Fatty acid desaturation-defective mutants of an arachidonic-acid-producing fungus, *Mortierella alpina* 1S-4

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Three mutants, which were defective in the desaturation of fatty acids, were isolated from an arachidonic-acid-producing fungus, *Mortierella alpina* 1S-4, after treating wild-type spores with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. They were designated Mut44, Mut48 and Mut49. Mut44 was a mutant with low $\Delta 5$ -desaturase activity. It accumulated a high level of dihomog- γ -linolenic acid (DGLA) (28.6%, w/w) but a low level of arachidonic acid (Ara) (10.6%), compared with the wild type, which had levels of 6.3 and 47.0%, respectively. Mut48 was unable to desaturate oleic acid (18:1) to linoleic acid (18:2), i.e. $\Delta 12$ -desaturation, and therefore a large amount of 18:1 (49.5%) accumulated and no fatty acid of the ω -6 family was detected. In addition, several fatty acids of the ω -9 family, such as 5,8,11-*cis*-eicosatrienoic acid, were found. In Mut49, 18:2 (46%) accumulated markedly, but only small amounts of DGLA and Ara were detected. Thus, Mut49 was considered to be defective in $\Delta 6$ -desaturation. These mutants showed a somewhat longer lag phase than the wild type on cultivation at both 28 and 12 °C.

Introduction

During the course of a study on the production of polyunsaturated fatty acids (PUFAs), such as dihomog- γ -linolenic acid (DGLA), arachidonic acid (Ara), 5,8,11,14,17-*cis*-eicosapentaenoic acid (EPA) and so on, by micro-organisms, we have found that many members of the *Mortierella* subgenus, especially the soil isolate *Mortierella alpina* 1S-4, are potent producers of these PUFAs (Yamada *et al.*, 1987; Shimizu *et al.*, 1988a, b, 1989; Shinmen *et al.*, 1989). However, how these PUFAs are produced has remained unclear. In order to obtain further details on their biosynthesis by this fungus, with a view to the possibility of increasing their production, we concentrated on the isolation of PUFA biosynthesis mutants.

In this paper, we report the isolation and character-

ization of three mutants of *M. alpina* 1S-4, which are considered to be completely defective (or to have low activities) in $\Delta 5$ -, $\Delta 6$ - and $\Delta 12$ -desaturation, respectively.

Methods

Chemicals. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) and triolein were obtained from Nacalai Tesque. Phospholipids were obtained from the Serdary Research Laboratory, London, Canada. All authentic fatty acids were obtained from Sigma except for Mead acid, which was purchased from Biomol Research Laboratories, Plymouth Meeting, PA, USA. All other reagents were of analytical grade.

Mutagenesis with MNNG. *M. alpina* 1S-4 (Yamada *et al.*, 1987; Shimizu *et al.*, 1988b) was inoculated onto Czapek agar medium (0.2% NaNO₃, 0.1% K₂HPO₄, 0.05% MgSO₄·7H₂O, 0.05% KCl, 0.001% FeSO₄·7H₂O, 3%, w/v, sucrose and 2%, w/v, agar, pH 6.0) and allowed to sporulate at 28 °C for 2 weeks. Spores were collected by pouring 50 ml sterile water containing a drop of Tween 40 into the medium, followed by filtration through 4-layers of gauze and centrifugation of the filtrate at 5000 r.p.m. for 10 min. The spores thus obtained were suspended in 50 mM-Tris/maleate buffer (pH 7.5) at a concentration of approximately 1×10^6 ml⁻¹ and then exposed to MNNG (100 µg ml⁻¹) at 28 °C with reciprocal shaking for 20 min. This resulted in survival rates of 0.1–5%. Three millilitres of 10% (w/v) sodium thiosulphate was added, followed by centrifugation at 5000 r.p.m. for 10 min and two washes with sterile distilled water.

Isolation of mutants. The MNNG-treated spores were inoculated into medium GY (2%, w/v, glucose and 1%, w/v, yeast extract, pH 6.0) and then incubated at 12 °C (or 6 or 15 °C) for 2–3 d. Each culture was

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Abbreviations: Ara, arachidonic acid; DGLA, dihomog- γ -linolenic acid; EPA, 5,8,11,14,17-*cis*-eicosapentaenoic acid; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; TG, triglycerides; 18:2 ω 9, 6,9-*cis*-octadecadienoic acid; 18:3 α , α -linolenic acid; 18:3 γ , γ -linolenic acid; 18:4,6,9,12,15-*cis*-octadecatetraenoic acid; 20:2 ω 9, 8,11-*cis*-eicosadienoic acid; 20:3 ω 3, 11,14,17-*cis*-eicosatrienoic acid; 20:3 ω 9, 5,8,11-*cis*-eicosatrienoic acid; 20:4 ω 3, 8,11,14,17-*cis*-eicosatetraenoic acid.

Fig. 1. Gas chromatograms of the fatty acid methyl esters obtained from *M. alpina* 1S-4 (wild type) and mutants Mut44, Mut48 and Mut49, defective in $\Delta 5$ -, $\Delta 12$ - and $\Delta 6$ -desaturation, respectively. The shaded peaks indicate the most abundant components.

filtered through a glass filter (pore diam., 20–30 μm) and the filtrate was then centrifuged to collect the non-germinated spores (about 1–10% of the total treated spores). The spores thus obtained were then plated on a Petri dish (90 mm diam.) containing an agar medium (1% glucose, 0.5% yeast extract, 0.005% Triton X-100 and 2% agar), followed by incubation at 28 °C for 2 d, when colonies appeared. About 100 colonies for each mutagenesis were randomly picked up and each of them was grown in liquid culture as described below. After incubation at 28 °C for 5 d, the cells were harvested, dried at 100 °C, and their fatty acid compositions analysed by GLC on a glass column (3 mm $\times$ 2 m) packed with 5% Advanced DS on 80/100 mesh Chromasorb W (Nishio Kogyo). The column temperature was 190 °C and other conditions were as given in detail previously (Shimizu *et al.*, 1991a).

Liquid culture. *M. alpina* 1S-4 (or a mutant) was inoculated as a spore suspension into a 10 ml Erlenmeyer flask containing 2 ml medium GY, and then the culture was incubated at 28 °C with reciprocal shaking (120 strokes min^{-1}) for 1 week, unless stated otherwise. The mycelial cells were harvested by suction filtration, washed with distilled water, and then dried at 100 °C overnight for subsequent fatty acid analysis by GLC as described above.

Lipid analysis. Extraction and fractionation into triglycerides and phospholipids were performed as described previously (Shimizu *et al.*, 1991a), except that the bands of lipids were detected under ultraviolet light (366 nm) after spraying with 0.01% premulin dissolved in acetone–water (4:1, v/v), and that methyl heptadecanoate was used as an internal standard.

Other methods. Fungal growth was measured by determining the mycelial weight after drying at 100 °C overnight. Fatty acids were identified by comparing their retention times on GLC with those of authentic standards. When authentic standard fatty acids were not commercially available, the fatty acids were isolated by reverse phase HPLC (Shimizu *et al.*, 1991a) and their structures were determined on the basis of both proton NMR and mass spectra of the corresponding

picolinyl esters prepared as described by Christie *et al.* (1986). The conditions for mass spectrometry and proton NMR were as described previously (Shimizu *et al.*, 1991a).

Results

Selection and characterization of mutants

Based on the assumption that mutants defective in fatty acid desaturases would be low-temperature sensitive, as in the case of the fatty acid desaturation-defective mutants of the cyanobacterium *Synechocystis* PCC6803 (Wada & Murata, 1989), we focussed on the isolation of low temperature sensitive mutants. In order to increase the proportion of low temperature sensitive mutants, MNNG-treated spores were first incubated in medium GY at various temperatures lower than the physiological one for the wild type (28 °C), as described in Methods. On testing about 1300 colonies, we found three such mutants, designated Mut44, Mut48 and Mut49. These mutants were not distinguishable from the wild type in morphology, but their fatty acid profiles were apparently different from that of the wild type (Fig. 1). Mut44 accumulated a significantly high percentage content of DGLA (28.6%, w/w) but a low Ara content (10.6%), compared with those in the case of the wild type, which were 6.3 and 47.0%, respectively. This phenomenon was similar to that previously reported for the inhibition of desaturation of DGLA to Ara by sesamin, that is,

Table 1. Fatty acid profiles of Mut48 and Mut49 grown in medium supplemented with fatty acids

Cells were cultured in duplicate in a medium containing 1% glucose and 0.5% yeast extract (pH 6.0) at 28 °C for 1 d, and then methyl linoleate or methyl γ -linolenate was added to one culture as an albumin complex, which was prepared by sonicating 10 mg of the fatty acid methyl ester with 1 ml sterile 5% (w/v) bovine serum albumin for 20 min at a concentration of 0.5 mg (ml culture broth)⁻¹. The values are means of two determinations. The control represents cells grown without the addition of fatty acids.

Fatty acid	Fatty acid content (% w/w)			
	Mut48		Mut49	
	Control	+18:2	Control	+18:3 γ
16:0	14.6	12.2	16.3	13.8
18:0	7.4	6.9	11.8	11.3
18:1	54.5	54.2	10.7	18.4
18:2	—	—	43.0	32.4
18:2 ω 9	10.9	11.8†	—	—
18:3 γ /20:0*	0.8	1.0	1.7	2.7
20:1	2.3	2.7	0.6	1.1
20:2	—	—	1.8	2.4
20:2 ω 9	1.6	2.0	—	—
20:3 ω 9	3.7	3.8	—	—
DGLA/22:0*	1.6	1.1	1.8	1.7
20:3	—	—	5.0	3.4
Ara	—	2.2	5.0	9.5
24:0	2.6	2.1	2.3	3.3

—, Undetectable.

* These pairs of fatty acids were not separated under the GLC conditions used. Both were present as minor components, as judged on reverse phase HPLC, in all cases except in Mut48 grown without linoleic acid addition, in which only 20:0 and 22:0 were found.

† Includes linoleic acid.

Δ 5-desaturation (Shimizu *et al.*, 1991b). Therefore, Mut44 was considered to be a mutant with low Δ 5-desaturase activity. The fatty acid composition of Mut48 included 18:1 (49.5%) as the most abundant fatty acid component and there was no fatty acid of the ω -6 family in its mycelia. It was striking that several fatty acids of the ω -9 family were accumulated. The C_{20:3} fatty acid was identified as 5,8,11-*cis*-eicosatrienoic acid (20:3 ω 9) by comparing its mass spectra and proton NMR with those of the authentic compound. The remainder were identified on the basis of proton NMR and mass spectra of the corresponding picolinyl esters as 6,9-*cis*-octadecadienoic acid (18:2 ω 9) and 8,11-*cis*-eicosadienoic acid (20:2 ω 9). In Mut49, it was found that 46% of the total mycelial fatty acids was 18:2 and that the amounts of downstream fatty acid metabolites, i.e. 18:3 γ , DGLA and Ara, were very small compared with those in the case of the wild-type. As shown in Table 1, Mut48 produced Ara on cultivation in medium supplemented with 18:2.

Similarly, the percentage content of Ara in Mut49 was double that of the control on growth in a medium supplemented with 18:3 γ . These observations suggested that Mut48 is only defective in Δ 12-desaturase, the other elongation and desaturation activities being retained, and that only Δ 6-desaturase in Mut49 was defective.

Lipid composition

The lipid composition of the mutants and the wild type grown at 28 and 12 °C are compared in Table 2. The major lipids of all strains were triglyceride (TG), phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylserine (PS). In the wild type grown at 28 °C, the relative content of TG was 82 mol%, and that of PC, PE and PS was 7, 8 and 3 mol%, respectively. The lipid composition of Mut44 and Mut49 were almost the same as that of the wild type, but that of Mut48 included a somewhat higher ratio of phospholipids. At 12 °C, all strains contained a somewhat higher ratio of phospholipids, as compared with that in cells grown at 28 °C.

Fatty acid composition on growth at low temperature

The fatty acid composition of the mutants and wild type grown at 28 or 12 °C is given in Table 3. Regardless of the growth temperature, the wild type contained the highest level of Ara (44–46%, w/w), whilst Mut44, Mut48 and Mut49 contained the highest levels of DGLA (32–34%), 18:1 (46–50%) and 18:2 (25–32%), respectively. Mut44, like the wild type, accumulated several fatty acids of the ω -3 family, i.e. 6,9,12,15-*cis*-octadecatetraenoic acid (18:4), 8,11,14,17-*cis*-eicosatetraenoic acid (20:4 ω 3) and EPA, when grown at 12 °C. It is notable that the percentage content of 20:4 ω 3 in Mut44 (6.0%) was much higher than that in the case of the wild type (0.4%), whilst EPA was decreased. In Mut48, there was no significant change in the fatty acid components when grown at a lower temperature, except for a marked increase in the 20:3 ω 9 content from 9.3% at 28 °C to 18.2% at 12 °C. In addition to the high content of 18:2, Mut49 showed some interesting changes in its fatty acid profile, as follows. Firstly, there was an unknown fatty acid, which was considered from mass spectra to be a C_{20:3} fatty acid. However, its retention time on GLC was different from those of DGLA and 20:3 ω 9. Secondly, the proportion of Ara increased from 6.3 to 22.7% at 12 °C. Thirdly, there was an additional C_{20:4} fatty acid when it was grown at 12 °C. This fatty acid was entirely separated from Ara and 20:4 ω 3 on high-resolution capillary GLC (Rosco Silar-5CP, 0.25 mm $\times$ 50 m; Nihon Chromato Works) (data not shown). The structures of these unknown PUFAs are being determined.

Table 2. Lipid composition of the fatty acid desaturation-defective mutants of *M. alpina* 1S-4

All strains were grown in medium GY at either 28 °C for 7 d or 12 °C for 10 d. The values are means of two independent determinations. Other minor components such as sterols, steroyl esters and glycolipids were not included for the calculation.

Lipids	Lipid composition (mol %)							
	12 °C				28 °C			
	1S-4	Mut44	Mut48	Mut49	1S-4	Mut44	Mut48	Mut49
TG	76	80	66	72	82	84	76	83
PE	10	8	14	10	7	6	9	6
PC	10	9	14	13	8	7	11	8
PS	4	3	6	5	3	3	4	3

Table 3. Comparison of fatty acid composition of the fatty acid desaturation-defective mutants of *M. alpina* 1S-4

All strains were grown in medium GY at either 28 °C for 7 d or at 12 °C for 6 d after preincubation for 1 d at 28 °C. All values are means of two determinations.

Fatty acid	Fatty acid content (% w/w)							
	12 °C				28 °C			
	1S-4	Mut44	Mut48	Mut49	1S-4	Mut44	Mut48	Mut49
16:0	8.6	6.9	4.6	5.8	11.1	10.8	9.9	12.7
18:0	8.9	9.9	8.0	10.2	5.3	7.8	6.1	10.7
18:1	10.3	11.8	46.4	8.7	13.7	11.1	49.3	15.3
18:2	5.4	4.2	—	25.5	7.4	5.6	—	31.8
18:2 ω 9	—	—	12.1	—	—	—	12.1	—
18:3 γ /20:0*	4.9	9.5	0.7	2.3	4.6	9.6	0.7	1.7
18:3 α /20:1†	0.9	1.4	3.6	2.7	1.1	1.1	2.7	1.0
18:4	0.2	1.0	—	—	—	—	—	—
20:2	1.0	1.1	—	6.1	0.6	1.0	—	2.1
20:2 ω 9	—	—	3.2	—	—	—	2.1	—
DGLA/22:0*	7.8	33.9	1.5	1.7	6.5	32.8	2.2	2.9
20:3 ω 9	—	—	18.2	—	—	—	9.3	—
20:3‡	—	—	—	6.4	—	—	—	7.8
20:3 ω 3/Ara§	45.4	8.1	—	22.7	44.4	11.7	—	6.3
20:4 ω 3	0.4	6.0	—	—	—	—	—	—
20:4‡	—	—	—	3.2	—	—	—	—
24:0	2.2	3.9	1.7	3.3	5.2	8.5	5.6	7.8
EPA	4.1	2.4	—	1.5	—	—	—	—

—, Undetectable.

* Both were found in all strains except for Mut48, in which 18:3 γ and DGLA were not detected.

† Only 20:1 was found in all strains on growth at 28 °C but 18:3 α was found as an additional fatty acid on growth at 12 °C, except in the case of Mut48, in which 18:3 α was not detected even at this temperature.

‡ These fatty acids were determined to be eicosatrienoic acid (20:3) and eicosatetraenoic acid (20:4), respectively, but the positions of the double bonds have not yet been determined.

§ Trace amounts of 20:3 ω 3 were detected on growth at 12 °C.

Growth characteristics and fatty acid content

Each of the three mutants was inoculated as a spore suspension into medium GY and grown at either 28 or 12 °C after preincubation at 28 °C for 1 d. All the

mutants grew somewhat more slowly, especially at 12 °C, than the wild type (Fig. 2). At 12 °C the lag phase for the wild type was about 1 d, whilst that for the mutants were about 2 d. The total mycelial fatty acid content corresponded to the growth rate, but reached a plateau more

Fig. 2. Growth and total mycelial fatty acid content of *M. alpina* 1S-4 and mutants. Growth (○) is expressed as mg dry mycelial mass (ml culture broth)⁻¹ and total mycelial fatty acids (□) are expressed as mg fatty acids (g dry mycelial mass)⁻¹. All values shown are means of two determinations.

slowly than the growth rate. All mutants accumulated almost the same level of fatty acids when grown at 28 °C for 5 d or at 12 °C for 7 d.

Discussion

As far as we know, this paper is the first to report the derivation of mutants defective in fatty acid desaturation of an Ara-producing fungus. Based on the fatty acid profiles, the mutants reported here are considered to be defective in $\Delta 5$ -, $\Delta 12$ - and $\Delta 6$ -desaturation. In Mut44 and Mut49, which are considered to be defective in $\Delta 5$ - and $\Delta 6$ -desaturation, respectively, Ara still existed. The occurrence of Ara may be explained by either of the following assumptions. The explanation for the lack of desaturation in the mutants is that mutation could occur in the genes encoding desaturases. It is possible that the $\Delta 5$ - or $\Delta 6$ -desaturase produced from the respective modified gene still retained some activity. Another possible assumption is that there could be two kinds of desaturases for desaturation at one position in the fatty acids, i.e. one requiring acyl-CoA ester and another requiring lipid-linked fatty acids (Pugh & Kates, 1977; Kamisaka *et al.*, 1990). The formation of 20:3 ω 9 in Mut48 would occur through the same sequential reactions suggested by Fulco & Mead (1959), i.e. 18:1 was desaturated at the $\Delta 6$ -position into 18:2 ω 9 followed by elongation and $\Delta 5$ -desaturation to 20:3 ω 9. The reason for the marked increases in the levels of 20:3 ω 9 and Ara in Mut48 and Mut49, respectively, on the temperature shift to 12 °C (Table 3), is still unclear, but it is possible that $\Delta 6$ -desaturase becomes more active at low temperature, or that the mutation of $\Delta 6$ -desaturation in Mut49 was temperature sensitive.

It has been suggested that one of the roles of PUFAs is the maintenance of the proper fluidity of biological

membranes, especially at low temperatures (Chapman, 1975). Though the mutants reported here germinated somewhat slower than the wild type at low temperature (12 °C; data not shown), they grew as well as the wild type. This observation may suggest that Ara and other ω -6 fatty acids are not absolutely required for normal growth of this fungus, and that the other unusual PUFAs, such as 20:3 ω 9 in Mut48 or the unknown fatty acids in Mut49, may compensate for their requirement of fatty acids for the maintenance of proper membrane fluidity.

Like the wild type, all the mutants accumulated a relatively high level of total fatty acids, and therefore they are promising with regard to their use in the fermentative production of fatty acids. Also, these mutants may be useful for cloning of genes encoding fungal-type desaturases by means of complementation of the mutants.

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