to serve as structural components of cell membranes and, when present as storage products, as a potential source of energy and metabolic precursors. Fatty acids provide the hydrophobic interior of all cell membranes, forming an impermeable barrier to water and polar molecules, and separating the cell contents from the extracellular medium. The physical properties of the membrane are determined by the individual lipids therein, the fatty acid components of the lipids and their interaction with sterols and with proteins, which may be enzymes or part of the cytoskeletal material [1]. Modification of plant membrane lipid composition and distribution is often in evidence in the interactions of plants with their environment, especially as chemical changes in the composition of fatty acids. The fatty acid compositions of microalgae have been shown to change in response to changes in salinity [2–6]. Green unicellular microalgae of the genus *Dunaliella* are well known for their capability to grow at high salinities up to salt-saturated water. The two isolates used in the present work were obtained from Antarctic hypersaline lakes, where salinities were >200‰ and were subsequently maintained in culture in medium containing 1.6 M NaCl for more than three years before being used for the experiments reported here. Although there are some reports of salinity effects on fatty acid composition of total cellular lipids in microorganisms, there are few reports comparing of the effects of high ionic and molecular osmotic pressures on cellular fatty acid production of microalgae. For this purpose, we have examined the effects of high ionic, molecular, and a mixture of ionic and molecular osmotic pressures at an identical total osmotic pressure in the growth medium on the cellular fatty acid composition of two *Dunaliella* isolates originating from Antarctic hypersaline lakes.

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RESULTS AND DISCUSSION

The cellular fatty acid composition of total lipids in the two *Dunaliella* isolates during exponential growth in high ionic (NaCl or KCl) and molecular (sorbitol) osmotic pressures is shown in Table 1. The major fatty acids present in cultures of isolates LS21F and LL23B grown in medium with 1.6 M NaCl were linolenic, palmitic, oleic, hexadecatetrienoic and linoleic acids. Although there were 12 molecular species of C_{20} and C_{22} fatty acids identified in the two isolates, together they only accounted for less than 3% of the total. The fatty acid composition is typical of most green algae, with C_{16} and C_{18} polyunsaturated fatty acids (PUFA) being the most abundant. The fatty acid pattern of these *Dunaliella* spp. are thus similar to those reported for *D. tertiolecta* and *D. parva* [6–8] and six *Dunaliella*

Table 1. Fatty acid composition of LL23b and LS21F cells grown in modified PHK media. (SAFA: total saturated fatty acids, MUFA: total monounsaturated fatty acids, PUFA: total polyunsaturated fatty acids).

	LL23B						LS21F							
Fatty acid	Na	±	SNa	±	S	±	Na	±	SNa	±	S	±	KNa	±
14:0	0.8	0.3	0.7	0.1	0.3	0.2	0.1	0.2	0.2	0.2	0.4	0.3	0.2	0.0
14:1ω5	0.8	0.1	1.2	0.5	1.1	0.1	0.8	0.2	0.9	0.2	0.8	0.2	0.8	0.0
16:0	18.4	4.5	18.1	0.9	13.0	0.5	19.4	1.3	25.4	6.3	27.0	1.1	27.4	0.5
16:1 *trans*	0.0	0.0	0.1	0.0	0.0	0.0	0.1	0.1	0.2	0.3	0.1	0.1	0.1	0.0
16:1ω7	3.0	0.4	6.6	0.5	5.5	0.4	1.9	0.4	2.3	0.6	3.0	0.5	4.5	0.7
16:2ω9	0.6	0.2	0.4	0.0	0.4	0.1	1.7	0.2	1.8	0.2	1.5	0.2	1.0	0.1
16:ω6	0.3	0.0	0.5	0.0	0.5	0.0	0.2	0.0	0.1	0.1	0.2	0.0	0.3	0.0
16:3ω3	2.0	0.1	1.5	0.0	1.5	0.1	3.1	0.3	3.0	0.8	2.8	0.1	2.5	0.3
16:4ω3	10.7	2.2	12.7	0.9	15.2	0.8	9.0	0.2	6.9	1.4	5.6	0.5	9.1	0.3
18:0	0.8	0.2	0.7	0.1	0.6	0.0	1.0	0.0	1.3	0.4	1.8	0.2	0.9	0.0
18:1ω9	12.3	2.9	8.5	0.1	6.6	0.3	19.5	2.1	20.5	2.6	19.1	1.4	9.9	0.5
18:1ω7	1.4	0.3	2.2	0.0	2.3	0.1	1.3	0.0	1.4	0.5	1.4	0.3	1.3	0.0
18:2ω9	1.8	0.3	1.7	0.0	1.6	0.2	1.3	0.5	1.7	0.5	1.9	0.1	1.9	0.0
18:2ω6	6.5	0.1	4.5	0.1	4.0	0.6	11.3	1.0	9.7	0.5	9.4	0.5	7.6	0.2
18:3ω6	2.0	0.1	2.7	0.0	2.8	0.2	1.3	0.0	1.4	0.4	1.7	0.2	1.8	0.1
18:3ω3	32.4	3.4	29.5	1.0	37.1	2.3	23.4	0.7	18.0	3.3	16.2	1.0	22.1	0.8
18:4ω6	0.4	0.1	0.6	0.0	0.2	0.0	0.1	0.0	0.2	0.1	0.4	0.2	0.7	0.2
18:4ω3	2.0	0.1	1.1	0.0	1.2	0.1	2.3	0.2	1.8	0.0	1.6	0.1	3.0	0.0
20:0	0.1	0.2	0.2	0.0	0.1	0.0	0.1	0.0	0.1	0.1	0.2	0.0	0.1	0.1
20:1ω9	0.1	0.1	0.1	0.0	0.2	0.1	0.1	0.0	0.1	0.1	0.2	0.0	0.2	0.0
20:2ω6	0.1	0.1	0.2	0.1	0.2	0.1	0.2	0.0	0.2	0.1	0.3	0.3	0.2	0.0
20:3ω3	0.1	0.1	0.3	0.1	0.3	0.0	0.1	0.0	0.2	0.2	0.3	0.3	0.2	0.0
20:4ω6	0.2	0.1	0.4	0.2	0.4	0.2	0.1	0.1	0.2	0.3	0.5	0.6	0.2	0.0
20:5ω3	0.1	0.0	0.3	0.1	0.2	0.2	0.1	0.0	0.1	0.1	0.1	0.1	0.2	0.0
22:0	0.1	0.0	0.1	0.0	0.2	0.1	0.0	0.0	0.1	0.0	0.1	0.1	0.1	0.1
22:1ω9	0.1	0.0	0.2	0.1	0.1	0.0	0.0	0.0	0.1	0.0	0.1	0.0	0.1	0.1
22:2ω6	0.1	0.0	0.2	0.0	0.2	0.1	0.1	0.0	0.1	0.0	0.1	0.1	0.1	0.0
22:4ω6	0.1	0.0	0.3	0.1	0.3	0.2	0.1	0.0	0.1	0.0	0.1	0.0	0.2	0.0
22:6ω3	0.1	0.0	0.3	0.0	0.2	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0
24:1ω11	0.1	0.0	0.2	0.1	0.2	0.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0
Unknown	2.8	1.8	3.9	0.7	3.5	1.0	1.4	0.7	2.1	1.5	3.1	0.0	3.3	0.6
SAFA	20.2		19.8		14.1		20.6		27.0		29.5		28.7	
MUFA	17.7		19.0		16.1		23.7		25.5		24.8		16.9	
PUFA	62.1		61.2		69.8		55.7		47.5		45.8		54.4	
ω3PUFA	47.3		45.7		55.7		37.9		30.0		26.7		37.1	

isolates from the Dead Sea [9], but are different from that of the extremely halophilic species *D. salina* [2, 10, 11] and *D. bardawil* [12], in both the molecular species of fatty acids present and their relative amounts.

Fatty acid composition was also determined for the isolates grown in modified medium with either 1.6 M NaCl (Na, as a control), 1.6 M sorbitol (S), 1.2 M sorbitol plus 0.4 M NaCl (SNa) or 1.2 M KCl plus 0.4 M NaCl (KNa). There were no significant differences in fatty acid variations of the two isolates grown in the different cultures. The compositions obtained differed only in the relative amount of individual fatty acids, but showed different patterns, in relation to the various mixes of osmotica, in the two isolates. LL23B had six major fatty acids in cells grown in NaCl (Na culture), which accounted for 83.3% in total, with the order of abundance being 18:3ω3, 16:0, 16:4ω3. 18:1ω9, 18:2ω6, and 16:1ω7. When sorbitol was used to replace 75% of the NaCl (1.2 M sorbitol, SNa culture) or all of the NaCl (1.6 M sorbitol, S culture) in the growth medium, the six major fatty acids accounted for 79.9% in the SNa culture and 81.5% in the S culture. However, the order of abundance changed; 16:1ω7 was more abundant than 18:2ω6 in the SNa culture and 16:4ω3 became the second and 16:1ω7 became the fifth most abundant fatty acid in the S culture. Comparing the two sorbitol containing cultures with the control, 16:0, 18:1ω9 and 18:2ω6 decreased, whilst 16:1ω7, 16:4ω3, and 18:3ω3 increased. There were also large variations in S and SNa cultures in the relative amounts of the major groupings of fatty acid components. Thus, fatty acid unsaturation increased in the sorbitol culture compared with the control with total unsaturated fatty acids comprising 79.8% of total fatty acids in Na

culture, 80.2% in the SNa culture and 85.9% in the S culture. The ratio of ω6 to ω3 fatty acids was 1.31 in the Na cells, 1.34 in SNa cells and 1.25 in S cells.

Isolate LS21F had five major fatty acids in cells grown in NaCl medium (as a control) which accounted for 82.5% in total. The order of abundance was 18:3ω3, 18:1ω9, 16:0, 18:2ω6, and 16:4ω3. When 1.2 M and 1.6 M sorbitol was used to replace NaCl in the medium, these five fatty acids accounted for 80.5% in the S culture and 77.4% in the SNa culture. Palmitic acid became the most abundant fatty acid and 18:3ω3 dropped to the third most abundant fatty acid. By comparing the two sorbitol containing cultures with the control, 16:0 increased whilst 18:3ω3 and 16:4ω3 decreased. Both S and SNa cultures showed a similar pattern in major fatty acid abundance and differed only by moderate variations in the relative amounts of the components. Cultures of isolate LS21F grown with some Na replaced with K (KNa cultures) had six major fatty acids which together accounted for 76% of the total with the order of abundance being 16:0, 18:3ω3, 18:1ω9, 16:4ω3, 18:2ω6 and 16:1ω7. In comparison with the control, 16:0 and 16:1ω7 increased by 8% and 2.6%, respectively, 18:1ω9 decreased by 10% and the proportions of other fatty acids were only slightly changed. Fatty acid unsaturation decreased in sorbitol-grown cultures; unsaturated fatty acids were 79.4% of total fatty acids in Na cells, 73% in SNa cells, 70.5% in S cells and 71.3% in KNa cells. The ratio of ω6 to ω3 fatty acids was 1.47 in Na and KNa cells, 1.58 in SNa cells and 1.71 in S cells.

The degree of unsaturation of the fatty acids is important in determining the fluidity and conformation of cellular membranes and in providing the correct environment for membrane functions. Any changes in the fatty acid composition can be interpreted in terms of regulation of membrane fluidity [13] and in modification of membrane conformation to adapt to a new growth environment, since fatty acids mainly integrate into cellular membranes when cell are at mid-log phase growth. Under these conditions, partitioning of fatty acids into storage triacylglycerols will be minimal. The two *Dunaliella* isolates used here showed modification of their fatty acid composition when using sorbitol to replace 75% or 100% of the NaCl in the growth medium. It is not clear why the two isolates had reverse responses of fatty acid unsaturation to growth in high sorbitol medium. Perhaps this reflects the possibility that they are two different *Dunaliella* species and have their own, species-specific responses to sorbitol, which needs further investigation.

These *Dunaliella* isolates from Antarctic hypersaline lakes usually need a few days of adaptation to normal seawater medium (0.4 M NaCl) when directly transferred from 1.6 M NaCl medium before growth commences. However, they can adapt, without an apparent lag in growth, to 1.6 M sorbitol medium without NaCl immediately after being transferred to the medium (strictly speaking, there are still trace amounts of Na^+ and Cl^- present as contaminants in the other chemicals of the medium). There were significant differences in growth rates of the two isolates grown in the different cultures ($p < 0.05$) except SNa and N cultures of LS21F. The division rates of the LS21F cells grown in 75% and full sorbitol media were 38% and 110% faster, respectively, than those of cells grown in 1.6 M NaCl medium (Table 2). Corresponding increases in division rate for LL23B, relative to the control, were 89% faster for sorbitol + NaCl and 53% faster for sorbitol alone (Table 2). LL23B cells could not grow in medium with 1.2 M KCl plus 0.4 M NaCl and neither of the two isolates could survive in medium with only 1.6 M KCl. When LS21F was transferred to medium containing 1.2 M KCl plus 0.4 M NaCl (KNa culture), there was a lag period of two weeks and once growth started, the growth rate was 73% that of the control. These observations suggest that high ionic concentrations, principally Na^+ and Cl^-, are not mandatory requirements of culture media for those so called "halophilic" species to grow well. Indeed, high concentrations of Na^+ and Cl^- inhibit cell metabolism and growth, while high K^+ concentration has a higher inhibitory effect on cell growth than high Na^+ concentration. By replacing ionic osmotic pressure partially or fully with molecular osmotic pressure in the growth environment, the cells can show better performance in terms of growth rate and photosynthesis [14], changes which are presumably linked to modifications of cellular fatty acid composition and other cell components. It is clear that the proper growth environment necessary for these "halophilic" species growth is a certain level of osmotic pressure in the medium, rather than a high salt concentration *per se*.

Table 2. Growth rates (as divisions per day) of the two *Dunaliella* sp. isolates grown with various osmotica in the medium. Values are means ± range from two experiments.

Osmoticum in the medium (final concentration 1.6M)	ISOLATE	
	LS21F	LL23B
Na^+	0.060 ± 0.006	0.073 ± 0.006
Na^+ + Sorbitol	0.083 ± 0.003	0.138 ± 0.002
Sorbitol only	0.126 ± 0.020	0.112 ± 0.002
K^+ + Na^+	0.044 ± 0.002	no growth

EXPERIMENTAL

Species, medium and growth conditions. The species of green unicellular microalga used in this study, identified as *Dunaliella* spp. by Dr G. Hallegraeff, University of Tasmania, was isolated from Lake Stinear (isolate LS21F) and Lebed Lake (isolate LL23B) in the Vestfold Hills, Australian Antarctic territory. At the time of isolation, the salinity was 200‰ in Lake Stinear and 216‰ in Lebed Lake. The isolates were

grown in PHK medium, a modified "D" medium [[15] and Dr G. Kelly, Queensland University of Technology, personal communication]. The concn of NaCl, KCl and sorbitol were adjusted to 1.6 M by changing the concn of NaCl in PHK medium. Cultures were inoculated to *ca* 5×10^4 cells ml^{-1} using those cells grown in 1.6 M NaCl PHK medium at 5°. The cells grown in 1 l conical flask with 500 ml medium at 5° under continuous illumination provided from cool white fluorescent lamps and given a photon flux at the culture surface of 45 $\mu E\, m^{-2}\, s^{-1}$.

Growth rates Cell growth was monitored by periodical cell counts using an improved Neubauer haemocytometer. Growth rate, k, during exponential phase was calculated from the linear portion of plots of $\log_{10}$ (cell number/mL; N) vs time (t) as $k = [\log(\Delta N)]/\Delta t$. Division rates as divisions per day are given by $3.322 \times k$ [16].

Extraction of lipids for fatty acid analysis. Lipids were extracted by a modified method from ref. [17]. Cells (200×2 ml) in exponential growth phase ($\sim 5 \times 10^6$) were filtered onto glass fibre filters. Filtered cells were then extracted immediately with a mixt. of 8 ml dist. H_2O, 10 ml $CHCl_3$ and 20 ml MeOH, and sonicated for 10 min. $CHCl_3$ (10 ml) and dist. H_2O (10 ml) were added sequentially to the extract and sonicated for 10 min, respectively. The resulting soln was filtered under vacuum through a glass fibre filter and the filtrate separated by centrifugation. The lower layer of $CHCl_3$ was transferred and evapd under red. pres.

Fatty acid methylation. Fatty acids were converted to their ME esters prior to analysis by capillary GC. A method modified from ref. [18] was used for methylation. Total lipids were placed in a Teflon stoppered tube and methylated in 5 ml of a mixt. of BF_3 (14% BF_3 in MeOH), benzene and MeOH (5 : 4 : 11). The tube was subsequently flushed with N_2 and sealed tightly before being heated at 90° for 30 min. After cooling, fatty acid ME esters (FAME) were extracted with GC-grade hexane and analysed by GC immediately.

Fatty acid analysis. Analysis of FAME was carried out using a fused silica capillary column (J&W Scientific, DB-23, 0.25 mm film thickness, $30\,m \times 0.25$ mm I.D.) and a FID. N_2 was the carrier gas. Initial column temp. was 100° and increased to 160° at 10° min^{-1}, then programmed to 220° at 2.5° min^{-1} and finally heated to 240° at 10° min^{-1}. Injector and FID detector temp. were 250° and 280°, respectively. Samples of FAME (1 μl) were injected though a JADE valve. FAMEs were identified by chromatography by comparison with authentic standards (Sigma). For some compounds, standards were not available; in this case, identities were confirmed by GC-MS.

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