Isolation and Characterization of a Δ9-Desaturation-Defective Mutant of an Arachidonic Acid-Producing Fungus, *Mortierella alpina* 1S-4

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ABSTRACT: A mutant, considered to be defective in Δ9-desaturation, of an arachidonic acid-producing fungus, *Mortierella alpina* 1S-4, was isolated after treating the wild-type spores with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine. The mycelial FA of this mutant included 38% stearic acid, the level being only 5% for the wild type. Upon growth at 24–28°C, its mycelial lipids included a high level (up to 50 mol% of total mycelial lipids) of FFA, of which about 90 mol% was stearic acid. However, the level of FFA was markedly decreased with a concomitant increase in the TAG level when the mutant was grown at 20°C or lower, or when it was grown in a culture medium supplemented with PUFA.

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KEY WORDS: Δ9-Desaturation, free fatty acids, *Mortierella*, mutation.

During the course of a study on the production of PUFA, such as dihomo-γ-linolenic acid (DHGA), arachidonic acid (AA), 5(*Z*),8(*Z*),11(*Z*),14(*Z*),17(*Z*)-eicosapentaenoic acid and so on, by microorganisms, we found that many members of the subgenus *Mortierella* are potent producers of these PUFA (1–4). Among them, one soil isolate, *Mortierella alpina* 1S-4, was considered to be the best producer. However, the mechanism by which these PUFA are produced has remained unclear. In order to obtain further details of their biosynthesis by this fungus, with a view to increasing their production, we have concentrated on the isolation of PUFA-biosynthesizing mutants.

In previous papers (5–7), we reported the isolation of three mutants considered to be completely defective (or to have low activity) in Δ6-, Δ12-, or Δ5-desaturase. Several n-9 FA, such as 5(*Z*),8(*Z*),11(*Z*)-eicosatrienoic acid (Mead acid), were found in the Δ12-desaturation-defective mutant, and two nonmethylene-interrupted FA, 5(*Z*),11(*Z*),14(*Z*)-eicosatrienoic acid and 5(*Z*),11(*Z*),14(*Z*),17(*Z*)-eicosatetraenoic acid, were detected in the Δ6-desaturation-defective mutant (8). These FA have never been detected in the wild type. The formation of these FA was considered to be due to the conversion of oleic acid (in the Δ12-desaturation-defective mutant) or linoleic acid (in the Δ6-desaturation-defective mutant) by the enzymes catalyzing the reactions that follow the defective step.

The FA desaturase and elongase genes of *M. alpina* for PUFA biosynthesis have been cloned (9–12). As far as the Δ9-desaturase is concerned, we reported the cloning and heterologous expression of *M. alpina* 1S-4 Δ9-desaturase gene (13). Wongwathanarat *et al.* (14) demonstrated that some strains of *M. alpina* sp. had two distinct Δ9-desaturase genes, *ole1* and *ole2*. The *ole1* gene was expressed in all strains of *M. alpina*, whereas the *ole2* gene was only detected in one of the six strains of *M. alpina* (14).

In this paper, we report the isolation and characterization of a mutant of *M. alpina* 1S-4, which is considered to have low Δ9-desaturation activity. This mutant showed several characteristics different from those of the mutants reported previously with respect to its lipid composition.

MATERIALS AND METHODS

Chemicals. [1-^{14}C]Stearic acid (18:0) (2.07 GBq/mmol), [1-^{14}C]oleic acid (18:1) (1.92 GBq/mmol), [1-^{14}C]linoleic acid (18:2) (1.89 GBq/mmol), and [2-^{14}C]dihomo-γ-linolenic acid (20:3) (2.13 GBq/mmol) were purchased from Amersham (Little Chalfont, Buckinghamshire, United Kingdom). All other chemicals used were commercially available and as described previously (7,15).

Microorganisms. Mortierella alpina 1S-4 and its mutants, Mut 48, Mut 49 and S14, which are defective in Δ12-, Δ6-, and Δ5-desaturation activity, respectively (7,16), were used. *Mortierella alpina* 1S-4 was stocked in the AKU culture collection (Department of Agriculture, Kyoto, University) as 3998.

Mutagenesis and isolation of mutants. Mutagenesis and the selection of mutants were performed essentially by the same methods as described previously (7). In brief, *M. alpina* 1S-4 spores were exposed to *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG), and then the selection of mutants was performed by analyzing the FA of vegetative mycelia after the growth of these MNNG-treated spores for a few days. Mutants with FA compositions different from that of the wild type were picked up and stored on a potato dextrose agar medium.

Liquid culture. Mortierella alpina 1S-4 and its mutants were each inoculated as a spore suspension into 10-mL

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Erlenmeyer flasks containing 2 mL of medium GY [2% (wt/vol) glucose and 1% (wt/vol) yeast extract, pH 6.0], followed by incubation with reciprocal shaking (120 strokes min^{-1}) at 28°C for 1 wk, unless otherwise noted.

Preparation of cell-free extracts. This was done as described previously (17). Protein concentrations were measured by the method of Bradford (18).

Assay of desaturation activity. The desaturation activity was measured using a labeled FA as a substrate (19,20). Each reaction mixture (1 mL) comprised 250 µmol sucrose, 100 µmol potassium phosphate buffer (pH 7.4), 45 µmol NaF, 150 µmol KCl, 1.5 µmol glutathione, 0.5 µmol nicotinamide, 5 µmol $MgCl_2$, 7.5 µmol ATP, 0.4 µmol CoA, 1.5 µmol NADH, 100 µmol FA (18:0, 18:1, 18:2, or 20:3 containing 3.7 KBq of the respective labeled FA) and the cell-free extract (1 mg protein). The reaction mixtures were incubated at 28°C for 30 min and terminated by the addition of 5 mL of ethanol and 1 mL of 4 N KOH. Procedures for saponification of reaction mixtures, extraction of FA, preparation of FAME and silica gel/silver nitrate TLC, and the quantitative analysis of radioactive FAME were as described previously (20).

Analysis of FA and lipids. The fungal cells were harvested by suction filtration and then dried at 100°C overnight. The dried cells were directly transmethylated with 10% methanolic HCl, and the resultant FAME were extracted with *n*-hexane and then analyzed by GLC. The column used was a glass one (3 mm × 3 m) packed with Advance DS (Nishio Kogyo, Tokyo, Japan). The column temperature was 195°C, and the other conditions were as given in detail previously (20). Fungal lipids were extracted with $CHCl_3/CH_3OH$ (2:1, vol/vol) according to Folch *et al.* (21). The lipids were separated into individual lipid classes by TLC, and the FA composition of each lipid class was analyzed by GLC as described previously (20). Lipid compositions were calculated from the amounts of total FA of individual lipid classes as recommended by Christie *et al.* (22). Heptadecanoate was usually added before transmethylation as an internal standard.

Determination of the positional distribution of FA in phospholipids. PC and PE were isolated from the total lipids by TLC as mentioned above, extracted with $CHCl_3/CH_3OH$ (2:1, vol/vol), and then concentrated with a rotary evaporator. The extracted PC and PE were hydrolyzed with a snake venom (*Ophiophagus hannah*) that contains phospholipase A_2, and FFA liberated from the *sn*-2 position of phospholipids were isolated by TLC and analyzed as to the FA composition by GLC (23).

Other methods. Fungal growth was measured by determining the mycelial weight after drying at 100°C overnight. All values shown in the figures and tables are the means of three independent determinations.

RESULTS

Isolation and characterization of the Δ9-desaturation-defective mutant. After treatment with 0.1 mg/ml of MNNG as a final concentration for 20 min, spore survival was approximately 0.01%. Through analysis of the FA composition of the 2,000 colonies of the vegetative mycelia derived from the MNNG-treated spores, we obtained a mutant that has a high stearic acid (18:0) level. This mutant (designated as T4) was not distinguishable from the wild type in morphology, but its FA profile was apparently different. The major (>1%) mycelial FA of T4 were palmitic acid (16:0, 8.2%), 18:0 (38%), oleic acid (18:1n-9, 5.2%), linoleic acid (3.9%), γ-linolenic acid (11.9%), DHGA (1.1%), and AA (31.7%); the corresponding values for the wild type were 11.1, 5.3, 13.7, 7.4, 4.6, 6.5, and 44.5%, respectively.

TABLE 1
Desaturation Activities of *Mortierella alpina* 1S-4 and Its Mutants

	Desaturase activities (pmol/min/mg)[a]			
Strain	Δ9	Δ12	Δ6	Δ5
1S-4	10.5	7.0	7.7	9.2
S14	11.1	9.0	7.8	<1.0
Mut48	10.8	1.0	7.8	14.3
Mut49	10.2	4.1	<0.5	11.4
T4	<0.5	7.5	7.9	10.3

[a]All activities were measured under the conditions given in the Materials and Methods section. Values are means of three independent assays (SD = ± 7%).

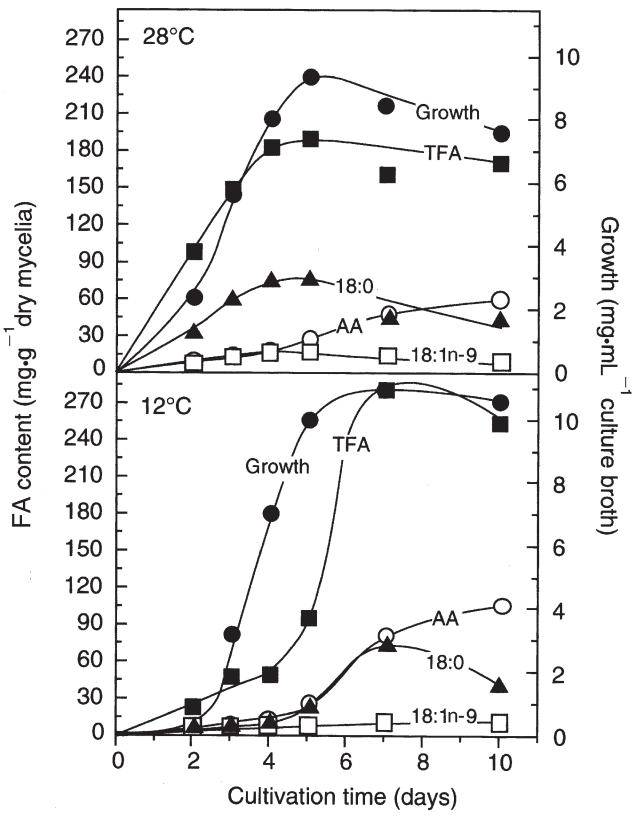

FIG. 1. Changes with time in mycelial concentrations of some FA in *Mortierella alpina* 1S-4 T4. The fungus was grown as described in the Materials and Methods section at 28°C for 1 d, after which the temperature was shifted to 12°C or kept at 28°C, and then growth was continued for the times indicated. "Cultivation time" includes the first day at 28°C. AA, arachidonic acid; TFA, total FA.

TABLE 2
FA Compositions of the Major Lipids in *M. alpina* 1S-4 and the Δ9-Desaturase-Defective Mutant on Growth at 28°C

Fraction[a]	Lipid comp.[a] (mol%)	FA composition (mol%)[b] 16:0	18:0	18:1n-9	18:2n-6	18:3n-6 20:0[c]	20:1n-9	20:2n-6	DHGA 22:0[c]	AA	24:0
T4											
TAG	13.5	17.2	55.7	4.7	1.6	12.0	—[d]	—	2.6	6.0	0.2
FFA	51.4	9.1	88.0	0.6	—	1.3	—	—	0.6	0.5	—
DAG	19.9	16.0	30.1	3.2	3.9	25.6	—	—	1.7	19.4	—
PE	7.2	11.4	26.6	4.2	4.5	26.4	—	—	2.7	24.2	—
PC	4.3	10.3	19.2	1.4	5.0	35.7	—	—	1.5	26.8	—
PS	3.7	23.2	38.5	4.7	5.2	12.1	—	—	3.2	13.2	—
Wild type											
TAG	81.1	23.7	8.7	18.3	8.5	3.5	1.2	0.6	5.8	28.0	1.7
FFA	3.1	26.6	16.5	21.7	5.4	2.6	—	3.2	4.5	15.9	3.6
DAG	3.5	15.8	6.4	31.1	10.1	11.8	0.6	0.7	3.4	20.2	—
PE	3.9	20.2	5.6	22.8	12.1	12.4	1.5	1.3	1.6	22.5	—
PC	6.1	17.9	2.5	7.3	16.9	5.4	0.5	5.2	1.0	43.4	—
PS	2.2	34.2	6.7	21.0	17.5	4.3	0.4	1.5	2.6	11.8	—

[a]Lipid composition. Other minor lipids, such as sterols, sterol esters, and glycolipids, were not included for the calculation.
[b]All values are means calculated from three separate cultures. Abbreviations: 16:0, palmitic acid; 18:0, stearic acid; 18:1n-9, oleic acid; 18:n-6, linoleic acid; 18:3n-6, linolenic acid; 20:0, arachidic acid; 20:1n-9, 11-eicosenoic acid; 20:2n-6, 11,14-eicosadienoic acid; DHGA, dihomo-γ-linolenic acid; 22:0, behenic acid; AA, arachidonic acid; 24:0, lignoceric acid.
[c]The first FA (18:3n-6 or DHGA) of the paired FA was the major (more than 90%) component.
[d]—, Undetectable.

Desaturation activities of strain T4 were compared with those of the parental strain 1S-4 and other mutants previously reported. The Δ9-desaturation activity in T4 was about 20-fold less than that of other strains; the other desaturation activities were retained (Table 1). These results suggest that T4 is only defective in Δ9 desaturation.

Time courses of changes in FA contents. Representative changes with time of the mycelial concentrations of FA are shown in Figure 1. When growth proceeded at 28°C, fungal growth in terms of dry mycelial mass and the concentrations of total mycelial FA usually reached a plateau in about 5 d, the maximal values being lower than those for growth at 12°C. At this growth temperature, the concentration of 18:0 increased markedly from 2 to 4 d cultivation, while the increase was much slower on growth at 12°C. The level of the final product of the n-6 FA biosynthetic route, AA, increased gradually with time, its concentration being higher than that of 18:0 throughout the cultivation at 12°C.

Distribution of FA among major lipids. Upon cultivation at 28°C, the mycelial lipids of the T4 strain included markedly high levels of FFA and DAG, as compared to those of the wild type (Table 2). FFA accounted for as much as 51 mol% of the total mycelial lipids, the FA composition including about 90 mol% 18:0. The high FFA level remained unchanged even when the cultivation time was extended to 10 d (data not shown). About 90 mol% of 18:0 accumulated in this mutant was found in TAG, FFA, and DAG, in the ratio of 26:50:14. Although the AA level was only about 6% in TAG, the levels in DAG, PE, PC, and PS were much higher.

Effect of temperature on the lipid composition. The high FFA level found with growth at 28°C decreased markedly on growth at low temperature (Fig. 2). The critical temperature for FFA accumulation was found to be within the range of 20–24°C. Compared with growth at 24°C, the fungus grown at 20°C showed a markedly reduced level of FFA, but its mycelial FA composition did not differ significantly (data not

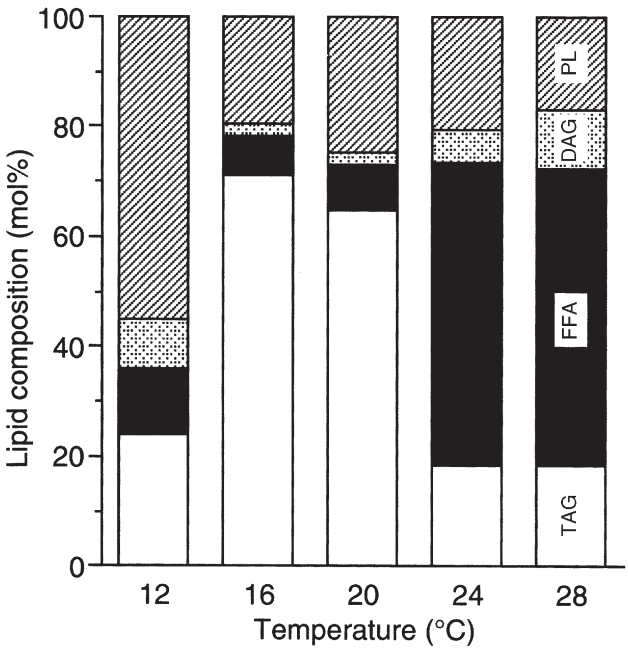

FIG. 2. Effect of growth temperature on the lipid composition of *M. alpina* 1S-4 T4. The fungus was grown in 100-mL Erlenmeyer flasks containing 20 mL of medium GY at 28°C for 2 d, after which the temperature was shifted to the temperature indicated, and then growth was continued for 5 d. PL, polar lipids (mainly phospholipids); for other abbreviation see Figure 1.

TABLE 3
Positional Distribution of FA in the Phospholipids of *M. alpina* 1S-4 and the Δ9-Desaturase-Defective Mutants

		FA composition (mol %)[a]								
Strain	Position	16:0	18:0	18:1n-9	18:2n-6	18:3n-6 20:0[b]	20:1n-9	20:2n-6	DHGA 22:0[b]	AA
PC[c]										
1S-4	*sn*-1	35.7	5.6	7.1	5.2	2.9	0.8	—[d]	0.2	42.5
	sn-2	6.2	4.1	8.6	27.7	3.9	—	0.3	3.1	46.2
T4	*sn*-1	20.2	29.9	2.4	1.7	22.4	—	—	0.5	23.0
	sn-2	8.0	12.2	3.0	5.3	40.9	—	—	1.5	29.1
PE[c]										
1S-4	*sn*-1	34.1	7.6	40.0	7.6	2.6	2.5	0.2	1.0	4.4
	sn-2	6.1	4.7	13.4	20.7	11.3	—	—	2.2	41.8
T4	*sn*-1	21.6	58.3	2.8	2.2	10.8	0.1	—	1.1	3.2
	sn-2	6.2	8.6	4.9	5.0	28.8	0.2	—	2.7	43.5

[a]All values are means calculated from three separate cultures. For abbreviations see Tables 1 and 2.
[b]The first FA (18:3n-6 or DHGA) of the paired FA was the major (more than 90%) component.
[c]PC and PE were derived from fungi grown at 28°C for 7 d.
[d]—, Undetectable.

shown). The phospholipid level was markedly high on growth at 12°C, but it decreased to about the same level as for growth at 16°C on growth for a further 3 d (data not shown).

Positional distribution of FA in phospholipids. Determination of the FA at each position of phospholipids revealed that 16:0 and 18:0 of PE and PC predominantly exist at the *sn*-1 position in the T4 strain (Table 3). Also, in the wild type, 16:0 was predominantly found at the *sn*-1 position in both PE and PC, but instead of 18:0 (the most abundant FA found at the *sn*-1 position of both PE and PC in the T4 strain), 18:1n-9 was the most abundant FA at the *sn*-1 position of PE. The FA at the *sn*-2 position of PE and PC in the T4 strain included more than 75 mol% PUFA.

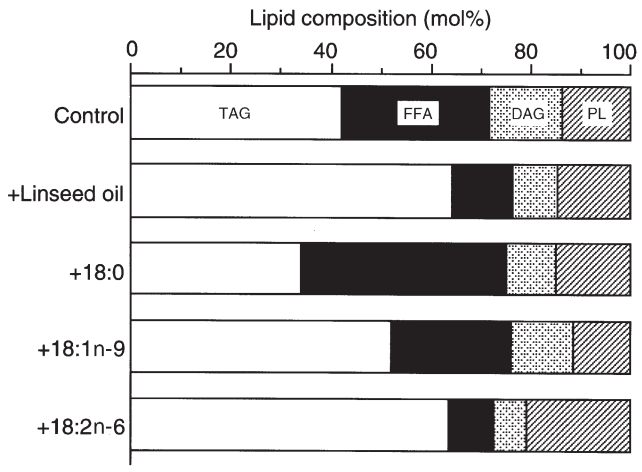

FIG. 3. Effects of FA and linseed oil on the lipid composition of *M. alpina* 1S-4 T4. The fungus was grown in a 50-mL Erlenmeyer flask containing 10 mL of medium GY at 28°C for 1 d, after which the linseed oil methyl ester (1% vol/vol culture broth) or the methyl ester of the FA (0.5% vol/vol culture broth) indicated was added, and then growth was continued for a further 6 d. For abbreviations see Figures 1 and 2.

Effects of FA on the lipid composition. According to the preceding results on positional distribution analysis of FA, it was postulated that FFA were accumulated in the T4 strain because of a lack of PUFA, which are the major compositional FA found at the *sn*-2 position. Therefore, we examined the effects of some FA on fungal lipid composition. Linseed oil was selected as an alternative source for α-linolenic acid since the fungus could not grow when a pure acid was used. As shown in Figure 3, the addition of 18:2n-6 or linseed oil methyl ester (a mixture of 10% 16:0, 4% 18:0, 10% 18:1n-9, 18% 18:2n-6, and 58% α-linolenic acid) was found to decrease the FFA level markedly, but 18:1n-9, which was efficiently incorporated into the fungal cells, could not decrease the FFA level as effectively as 18:2n-6 or linseed oil did. Also, 18:0 and 16:0 (data not shown) did not decrease the FFA level, but their effects were unclear since these FA crystallized under the growth temperature used and most of them remained in the culture medium throughout the cultivation.

DISCUSSION

Although Δ9-desaturase-defective mutants have been isolated from some organisms, such as Chinese hamster ovary cells (24) and *Saccharomyces cerevisiae* (25), we are the first to report such a mutant of an AA-producing fungus. The Δ9-desaturase-defective T4 strain obtained here differed from these mutants in that it does not require exogenous unsaturated FA for normal growth. The T4 mutant is considered to be leaky, as it produced AA and some other metabolites of Δ9-desaturation. Differing from the Δ12- and Δ6-desaturase-defective mutants (5,8), its mycelial FA did not include any FA other than those found in the wild type. This observation indicates that the accumulated 18:0 cannot serve as a substrate for other enzymes, such as Δ12-desaturase, involved in PUFA biosynthesis, and that the first double bond needs to be inserted at the Δ9-position.

In the T4 mutant, the compositions of 18:0 (38%) and γ-linolenic acid (11.9%) in the total FA were higher than those of wild-type strain. The high content of 18:0 was due to low activity of Δ9-desaturase in the T4 mutant. In general, γ-linolenic acid is readily converted to DHGA by FA elongase in the wild-type strain. The high content of γ-linolenic acid in the T4 mutant may be explained by lower activity of FA elongase using γ-linolenic acid as a good substrate.

The mutant is also different from the mutants reported previously (7) in its lipid composition, which included markedly high levels of FFA and DAG, but a low TAG level. Because 90 mol% of the FFA was 18:0, the accumulation of FFA was considered to be due to the conversion of 18:0 derivatives (presumably the CoA-esters) to free acid. The slower growth of the T4 mutant than that of *M. alpina* 1S-4 may be due to potential toxicity of the accumulated FFA. Several routes, such as esterification to glycerol 3-phosphate and subsequent esterification to monoacylglycerol 3-phosphate to form PA, esterification to DAG to form TAG, hydrolysis to a FA, and desaturation to 18:1n-9, would be involved in the conversion of 18:0-CoA. Analysis of the positional distribution of FA in PC and PE revealed that 18:0 existed predominantly at the *sn*-1 position, with PUFA being at the *sn*-2 position. According to these observations, we suppose that 18:0-CoA was converted to a free acid because there was not enough PUFA to be esterified at the *sn*-2 position of monoacylglycerol 3-phosphate, which resulted in the accumulation of 18:0-CoA. This speculation is supported in part by the observation that FFA decreased with the addition of PUFA to the culture medium (Fig. 3). However, further work is required to prove this speculation, and to determine the means by which the FFA level decreased upon lowering of the growth temperature, though there was no significant change in the FA composition. Also, the mechanism of γ-linolenic acid accumulation in this mutant remains to be clarified.

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REFERENCES

1. Shimizu, S., H. Kawashima, Y. Shinmen, K. Akimoto, and H. Yamada, Production of Eicosapentaenoic Acid by *Mortierella* Fungi, *J. Am. Oil Chem. Soc. 65*:1455–1459 (1988).
2. Shimizu, S., Y. Shinmen, H. Kawashima, K. Akimoto, and H. Yamada, Fungal Mycelia as a Novel Source of Eicosapentaenoic Acid, Activation of Enzyme(s) Involved in Eicosapentaenoic Acid Production at Low Temperature, *Biochem. Biophys. Res. Commun. 150*:335–341 (1988).
3. Shinmen, Y., S. Shimizu, K. Akimoto, H. Kawashima, and H. Yamada, Production of Arachidonic Acid by *Mortierella* Fungi: Selection of a Potent Producer and Optimization of Culture Conditions for Large Scale Production, *Appl. Environ. Microbiol. 31*:11–16 (1989).
4. Yamada, H., S. Shimizu, and Y. Shinmen, Production of Arachidonic Acid by *Mortierella alpina* 1S-5, *Agric. Biol. Chem. 51*:785–790 (1987).
5. Jareonkitmongkol, S., H. Kawashima, S. Shimizu, and H. Yamada, Production of 5,8,11-*cis*-Eicosatrienoic Acid by a Δ12-Desaturase-Defective Mutant of *Mortierella alpina* 1S-4, *J. Am. Oil Chem. Soc. 69*:939–944 (1992).
6. Jareonkitmongkol, S., H. Kawashima, N. Shirasaka, S. Shimizu, and H. Yamada, Production of Dihomo-γ-linolenic Acid by a Δ5-Desaturase-Defective Mutant of *Mortierella alpina* 1S-4, *Appl. Environ. Microbiol. 58*:2196–2200 (1992).
7. Jareonkitmongkol, S., S. Shimizu, and H. Yamada, Fatty Acid Desaturation-Defective Mutants of an Arachidonic-Acid-Producing Fungus, *Mortierella alpina* 1S-4, *J. Gen. Microbiol. 138*:997–1002 (1992).
8. Jareonkitmongkol, S., S. Shimizu, and H. Yamada, Occurrence of Two Nonmethylene-Interrupted Δ5-Polyunsaturated Fatty Acids in a Δ6-Desaturase-Defective Mutant of the Fungus, *Mortierella alpina* 1S-4, *Biochim. Biophys. Acta 1167*:137–141 (1993).
9. Sakuradani, E., M. Kobayashi, and S. Shimizu, Δ6-Fatty Acid Desaturase from an Arachidonic Acid-Producing *Mortierella* Fungus: Gene Cloning and Its Heterologous Expression in a Fungus, *Aspergillus, Gene 238*:445–453 (1999).
10. Sakuradani, E., M. Kobayashi, and S. Shimizu, Identification of Δ12-Fatty Acid Desaturase from Arachidonic Acid-Producing *Mortierella* Fungus by Heterologous Expression in the Yeast *Saccharomyces cerevisiae* and the Fungus *Aspergillus oryzae*, *Eur. J. Biochem. 261*:812–820 (1999).
11. Michaelson, L.V., C.M. Lazarus, G. Griffiths, J.A. Napier, and A.K. Stobart, Isolation of a Δ5-Fatty Acid Desaturase Gene from *Mortierella alpina, J. Biol. Chem. 273*:19055–19059 (1998).
12. Parker-Barnes, J.M., T. Das, E. Bobik, A.E. Leonard, J.M. Thurmond, L.T. Chuang, Y.S. Huang, and P. Mukerji, Identification and Characterization of an Enzyme Involved in the Elongation of n-6 and n-3 Polyunsaturated Fatty Acids, *Proc. Natl. Acad. Sci. USA 97*:8284–8289 (2000).
13. Sakuradani, E., M. Kobayashi, and S. Shimizu, Δ9-Fatty Acid Desaturase from Arachidonic Acid-Producing Fungus: Unique Gene Sequence and Its Heterologous Expression in a Fungus, *Aspergillus, Eur. J. Biochem. 260*:208–216 (1999).
14. Wongwathanarat, P., L.V. Michaelson, A.T. Carter, C.M. Lazarus, G. Griffiths, A.K. Stobart, D.B. Archer, and D.A. MacKenzie, Two Fatty Acid Δ9-Desaturase Genes, *ole1* and *ole2*, from *Mortierella alpina* Complement the Yeast *ole1* Mutation, *Microbiology 145*:2939–2946 (1999).
15. Jareonkitmongkol, S., S. Shimizu, and H. Yamada, Production of Eicosapentaenoic Acid-Containing Oil by a Δ12-Desaturase-Defective Mutant of *Mortierella alpina* 1S-4, *J. Am. Oil Chem. Soc. 70*:119–123 (1993).
16. Jareonkitmongkol, S., E. Sakuradani, and S. Shimizu, A Novel Δ5-Desaturase-Defective Mutant of *Mortierella alpina* 1S-4 and Its Dihomo-γ-Linoleic Acid Productivity, *Appl. Environ. Microbiol. 59*:4300–4304 (1993).
17. Shimizu, S., S. Jareonkitmongkol, H. Kawashima, K. Akimoto, and H. Yamada, Inhibitory Effect of Curcumin on Fatty Acid Desaturation in *Mortierella alpina* 1S-4 and Rat Liver Microsomes, *Lipids 27*:509–512 (1992).
18. Bradford, M.M., A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein–Dye Binding, *Anal. Biochem. 72*:248–254 (1976).
19. Kawashima, H., K. Akimoto, T. Fujita, H. Naoki, K. Kinoshita, and S. Shimizu, Preparation of ^{13}C-Labeled Polyunsaturated Fatty Acids by an Arachidonic Acid-Producing Fungus *Mortierella alpina* 1S-4, *Anal. Biochem. 229*:317–322 (1995).
20. Shimizu, S., S. Jareonkitmongkol, H. Kawashima, K. Akimoto, and H. Yamada, Production of a Novel ω1-Eicosapentaenoic

Acid by *Mortierella alpina* 1S-4 Grown on 1-Hexadecene, *Arch. Microbiol. 156*:163–166 (1991).
21. Folch, J., M. Lees, and G.H. Sloane Stanley, A Simple Method for Isolation and Purification of Total Lipids from Animal Tissues, *J. Biol. Chem. 226*:497–509 (1957).
22. Christie, W.W., C. Nobler, and J.H. Moore, Determination of Lipid Classes by a Gas-Chromatography Procedure, *Analyst 95*:940–944 (1970).
23. Christie, W.W., Structural Analysis of Lipids by Means of Enzymatic Hydrolysis, in *Lipid Analysis*, edited by W.W. Christie, Pergamon Press, Oxford, 1982, pp. 162–163.
24. Chang, T.Y., and P.R. Vagelos, Isolation and Characterization of an Unsaturated Fatty Acid-Requiring Mutant of Cultured Mammalian Cells, *Proc. Natl. Acad. Sci. USA 73*:24–28 (1976).
25. Resnick, M.A., and R.K. Mortimer, Unsaturated Fatty Acid Mutants of *Saccharomyces cerevisiae, J. Bacteriol. 92*:597–600 (1966).

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