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Lipid and fatty acid composition of the green oleaginous alga *Parietochloris incisa*, the richest plant source of arachidonic acid

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Abstract

We have hypothesized that among algae of alpine environment there could be strains particularly rich in long chain polyunsaturated fatty acids (LC-PUFA). Indeed, the chlorophyte (Trebuxiophyceae) *Parietochloris incisa* isolated from Mt. Tateyama, Japan, was found to be the richest plant source of the pharmaceutically valuable LC-PUFA, arachidonic acid (AA, 20:4ω6). The alga is also extremely rich in triacylglycerols (TAG), which reaches 43% (of total fatty acids) in the logarithmic phase and up to 77% in the stationary phase. In contrast to most algae whose TAG are made of mainly saturated and monounsaturated fatty acids, TAG of *P. incisa* are the major lipid class where AA is deposited, reaching up to 47% in the stationary phase. Except for the presence of AA, the PUFA composition of the chloroplastic lipids resembled that of green algae, consisting predominantly of C_{16} and C_{18} PUFAs. The composition of the extrachloroplastic lipids is rare, including phosphatidylcholine (PC), phosphatidylethanolamine (PE) as well as diacylglyceryltrimethylhomoserine (DGTS). PC and PE are particularly rich in AA and are also the major depots of the presumed precursors of AA, 18:3ω6 and 20:3ω6, respectively.

Keywords: *Parietochloris incisa*; Trebuxiophyceae; Lipid and fatty acid composition; Algal lipids; Triacylglycerols; Arachidonic acid; LC-PUFA

1. Introduction

Long-chain polyunsaturated fatty acids (LC-PUFAs) of the ω3 family are quite abundant in microalgae. For example, *Porphyridium cruentum* (Cohen et al., 1988), *Nannochloropsis* sp. (Seto et al., 1984; Sukenik and Carmeli, 1989), *Phaeodactylum tricornutum* (Yongmanitchai and Ward, 1992; Molina Grima et al., 1999) and *Monodus subterraneus* (Cohen, 1994) were studied for their potential to produce eicosapentaenoic acid (EPA, 20:5ω3). Likewise, *Crypthecodinium cohnii* (Jiang et al., 1999) and *Chroomonas salina* (Henderson and MacKinlay, 1992) contain docosahexaenoic acid (DHA, 22:6ω3). However, ω6 LC-PUFAs are relatively rare. High contents of 20:3ω6 were not found in any organism. Arachidonic acid (AA, 20:4ω6) is almost excluded from the lipids of fresh water algae and in most marine species it does not account for more than a few per cent of total fatty acids (Thompson, 1996; Table 1). The only microalga reported to produce AA in significant quantities is the unicellular rhodophyte *P. cruentum* (Cohen, 1990). Under logarithmic growth, the major PUFA of this alga is EPA, but under unfavorable conditions, AA predominated, reaching 40% of total fatty acids. In other rhodophytes such as *Gracilaria* sp., the proportion of AA can be as high as 60% of total fatty acids; however, the dry weight content does not exceed 0.2% (Araki et al., 1990). When present, LC-PUFAs are predominantly located in the polar membranal lipids, whereas triacylglycerols (TAG) generally contain very little PUFAs (Cohen, 1999).

Detailed lipid analyses of relatively few species of green algae have been carried out, mainly of those that have been widely used as model systems for biochemical studies. For example, most of these present a fatty acid composition similar to that of higher plants, characterized by the predominance of C_{16} and C_{18} trienoic fatty acids (Thompson, 1996). We have isolated an alga from the snowy slopes of Mt. Tateyama in Japan. This strain

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was later identified as the Chlorophyte (Trebuxiophyceae) *P. incisa* comb. nov (Watanabe et al., 1996). We have studied the fatty acid and lipid composition of this species and found it to be one of the richest sources of AA. This finding is of particular interest since AA, as well as DHA, the major PUFAs of brain cell phospholipids (Hansen et al., 1997), have been shown to improve infant development (Koletzko and Brown, 1991; Agostoni et al., 1994). Indeed, addition of AA to newborn milk formula was recently recommended, especially for pre-term infants (Clandinin et al., 1992). In this paper, we discuss the unique fatty acid and lipid composition of this alga with respect to its resistance to low temperatures.

2. Results

AA is the major fatty acid of *P. incisa*, comprising 33.6% of total fatty acids in the logarithmic phase and 42.5% in the stationary phase (Table 2). Even higher proportions (up to 60%) were obtained under nitrogen starvation (Bigogno, 2000). Other major fatty acids were 16:0, 18:1 and 18:2. Among the minor fatty acids there were ω6 PUFAs such as 18:3ω6 and 20:3ω6 as well as PUFAs of the ω3 family, 16:3ω3, 18:3ω3 and 20:5ω3.

Both the fatty acid composition and the fatty acid content changed with the aging of the culture. The fatty acid content increased from 8.9% (of dry weight) at a cell concentration of 1.3 g (dry wt.) l^{-1} to 27.1% at 5.4 g l^{-1} (Fig. 1, top). The proportion of AA increased from 25.6 to 44.9%, respectively (Fig. 1, bottom). Concurrently, the proportions of 16:3ω3 and l8:3ω3 sharply decreased from 7.5 and 12.5% to 0.5 and 1.3%, respectively (not shown). Consequently, the content of AA increased dramatically from 2.3 to 12.2% (Fig. 1, top).

As in other chloroccoccales, the polar lipid profile of *P. incisa* includes the galactolipids, monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG), the sulfolipid, sulfoquinovosyldiacylglycerol (SQDG) and the phospholipids, phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylinositol (PI) and phosphatidic acid (PA) (Table 2). *P. incisa* contains also a betaine lipid, diacylglyceryltrimethylhomoserine (DGTS). Even in logarithmic cultures, TAG was the major lipid class, comprising 43% of total fatty acids (Table 2). In the stationary phase, its proportion increased even further to 77% at the expense of the polar lipids, except for DGTS, which was not significantly changed, becoming one of the major polar lipids (5%).

With the exception of the presence of AA, the fatty acid composition of MGDG was not too different from that of typical green algae such as *Chlorella* (Safford and Nichols, 1970). The major fatty acids in the logarithmic phase were characterized by the presence of the

Table 1
Major fatty acid composition of algae relatively rich in arachidonic acid

Species	Major fatty acids (% of total)														Ref.
	14:0	16:0	16:1	16:2	16:3	18:0	18:1	18:2	18:3 ω6	18:3 ω3	18:4 ω3	20:4 ω6	20:5 ω3	22:6 ω3	
Bacillariophyceae															
Thalassiosira pseudonana	15	10	29	5	6			1				14	15		[a]
Chlorophyceae															
Parietochloris incisa		10	2	1	1	3	16	17	1	2		43	1		[b]
Dinophyceae															
Amphidinium carteri	2	12	1	2		2	2	1	3		19	20		24	[a]
Phaeophyceae															
Desmarestia acculeata	4	12	2				7	6	10	2	16	19	19		[c]
Dictyopteris membranacea	6	20	1			2	14	14	11	2	11	11	9		[d]
Ectocarpus fasciculatus	2	17				1	13	4	15	1	23	11	13		[e]
Prasinophyceae															
Ochromonas danica	13	4				3	7	26	12	7	7	8			[f]
Rhodophyceae															
Gracilaria confervoides	8	18	3			1	16	2		1	1	46			[c]
Phycodrys sinuosa	5	22	5	2	1	3	5	1	1			44	2		[c]
Porphyridium cruenturn 1380-la		34	1			1	2	12		1		40	7		[g]

[a] Cobelas and Lechado, 1989.
[b] This work.
[c] Pohl and Zurheide, 1979.
[d] Hofman and Eichenberger, 1997.
[e] Makewitcz et al., 1997.
[f] Vogel and Eichenberger, 1992.
[g] Cohen, 1990.

ω3 fatty acids, 18:3ω3 (31.9%) and 16:3ω3 (20.5%). In the stationary phase, the proportion of these fatty acids decreased while that of their respective ω6 precursors, 18:2 and 16:2, increased. The analysis of individual components revealed indeed that the major molecular species of MGDG are the prokaryotic species 18:3ω3/16:3ω3 and 18:2ω6/16:2ω6 (Fig. 2, top). A similar shift from 18:3ω3 to 18:2 took place also in DGDG. The latter contained very little C_{16} PUFAs (Table 2) and had a relatively high proportion of 16:0 (16%) in the logarithmic phase, and even a higher proportion in the stationary phase (34%). In the logarithmic phase, AA was a major component of both galactolipids, however, in contrast to other ω6 fatty acids, its proportion decreased sharply at the stationary phase. The sulfolipid, SQDG, was rich in 16:0, 18:1ω7, 18:2 and 18:3ω3. In the stationary phase, the proportion of 18:3ω3 was drastically reduced, while that of 16:0 and 18:1ω7 increased.

PE and PC are the major polar lipids where ω6 PUFAs are deposited in *P. incisa*. In the logarithmic phase, the proportion of AA, in these lipids, reached 43 and 21%, significantly decreasing to 9.6 and 14.3%, respectively, in the stationary phase. Similar decreases in the proportion of AA were noted in all other polar lipids. PE was also extremely rich (18%) in 18:1ω7, a fatty acid, which is more common to bacteria (Asselineau, 1966) and relatively rare in algae. In the stationary phase, the proportion of 18:2 and 18:1ω7 increased in PC and PE at the expense of AA.

TAG, the major lipid of *P. incisa*, contained 43% AA, as well as 16:0, 18:1 and 18:2. In the stationary phase, the proportion of AA slightly increased to 47% at the expense of 16:0. Due to the sharp increase in the accumulation of TAG at the stationary phase (Table 2), the share of cellular AA that was deposited in TAG increased from 60% in the logarithmic phase to 90% in

Table 2
Fatty acids composition of the lipids of *Parietochloris incisa* in the logarithmic (L) and stationary (S) phases

Lipid class		Lipid distribution[a]	Fatty acid composition (% of total fatty acids)													
			16:0	16:1 ω11	16:1 ω7	16:2 ω6	16:3 ω3	18:0	18:1 ω9	18:1 ω7	18:2 ω6	18:3 ω6	18:3 ω3	20:3 ω6	20:4 ω6	20:5 ω3
Biomass	L		13.9	4.7	0.2	1.7	4.0	1.7	6.7	5.1	13.2	1.5	10.3	1.2	33.6	1.7
	S		10.1	1.8	tr	1.3	0.9	2.5	12.2	4.2	17.2	0.8	2.0	1.0	42.5	0.7
MGDG	L	22.1	1.9	1.0	–	8.5	20.5	0.4	3.6	0.8	14.5	0.8	31.9	0.3	13.9	1.1
	S	4.9	3.4	1.2	–	20.8	11.0	0.5	4.3	0.0	31.4	0.7	18.5	0.2	6.1	0.6
DGDG	L	14.2	16.0	1.1	1.1	1.4	1.7	1.9	5.8	4.0	26.0	1.5	18.6	0.7	18.1	1.4
	S	6.8	34.0	0.5	2.2	0.2	0.6	4.5	6.9	3.0	31.0	2.4	2.9	0.4	7.6	0.6
SQDG	L	4.0	36.0	0.3	–	–	–	2.3	3.9	13.0	21.2	1.1	19.4	–	2.7	–
	S	2.8	54.6	0.5	–	–	–	1.1	3.6	20.2	17.0	0.9	1.6	–	0.4	–
PG	L	2.2	26.4	3.0	–	0.6	tr	2.8	7.5	4.5	30.8	3.9	11.9	–	2.6	–
	S	0.2	31.5	9.6	–	2.7	1.5	4.2	12.8	5.0	16.8	1.1	1.7	–	2.1	–
PC	L	5.7	29.5	2.7	–	0.2	0.5	4.2	11.8	2.0	16.0	4.5	2.3	1.9	21.0	1.8
	S	0.7	31.2	1.4	–	–	–	5.5	8.9	15.4	21.1	2.6	1.4	0.7	9.6	–
DGTS	L	4.0	47.1	1.8	1.4	0.3	0.5	7.4	2.0	7.4	9.4	3.6	1.9	0.9	15.2	0.8
	S	4.6	30.0	0.4	0.8	2.6	0.6	3.9	5.4	2.8	34.2	3.3	3.5	0.4	10.0	0.6
PE	L	2.9	11.1	1.4	tr	0.2	1.3	3.9	4.2	18.2	4.8	1.2	3.0	4.1	43.2	1.7
	S	0.6	19.8	4.5	tr	0.3	4.8	7.1	4.3	24.7	11.2	1.5	1.8	2.0	14.3	–
PA	L	1.1	32.3	1.6	–	–	–	9.2	13.3	5.7	17.0	–	6.7	0.0	14.2	–
	S	1.4	32.8	1.2	0.6	0.7	0.2	3.2	10.8	3.7	34.8	–	3.4	0.4	8.3	–
PI	L	0.7	43.3	1.6	–	–	–	4.4	5.6	8.8	16.3	2.5	1.3	0.0	16.3	–
	S	0.5	42.4	1.4	–	–	–	4.0	11.2	5.7	22.0	1.3	0.7	0.4	9.9	–
TAG	L	42.9	13.3	0.5	tr	tr	0.4	3.7	15.3	6.8	10.4	1.1	1.0	1.5	43.0	1.0
	S	77.1	8.4	0.4	tr	tr	tr	3.1	18.0	4.0	14.1	0.7	0.4	1.1	47.1	0.7

[a] Lipid distribution (% of total fatty acids): – undetected; L, log phase; S, stationary phase; tr, trace levels (<0.1%); PA, phosphatidic acid; PI, phosphatidylinositol. The fatty acids 18:4ω3, 20:0, 20:1 and 20:2 were present at less than 1%. In PG, 16:1Δ3t constituted 5.8 and 10% (of fatty acids) in the log and stationary phases, respectively.

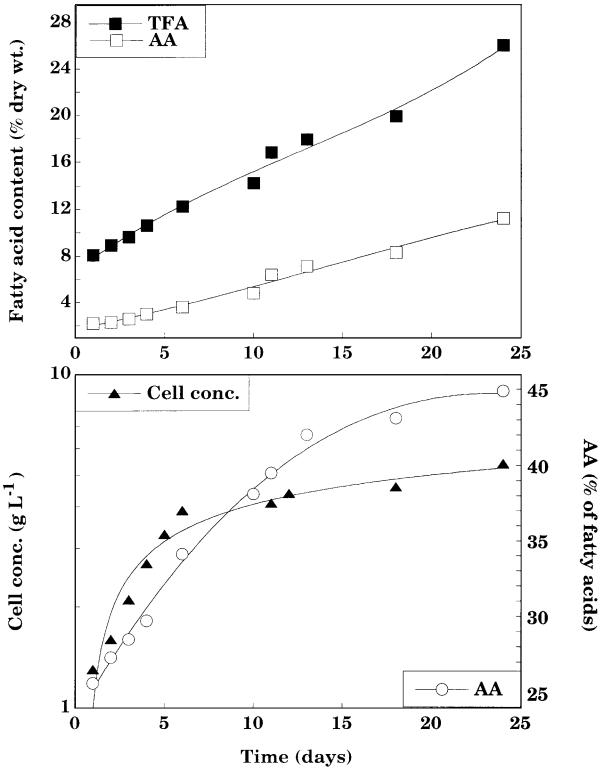

Fig. 1. Changes in cell concentration, proportion of arachidonic acid, and total fatty acid and arachidonic acid content, during batch cultivation of *Parietochloris incisa*. Fatty acid composition and content were determined by gas chromatography (GC) following transmethylation.

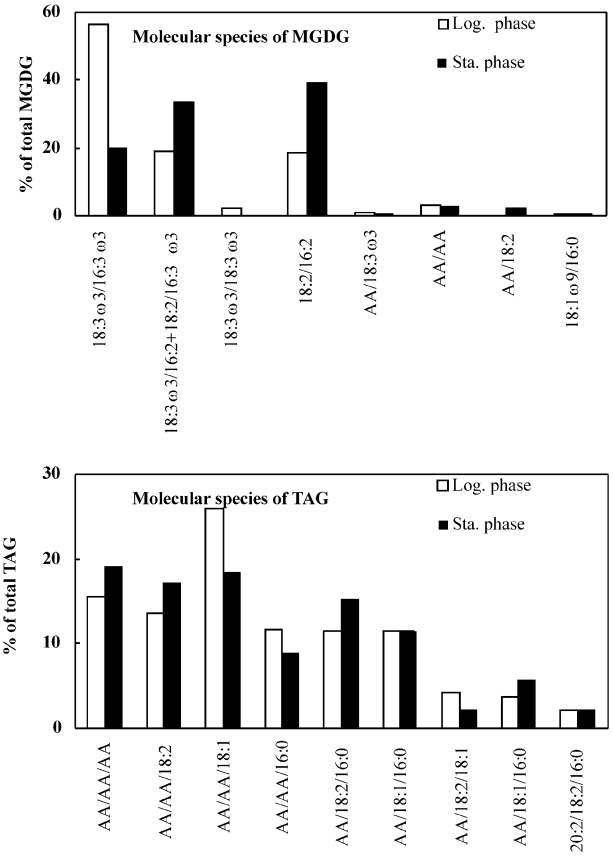

Fig. 2. Composition of the major molecular species of monogalactosyldiacylglycerol (MGDG; top) and triacylglycerols (TAG; bottom) of *Parietochloris incisa* in the logarithmic and stationary phases. Lipids were separated by 2D TLC and anaylzed by gas chromatography (GC) following transmethylation. Positional distribution was not determined.

the stationary phase. The major molecular species of TAG contain two arachidonyl moieties (AA/AA/16:0, AA/AA/18:1 and AA/AA/18:2) or even three (triarachidonin, AA/AA/AA) (Fig. 2, bottom). Only minor changes were noted on transfer to the stationary phase; the proportion of AA/AA/18:2 and AA/AA/AA increased at the expense of the more saturated species AA/AA/16:0 and AA/AA/18:1.

3. Discussion

The ability of cells to accumulate PUFAs is intrinsically limited in most algae, since these fatty acids are generally components of membranal lipids whose content is strictly regulated. Nevertheless, certain algal species can be induced to synthesize and accumulate extremely high proportions of TAG (Shifrin and Chisholm, 1981). In *Pavlova lutheri*, TAG represent 40% of total lipids in the logarithmic phase and 80% in the stationary phase (Eichenberger and Gribi, 1997). High TAG contents were found also in several ice algae such as *Nitzschia frigida* and *Melosira antartica* (Falk-Peterson et al., 1998). However, while PUFA-rich higher plant oils are rather common, algal TAG are generally characterized by saturated and monounsaturated fatty acids (Piorreck et al., 1984; Henderson and Sargent, 1989). This characteristic seems to be common to most algae studied for their potential to produce long chain PUFAs. The TAG of the eustigmatophyte *Nannochloropsis* sp., an EPA producer (Seto et al., 1984), contain mainly 14:0, 16:0 and 16:1 (Sukenik et al., 1993). A similar composition was found in another EPA producer, the diatom *P. tricornutum* (Yongmanitchai and Ward, 1992). Likewise, TAG of the DHA-rich cryptomonad *C. sauna* (Henderson and MacKinlay, 1992) are almost entirely made of C_{18} fatty acids. In contrast, TAG rich in AA were found in several rhodophytes. For example, the proportion of AA in TAG was reported to be 36% in *Chondrus crispus*, 49% in *Polysyphonia lanosa* (Pettitt et al., 1989) and 40–64% in various *Gracilaria* sp. (Araki et al., 1990), yet the TAG content of these algae is rather low, varying from 0.03 to 0.16% (of dry wt.; Araki et al., 1990). It appears thus that even in the few algae capable of producing PUFA-rich TAG, these TAG do not accumulate to a significant extent.

Cohen et al. (1988) and Cohen (1990) have shown that TAG of the red microalga *P. cruentum*, are unusually rich in AA. According to the prevailing dogma, plants accumulate TAG as a store of energy. However, our studies have shown that at low temperatures, *P. cruentum* can utilize AA of TAG for rapid biosynthesis of the eukaryotic-like molecular species of MGDG, which contains AA or EPA in both positions (Khozin-Goldberg et al., 2000). Radiolabelling of *P. cruentum* with acetate have shown that labeled EPA appeared in the chloroplast more than 10 h after the pulse (Khozin et al., 1997), suggesting that algae that are exposed to rapid changes of temperature would have difficulties in increasing their chloroplastic PUFA content, especially LC-PUFAs, at low temperatures by de novo synthesis. The protective role of PUFAs against the damaging effect of high light intensity and UV radiation, especially at low temperature, may also be important in the ability of the organism to survive and adapt in extreme environments (Whitelam and Codd, 1986). The capability to store LC-PUFAs in TAG as a buffer capacity would allow the organism to swiftly adapt to the rapidly changing environment. In order to test this hypothesis, we have collected several algal species from the soil of Mt. Tateyama. This place is characterized by a wide range of temperatures, from freezing to over 20 °C, and light intensity varying from normal to very high, due to snow reflection. Indeed, we have isolated a strain of *P. incisa* which is, to the best of our knowledge, the first reported alga capable of accumulating large quantities of TAG that are particularly rich in any PUFA. Nitrogen starvation induced the production of AA, resulting in an extremely high content of 21% (of dry wt.; Bigogno, 2000).

We have further speculated that the unique ability of *P. incisa* to simultaneously accumulate AA and TAG, allows this organism to utilize TAG as a reservoir of AA, for rapid membrane construction following sharp and fast changes in environmental conditions. This could explain the ability of this organism to retain much of its photosynthetic activity at low temperatures (data not shown). Indeed, when exponential cultures of *P. incisa* were labeled at 25° C with [1-^{14}C]arachidonic acid we have found that following a reduction in temperature, a rapid transfer of label from TAG to polar lipids took place, while at 25 °C, the level of radioactivity remained relatively stable in both TAG and polar lipids (Cohen et al., 2000; Bigogno et al., 2002a).

A shift from ω3 to ω6 PUFAs in chloroplastic lipids that ensues a transfer from the logarithmic to the stationary phase was reported to occur in many microalgae. For example, an increase in 20:4ω6 at the expense of 20:5ω3 was reported in *P. cruentum* (Cohen et al., 1988). Klyachko Gurvich et al. (1999) suggested that ω3 PUFAs are necessary for the maintenance of photosystem I (PSI) and that the accumulation of the ω6 precursors in the stationary phase enables the organism to rapidly produce ω3 PUFAs once growth conditions are resumed. In the stationary phase, the proportion of 18:2 in MGDG and DGDG of *P. incisa* increases at the expense of 18:3ω3. However, AA is not significantly converted to 20:5ω3.

The lipid composition of *P. incisa* is also unusual. The contemporary presence of DGTS, PC and PE is not very common. DGTS is abundant in many species of green algae such as *Dunaliella salina*, *Chlamydodmonas reinhardtii* and *Volvox carteri* and appeared to be located in non-plastidial membranes (Harwood and Jones, 1989; Thompson, 1996). DGTS resembles PC in some aspects and generally occurs when PC is either low or absent (Vogel and Eichenberger, 1992). The coexistence of these three lipids was reported to occur also in the EPA-producing eustigmatophytes *Nannochloropcis* sp. (Schneider and Roessler, 1994) and *Monodus subterraneus* (Nichols and Appleby, 1969). Generally however, DGTS appears with either PC or PE. For example, *C. reinhardtii* (Giroud et al., 1988) contains PE and DGTS, while PC and DGTS are found in *D. parva* (Evans et al., 1982) and *D. salina* (Norman and Thompson, 1985). The co-occurrence of these lipids in *P. incisa* appears to be related to the production of AA. We have recently shown (Bigogno et al., 2002b) that PC and DGTS are the major substrates for the sequential Δ12 and Δ6 desaturation of oleic acid to 18:3ω6, while PE and PC serve as substrates for the Δ5 desaturation of 20:3ω6 to AA.

The capacity for AA accumulation makes *P. incisa* one of the best candidates for large-scale production of AA. This PUFA was shown to be a major component of brain cell membranes, as well as of breast milk (Hansen et al., 1997). It was thus suggested that the diet of preterm infants that are not breast-fed should be implemented with AA by supplementation of newborn milk formula (Carlson et al., 1993; Boswell et al., 1996). The finding that most AA of *P. incisa* is deposited in TAG is of practical value since TAG are the preferred chemical form for the introduction of AA into baby formula.

4. Experimental

4.1. Growth condition

A culture of *P. incisa* was isolated from Mt. Tateyama in Japan (Watanabe et al., 1996). An axenic culture was obtained by means of successive dilutions and isolations of individual cell colonies grown on a solidified BG11 medium in Petri dishes (Stanier et al., 1971). Cultures were grown on BG11 medium, in 150-ml Erlenmeyer

flasks under an air/CO_2 (99:1) atmosphere. The flasks were placed in an incubator shaker at 25 °C and illuminated continuously from above at a light intensity of 115 μmol quanta m^{-2} s^{-1}.

4.2. Lipid extraction

Freeze-dried samples of *P. incisa* biomass were extracted with methanol containing 10% DMSO, by warming to 40 °C for 5 min and stirring at 40 °C for another hour. The mixture was centrifuged, the supernatant removed and the pellet was re-extracted with a mixture of hexane and ether (1:1, v/v). Diethyl ether, hexane and water were added to the combined supernatants, so as to form a ratio of 1:1:1:1 (v/v/v/v). The mixture was shaken and then centrifuged for 5 min at 35×100 rpm and the upper phase was collected. The water phase was re-extracted twice with a mixture of diethyl ether:hexane (1:1, v/v). The organic phases were combined and evaporated to dryness. The diethyl ether utilized in the extractions and the lipid analysis was peroxide-free and contained 0.01% butylated hydroxytoluene (BHT).

4.3. Lipid analysis

Polar lipids were separated by two-dimensional TLC using a solvent system of chloroform:methanol:water (65:25:4, v/v/v) for the first direction and of chloroform: methanol: 1 - ethylpropylamine:conc. ammonia (65:35: 0.5:5, v/v/v/v) for the second direction. Neutral lipids were resolved with petroleum ether:diethyl ether:acetic acid (80:20:1, v/v/v). MGDG and DGDG eluted from the silica gel plates were separated to their constituent molecular species by reversed-phase HPLC as previously described (Khozin et al., 1997).

4.4. Fatty acid analysis

Freeze-dried cells, lipid extracts, or individual lipids were transmethylated with 2% H_2SO_4 in methanol at 70 °C for 1 h. Heptadecanoic acid was added as an internal standard. Gas chromatographic analysis was performed according to Cohen et al. (1993). Fatty acid methyl esters were identified by co-chromatography with authentic standards (Sigma Co., St Louis) and by comparison of their equivalent chain length (Ackman, 1969). The data shown represent mean values with a range of less than 5% for major peaks (over 10% of fatty acids) and 10% for minor peaks, of at least two independent samples, each analyzed in duplicate.

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