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Production of 5,8,11-Eicosatrienoic Acid (Mead Acid) by a Δ6 Desaturation Activity-Enhanced Mutant Derived from a Δ12 Desaturase-Defective Mutant of an Arachidonic Acid-Producing Fungus, *Mortierella alpina* 1S-4

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Enhanced production of 5,8,11-eicosatrienoic acid (Mead acid, 20:3ω9) was attained by a mutant fungus, *Mortierella alpina* M209-7, derived from Δ12 desaturase-defective *M. alpina* Mut48. The 20:3ω9 production by M209-7 was 1.3 times greater than that by its parent strain, Mut48. This is thought to be due to its enhanced Δ6 desaturation activity, which was 1.4 times higher than that of Mut48. In both strains, 87 to 88% of the total lipids comprised triacylglycerol (TG) and 85% of 20:3ω9 was contained in TG. On optimization of the culture conditions for M209-7, earlier glucose feeding and shifting of the growth temperature from 28 to 19°C on the second day were shown to be effective. Under the optimal conditions with a 10-liter jar fermentor, 20:3ω9 production reached 1.65 g/liter of culture medium (corresponding to 118 mg/g of dry mycelia and 28.9% of total fatty acids), which is about twice that reported previously (0.8 g/liter).

5,8,11-Eicosatrienoic acid (Mead acid, 20:3ω9) is known as an unusual fatty acid that accumulates in essential fatty acid-deficient animals (6, 19). In essential fatty acid deficiency, 20:3ω9 is endogenously synthesized from oleic acid (18:1) through Δ5 and Δ6 desaturation and elongation, via 6,9-octadecadienoic acid (18:2ω9) and 8,11-eicosadienoic acid (20:2ω9), in place of synthesis of arachidonic acid (AA) from dietary linoleic acid (LA) (Fig. 1). Since 20:3ω9 was hardly found in a normal animal and not converted to any prostaglandin-like metabolite by cyclooxygenase, it was believed to have no important biological activity. However, 20:3ω9 was found recently in organs of normal animals, such as human umbilical cord (9), and the cartilage of young animals (1, 3), fetuses, and infants (3). Furthermore, through the lipoxygenase pathway, 20:3ω9 was converted to 3-series leukotrienes (LT), which had a biphasic effect on platelet aggregation (8, 18). More recently, it was reported that dietary supplementation of 20:3ω9 resulted in inhibition of LT B_4 synthesis in rats; probably through inhibition of LT A hydrolase (10), and that human neutrophil LT B_4 synthesis was reduced by 20:3ω9 more than by eicosapentaenoic acid (EPA), which has an anti-inflammatory effect (4). These findings suggest that 20:3ω9 effectively reduces inflammatory diseases and that 20:3ω9 may have an unknown physiological role in umbilical cord vessels, cartilage, etc.

Nevertheless, 20:3ω9 has not been as widely studied as analogous fatty acids, such as AA and EPA, probably due to its limited availability. As a method for chemical synthesis of 20:3ω9, only random reduction of AA was known (7), but this is not practical because AA is rather expensive and the yield is low. Previously, we isolated a Δ12 desaturase-defective mutant, Mut48 (14), from an AA-producing fungus, *Mortierella alpina* 1S-4 (22, 24). Mut48 is unable to convert 18:1 to LA and accumulates several fatty acids of the n-9 family, such as 18:2ω9, 20:2ω9, and 20:3ω9. We expected it to be a potent producer of 20:3ω9 and optimized the culture conditions for practical production, resulting in a 20:3ω9 productivity of 0.8 g/liter of culture medium (11). However, the productivity was much lower than that of AA (3.6 g/liter of culture medium) (22), EPA (1.9 g/liter) (21), and dihomo-γ-linolenic acid (DGLA) (3.2 g/liter) (12) by *M. alpina* 1S-4 and its mutants. We thought that the fungus had potential ability to accumulate a greater amount of 20:3ω9 and attempted to obtain another mutant with higher productivity. Here we report that a new mutant (designated M209-7) produced a greater amount of 20:3ω9 than Mut48 and that this was due to enhancement of its Δ6 desaturation activity. Furthermore, we optimized the culture conditions for this mutant and examined the distribution of mycelial fatty acids among the major lipid classes.

MATERIALS AND METHODS

Chemicals. Bovine serum albumin (BSA; fatty acid free) was purchased from Sigma (St. Louis, Mo.). Olive oil (9.9% palmitic acid, 2.0% stearic acid, 82.0% oleic acid, 5.1% LA, 1.0% arachidic acid) was purchased from Wako Pure Chemicals (Osaka, Japan). Adekanol, a polyether-type antifoaming agent, was purchased from Asahi Denka Industry (Tokyo, Japan). All other reagents were commercially available and obtained as described previously (14).

Mutagenesis and isolation of mutants. Mutagenesis and isolation of mutants were performed essentially as described previously (14). Spores of *M. alpina* Mut48 (14), which is a Δ12 desaturase-defective mutant derived from *M. alpina* 1S-4 (22, 24), were treated with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine. The M209-7 strain obtained from Mut48 is stocked in the AKU culture collection (Faculty of Agriculture, Kyoto University) as 3999-35. The mycelial fatty acids of the colony derived from each treated spore were analyzed by gas-liquid chromatography (GLC) as described below, and the strains with high contents of 20:3ω9 were stored on a potato dextrose agar slant medium.

Liquid culture. *M. alpina* Mut48 and its mutants were each inoculated as a spore suspension into a 100-ml Erlenmeyer flask containing 20 ml of medium (2% glucose, 1% yeast extract, pH 6.0) and then incubated with reciprocal shaking (120 rpm) at 28°C for 7 days. For jar fermentation, the fungus was precultured at 28°C for 3 days in 100 ml of the same medium and then the resultant culture was inoculated into 5 liters of another medium (2% glucose, 1% yeast extract, 0.1% olive oil, 0.01% Adekanol, pH 6.0) in a 10-liter jar fermentor

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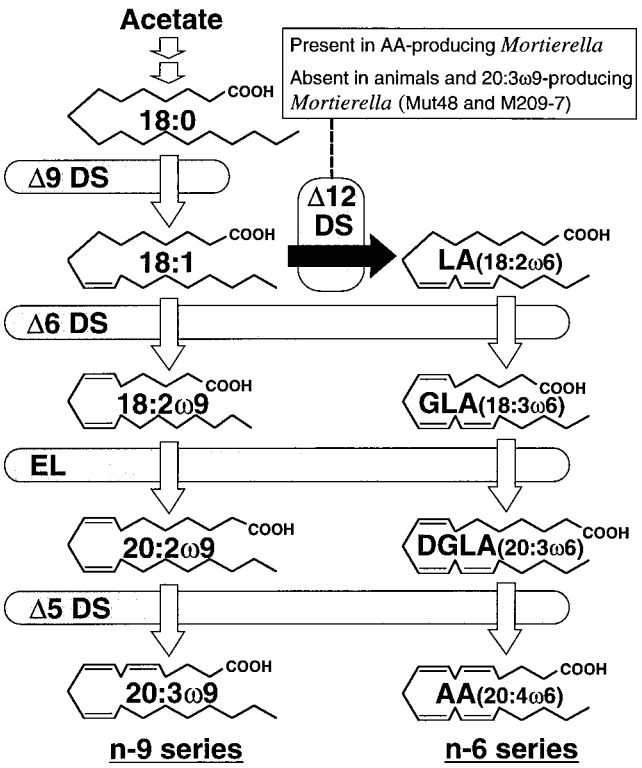

FIG. 1. Biosynthetic pathway for polyunsaturated fatty acids in animals and 20:3ω9-producing and AA-producing *Mortierella* fungi. Abbreviations: DS, desaturase; EL, elongase; 18:0, stearic acid; 18:1, oleic acid; 18:2ω9, 6,9-octadecadienoic acid; 20:2ω9, 8,11-eicosadienoic acid; 20:3ω9, 5,8,11-eicosatrienoic acid; LA(18:2ω6), linoleic acid; GLA(18:3ω6), γ-linolenic acid; DGLA(20:3ω6), dihomo-γ-linolenic acid; AA(20:4ω6), arachidonic acid.

(Able Co., Tokyo, Japan). For other conditions for jar fermentation, see Tables 1, 3, and 4, footnote *a*, and the legends to Fig. 2 and 3.

Fatty acid and lipid analyses. Mycelia were harvested by suction filtration, washed with water, and then dried at 100°C for 2 h for subsequent fatty acid analysis by GLC after transmethylation with methanolic HCl as described previously (20). Fungal lipids were extracted with the chloroform-methanol-water system described by Folch et al. (5). The lipids were separated into the triacylglycerol and polar lipid classes by thin-layer chromatography (20), and the fatty acid composition of each lipid class was analyzed by GLC as described above. The desaturation index, which reflects desaturation activity during cultivation, was expressed as the ratio of the amount of the substrate of desaturase to that of the product and further metabolites (17). For example, Δ5 desaturation activity during cultivation is expressed as the Δ5 desaturation index, i.e., the ratio of 20:2ω9 to 20:3ω9.

Analysis of Δ5 and Δ6 desaturation and elongation activities using washed mycelia. Since fatty acids belonging to the n-6 series are not synthesized and are thus undetectable in mycelia of Mut48 and M209-7 (Fig. 1), exogenous n-6 fatty acids can be used as a tracer. So we determined the Δ5 and Δ6 desaturation and elongation activities as the rate of conversion of the exogenous n-6 fatty acids to desaturated and elongated products. Mut48 and M209-7 were cultivated in a medium containing 2% glucose and 1% yeast extract, pH 6.0, at 28°C for 6 days. The resultant mycelia were washed with potassium phosphate buffer (0.1 M, pH 7.4) and then harvested by suction filtration. LA, γ-linolenic acid, and DGLA were used as substrates to measure Δ6 desaturation, elongation, and Δ5 desaturation activities, respectively. Each substrate was suspended in 5% BSA by sonication. A 1.0-mg substrate sample (100-μl BSA suspension) was added to 30 mg of wet mycelia in 1 ml of potassium phosphate buffer (0.1 M, pH 7.4) and shaken gently at 28°C for 4 h. The reaction was stopped by adding 4 ml of ethanol. The resultant reaction mixture was dried by centrifugal evaporation to obtain the dried mycelia, which were transmethylated and then analyzed by capillary GLC as described previously (15). The activities are represented as follows: Δ6 desaturation, the sum of GLA, DGLA, and AA synthesized per 1 hour per milligram of wet mycelia; elongation, the sum of DGLA and AA synthesized per 1 hour per milligram of wet mycelia; Δ5 desaturation, the amount of AA synthesized per 1 hour per milligram of wet mycelia.

Other methods. Fungal growth was measured by determining the mycelial weight after drying at 100°C overnight. The glucose concentration of the culture

TABLE 1. Comparison of the fatty acid compositions of *M. alpina* 1S-4 Mut48 and its mutant, M209-7[a]

Strain	20:3ω9 concn in:		Mycelial dry wt (g/liter of culture medium)	Total fatty acid concn (mg/g of dry mycelia)	Fatty acid composition (wt %)[b]							
	g/liter of culture medium	mg/g of dry mycelia			16:0	18:0	18:1	18:2ω9	20:0	20:1	20:2ω9	20:3ω9	22:0	24:0	Others
Mut48	1.18 ± 0.09	77.6 ± 0.9	15.2 ± 0.9	394 ± 9	5.9 ± 0.2	8.7 ± 0.2	40.9 ± 0.3	11.2 ± 0.3	0.9 ± 0.1	2.8 ± 0.1	3.0 ± 0.0	19.7 ± 0.2	3.0 ± 0.0	3.3 ± 0.2	0.6 ± 0.2
M209-7	1.53 ± 0.05	101 ± 3.5	15.1 ± 0.1	397 ± 14	5.1 ± 0.4	7.6 ± 0.3	37.0 ± 0.4	11.4 ± 0.3	0.8 ± 0.0	3.0 ± 0.1	3.0 ± 0.1	25.5 ± 0.3	3.1 ± 0.1	3.2 ± 0.5	0.5 ± 0.0

[a] The fungi were cultivated in a 10-liter jar fermentor containing 5 liters of medium (2% glucose, 1% yeast extract, 0.1% olive oil, 0.01% Adekanol, pH 6.0) at 28°C for 2 days and then at 20°C for 7 days with aeration at 1 vol/vol/min and agitation at 300 rpm. Glucose at 1.2, 0.6, and 0.2% was added on the second, fourth, and seventh days, respectively. The data are means ± standard deviations calculated from four separate cultures.

[b] Abbreviations: 16:0, palmitic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2ω9, 6,9-octadecadienoic acid; 20:0, AA; 20:1, 11-eicosenoic acid; 20:2ω9, 8,11-eicosadienoic acid; 20:3ω9, 5,8,11-eicosatrienoic acid; 22:0, behenic acid; 24:0, lignoceric acid.

TABLE 2. Comparison of the Δ5 and Δ6 desaturation and elongation activities of *M. alpina* Mut48 and M209-7[a]

Strain	Activity in pmol/h/mg of wet mycelia (amt[s] of fatty acid[s] formed)		
	Δ6 DS (GLA, DGLA, AA)	EL (DGLA, AA)	Δ5 DS (AA)
Mut48	507 ± 18 (364, 3, 140)	389 ± 17 (250, 139)	32 ± 5 (32)
M209-7	710 ± 26[c] (520, 59, 131)	368 ± 18 (220, 148)	49 ± 10 (49)

[a] A reaction mixture comprising 30 mg of wet mycelia and 1.0 ml of potassium phosphate buffer (0.1 M, pH 7.4) was incubated with 1.0 mg of substrate fatty acid suspended in 100 μl of BSA (5%) at 28°C for 4 h. To determine Δ6 desaturation, elongation, and Δ5 desaturation activities, LA, GLA, and DGLA were used as substrates, respectively.

[b] Activity is expressed as the amount of fatty acid(s) synthesized per hour per milligram of wet mycelia. The values in parentheses are the amounts of individual fatty acids formed. Each activity is expressed as follows: Δ6 desaturation, the sum of GLA, DGLA, and AA synthesized; elongation, the sum of DGLA and AA synthesized; Δ5 desaturation, the amount of AA synthesized. The data shown are means ± standard deviations calculated from three separate cultures. DS, desaturation; EL, elongation.

[c] $P < 0.01$ versus Mut48; other values are not significantly different ($P > 0.05$).

medium was measured as described by Werner et al. (23). Statistical evaluation was accomplished by using Student's two-tailed *t* test.

RESULTS

Isolation of a mutant with a high content of 5,8,11-eicosatrienoic acid (20:3ω9). Through analysis of the fatty acid composition of mycelia independently obtained from 2,000 *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine-treated spores of *M. alpina* Mut48 (14), we selected a mutant with a high content of 20:3ω9. This mutant (designated M209-7) was similar to the parent strain morphologically, but the percentage of the mycelial 20:3ω9 in M209-7 was 32.8%, while that in Mut48 was 23.5%, when the fungi were cultivated in 20 ml of medium at 28°C for 7 days (data not shown). We compared M209-7 to Mut48 under large-scale culture conditions in a 10-liter jar fermentor (Table 1). 20:3ω9 production by M209-7 (1.53 g/liter of culture medium on the ninth day) was about 1.3 times greater than that by Mut48 (1.18 g/liter), although the rates of glucose consumption and mycelial growth were similar (data not shown). The total fatty acid contents of these strains were also similar (394 to 397 mg/g of dry mycelia). The difference in 20:3ω9 production between M209-7 and Mut48 was mainly due to the difference in their fatty acid compositions, i.e., the percentage of 20:3ω9 in M209-7 (25.5%) was about 1.3 times that in Mut48 (19.7%). The percentages of other fatty acids were similar, except that of oleic acid (18:1) (Mut48, 40.9%; M209-7, 37.0%).

Δ5 and Δ6 desaturation and elongation activities. We compared the Δ5 and Δ6 desaturation and elongation activities of M209-7 with those of Mut48 by using their washed mycelia and n-6 fatty acids as tracers. Table 2 shows that the Δ6 desaturation activity of M209-7 (710 pmol/h/mg of wet mycelia) was 1.4 times that of Mut48 (507 pmol/h/mg). The elongation and Δ5 desaturation activities of the two strains were not significantly different. The incorporation of n-6 fatty acids into the mycelia was not different between the two strains, and the amount of each n-9 fatty acid in the mycelia changed little during the reaction (data not shown).

Fatty acid compositions of the lipid classes. The fatty acid compositions of the triacylglycerol (TG) and polar lipid (PL) classes in the mycelia of Mut48 and M209-7 are given in Table 3. In both strains, 87 to 88% (by mole) of the total lipids comprised TG. About 85% of 20:3ω9 was found in TG, and the remainder was in PL in both strains. When we compared the fatty acid compositions of TG and PL, the percentages of palmitic acid (16:0) and stearic acid (18:0) were found to be higher in TG than in PL and those of 18:2ω9 and 20:3ω9 were lower in TG, also in both strains.

Factors affecting 20:3ω9 production by M209-7. (i) Glucose feeding. M209-7 was cultivated in 5 liters of a medium containing 2% glucose and supplemented afterwards with another 2% glucose divided into two or three parts (Table 4). The production and contents of 20:3ω9 under the three sets of conditions were almost the same (1.51 to 1.62 g/liter of culture medium and 109 to 113 mg/g of dry mycelia, respectively). As for the percentage of 20:3ω9, however, earlier glucose feeding resulted in a higher percentage of 20:3ω9, i.e., the percentages of 20:3ω9 were 28.5% (condition C), 27.3% (B), and 26.6% (A). However, there was an opposite tendency for the percentages of 16:0 and 18:1 (A > B > C).

(ii) Growth temperature. Previously, we found that cultivation of Mut48 at 28°C and then at 12 to 20°C was suitable for 20:3ω9 production (11). Here, M209-7 was cultivated at 28°C for 2 days and then the growth temperature was shifted to 16 to 23°C (Fig. 2). The percentage of 20:3ω9 was highest at 19°C (28.9%) and decreased with an increase or decrease in temperature (Fig. 2a). On the contrary, the percentage of 18:1 was lowest at 19°C (32.9%) and increased with an increase or decrease in temperature. The percentage of 16:0 plus 18:0 was highest at 16°C and decreased with an increase in temperature. The percentages of other fatty acids were similar at 16 to 23°C (data not shown). Figure 2b indicates that the Δ5 desaturation

TABLE 3. Lipid and fatty acid compositions of *M. alpina* 1S-4 Mut48 and its mutant, M209-7[a]

Strain and fraction[b]	Lipid composition (mol%)	Fatty acid composition (mol%)[c]										
		16:0	18:0	18:1	18:2ω9	20:0	20:1	20:2ω9	20:3ω9	22:0	24:0	Others
Mut48												
TG	88 ± 2	8.9 ± 0.2	5.5 ± 0.2	47.5 ± 0.6	11.6 ± 0.3	0.1 ± 0.1	2.5 ± 0.3	2.9 ± 0.4	15.0 ± 0.7	2.9 ± 0.3	2.1 ± 0.6	1.0 ± 0.4
PL	12 ± 2	6.4 ± 0.3	3.9 ± 0.4	46.3 ± 0.7	14.2 ± 1.0	0.4 ± 0.1	2.5 ± 0.4	1.9 ± 0.3	19.9 ± 0.6	1.8 ± 0.4	1.9 ± 0.1	0.8 ± 0.3
M209-7												
TG	87 ± 1	6.9 ± 0.4	5.0 ± 0.3	40.4 ± 0.5	12.4 ± 0.5	0.9 ± 0.2	2.7 ± 0.1	3.3 ± 0.5	22.7 ± 0.5	2.9 ± 0.3	2.2 ± 0.2	0.6 ± 0.3
PL	13 ± 1	5.2 ± 0.3	4.3 ± 0.2	39.8 ± 0.7	14.9 ± 0.4	0.4 ± 0.1	3.0 ± 0.3	2.2 ± 0.2	25.5 ± 0.5	2.1 ± 0.2	2.3 ± 0.3	0.3 ± 0.3

[a] The fungi were cultivated in a 10-liter jar fermentor containing 5 liters of medium (2% glucose, 1% yeast extract, 0.1% olive oil, 0.01% Adekanol, pH 6.0) at 28°C for 2 days and then at 20°C for 6 days with aeration at 1 vol/vol/min and agitation at 300 rpm. Glucose at 1.2, 0.6, and 0.2% was added on the second, fourth, and seventh days, respectively. The data shown are means ± standard deviations calculated from three separate cultures.

[b] Minor lipids other than TG and PL, such as sterol, sterol ester, etc., were not included in the calculation.

[c] The abbreviations are defined in Table 1, footnote *b*.

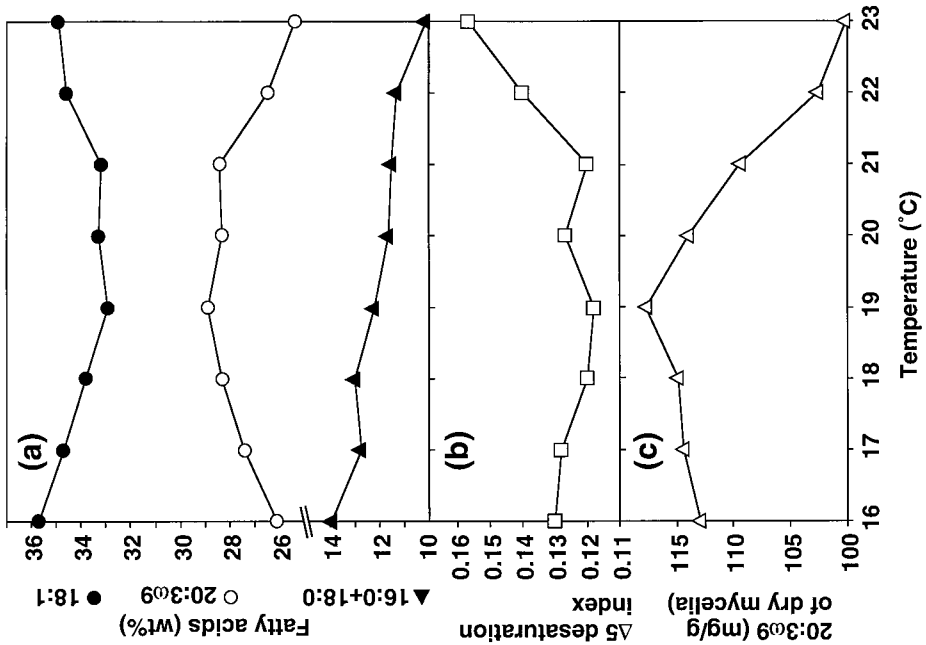

FIG. 2. Effects of a culture temperature shift from 28°C to 16 to 23°C. (a) Percentage of each mycelial fatty acid in the total fatty acids. (b) Δ5 desaturation index (20:2ω9/20:3ω9). (c) 20:3ω9 content. The fungus was cultivated in a 10-liter jar fermentor containing 5 liters of medium (2% glucose, 1% yeast extract, 0.1% olive oil, 0.01% Adekanol, pH 6.0) at 28°C for 2 days and then at 16 to 23°C for 7 days with aeration at 1 vol/vol/min and agitation at 300 rpm. Glucose (1%) was added once on each of the first and second days. The data shown are means calculated from two separate cultures.

index (20:2ω9/20:3ω9), which reflects the Δ5 desaturation activity (20), hardly changed (0.118 to 0.130) in the range of 16 to 21°C but increased to 0.157 with a temperature increase to 23°C. On the other hand, the Δ6 desaturation index [18:1/(18:2ω9 + 20:2ω9 + 20:3ω9)] was not measured because it is much influenced by the amount of 18:1 incorporated from the exogenous olive oil (the Δ5 desaturation index is not influenced because olive oil contains neither 20:2ω9 nor 20:3ω9) and thus cannot reflect the Δ6 desaturation activity in this case. The 20:3ω9 content was maximum at 19°C (118 mg/g of dry mycelia) and decreased gradually with decreasing temperature (113 mg/g at 16°C) and drastically with increasing temperature (100 mg/g at 23°C) (Fig. 2c). The curve for the change in 20:3ω9 production at 16 to 23°C (data not shown) was similar to that in Fig. 2c.

20:3ω9 production under optimal conditions. The conditions of supplementation of 1% glucose once each on the first and second days and a shift in the growth temperature from 28 to 19°C were the most suitable for 20:3ω9 production (Fig. 3). Figure 3a shows that mycelial growth became almost maximal on the fourth day. On the other hand, 20:3ω9 production increased throughout the cultivation and reached 1.65 g/liter of

TABLE 4. Effect of glucose feeding on 20:3ω9 production by *M. alpina* M209-7[a]

Condition	Glucose feeding (%) on:				20:3ω9 concn in:		Mycelial dry wt (g/liter of culture broth)	Total fatty acid concn (mg/g of dry mycelia)	Fatty acid composition (wt%)[b]										
	Day 1	Day 2	Day 4	Day 7	g/liter of culture broth	mg/g of dry mycelia			16:0	18:0	18:1	18:2ω9	20:0	20:1	20:2ω9	20:3ω9	22:0	24:0	Others
A	0	1.2	0.6	0.2	1.60 ± 0.07	109 ± 5	14.7 ± 1.1	410 ± 13	4.3 ± 0.1	8.3 ± 0.4	33.6 ± 0.2[c]	12.8 ± 1.1	1.2 ± 0.2	2.9 ± 0.1	3.7 ± 0.5	26.6 ± 0.3	2.6 ± 0.3	3.8 ± 0.6	0.2 ± 0.2
B	1	0.8	0	0.2	1.62 ± 0.08	113 ± 2	14.3 ± 0.7	414 ± 4	4.2 ± 0.1	8.1 ± 0.4	32.6 ± 0.4	13.1 ± 0.2	1.1 ± 0.2	2.9 ± 0.3	3.7 ± 0.3	27.3 ± 0.3	2.6 ± 0.2	4.1 ± 0.3	0.3 ± 0.3
C	1	1	0	0	1.51 ± 0.07	113 ± 4	13.4 ± 0.5	395 ± 11	3.8 ± 0.1[d]	8.4 ± 0.3	32.6 ± 0.5	12.6 ± 0.7	1.0 ± 0.5	1.6 ± 1.1	3.7 ± 0.1	28.5 ± 0.2[d]	2.5 ± 0.4	3.7 ± 0.5	1.6 ± 1.3

[a] The fungus was cultivated under the conditions given in Table 1, footnote *a*, except for glucose feeding, as indicated. The data shown are means ± standard deviations calculated from three separate cultures.
[b] The abbreviations are defined in Table 1, footnote *b*.
[c] $P < 0.05$.
[d] $P < 0.01$.

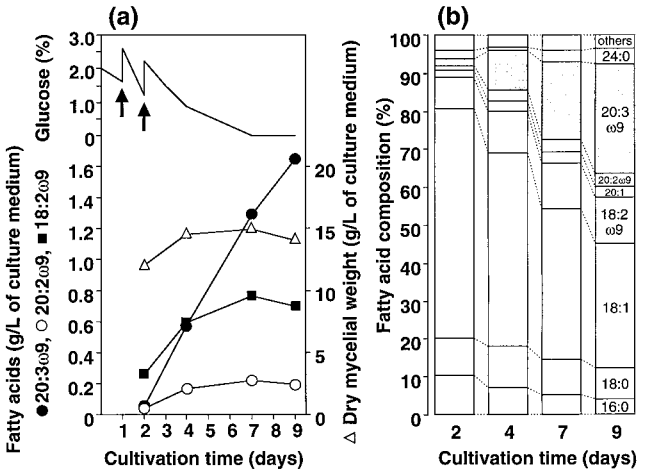

FIG. 3. Production of 20:3ω9 in a 10-liter jar fermentor. (a) Production of each fatty acid and glucose concentration in the medium. Glucose (1%) was added at the times indicated by the arrows. (b) Changes in mycelial fatty acid composition during cultivation. The fungus was cultivated in 5 liters of medium (2% glucose, 1% yeast extract, 0.1% olive oil, 0.01% Adekanol, pH 6.0) at 28°C for 2 days and then at 19°C for 7 days with aeration at 1 vol/vol/min and agitation at 300 rpm. The data shown are means calculated from two separate cultures. The abbreviations are defined in Table 1, footnote *b*.

culture medium on the ninth day (118 mg of 20:3ω9/g of dry mycelia). As for the fatty acid composition, 18:1 occupied over 60% of the mycelial fatty acids and the sum of C_{20} n-9 fatty acids comprised only 5.2% (20:3ω9, 2.2%) on the second day (Fig. 3b). This composition is similar to that of the supplemented olive oil. As the cultivation proceeded, the percentage of 20:3ω9 increased to 28.9% and that of 18:1 decreased to 32.9% on the ninth day. Saturated fatty acids, such as 16:0 and 18:0, had also decreased to 4.1 and 8.1% on the ninth day, respectively.

DISCUSSION

Previously, we obtained a Δ12 desaturase-defective mutant, Mut48, from an AA-producing fungus, *M. alpina* 1S-4 (11, 14). Mut48 accumulates some 20:3ω9, but its productivity of 20:3ω9 (0.8 g/liter of culture medium) is not sufficient. The present study shows that a new mutant, M209-7, is a more potent producer of 20:3ω9 than Mut48.

M209-7 and Mut48 are similar morphologically, in the rates of glucose consumption and growth, and in total fatty acid productivity but differ in mycelial fatty acid composition (Table 1); the percentage of 20:3ω9 in M209-7 (25.5%) was greater than that in Mut48 (19.7%). This increase in the percentage (about 1.3 times) led to an increase in 20:3ω9 production by M209-7 (1.56 g/liter of culture medium, about 1.3 times greater than the 1.18 g/liter produced by Mut48). The percentages of other fatty acids in the two strains were similar, except that of 18:1, i.e., the percentage of 18:1 in M209-7 (36.8%) was smaller than that in Mut48 (40.9%). Considering that the biosynthetic route is 18:1→18:2ω9→20:2ω9→20:3ω9 (Fig. 1), the rate of conversion of 18:1 to 20:3ω9 seemed to be faster in M209-7 than in Mut48. In this conversion, the Δ6 desaturation reaction (18:1→18:2ω9) was suggested to be faster in M209-7 because the percentage of only 18:1 changed drastically and those of 18:2ω9 and 20:2ω9 did not change.

To examine this assumption, we assayed the activities of Δ5 and Δ6 desaturation and chain elongation by using a washed-mycelium system. Table 2 shows that only Δ6 desaturation activity was higher in M209-7 than in Mut48. Among the various conversion steps, Δ6 desaturation is the key reaction in 20:3ω9 synthesis, because 18:1 seems to be a worse substrate for Δ6 desaturase than LA or α-linolenic acid (ALA); i.e., (i) large amounts of 18:1 are accumulated in Mut48 and M209-7, (ii) the parent strain (1S-4) does not desaturate 18:1 to 18:2ω9, regardless of the existence of 18:1 in the mycelia, (iii) even Mut48 does not desaturate 18:1 to 18:2ω9, and does not accumulate 20:3ω9, when grown with linseed oil containing LA and ALA (in this case, AA and EPA were formed in place of 20:3ω9) (16), and (iv) rat liver microsomal Δ6 desaturase prefers LA and ALA to 18:1 (2). Since we cannot find another difference between M209-7 and Mut48, the high productivity of 20:3ω9 by M209-7 appears to be due to its high Δ6 desaturation activity.

Analysis of the fatty acid profiles of the lipid classes revealed that most of the mycelial fatty acids comprised TG (87 to 88% of total fatty acids), as in *M. alpina* 1S-4 and its mutants (14, 16). While the percentage of 20:3ω9 was slightly lower in TG than in PL, 85% of the total 20:3ω9 was contained in TG. This feature is suitable for practical 20:3ω9 production.

On optimization of the culture conditions, earlier glucose feeding and shifting of the cultivation temperature from 28 to 19°C were shown to be effective for increasing the percentage of 20:3ω9. In particular, temperature control was important for 20:3ω9 production. The percentage of 20:3ω9 was highest at 19°C (Fig. 2a). The reduced percentage of 20:3ω9 at high temperature may be due to low desaturation activity, since the Δ5 desaturation index increased drastically at 22 to 23°C, suggesting that Δ5 desaturation activity was low. It is reasonable that desaturation is enhanced at low temperature to maintain membrane fluidity. On the other hand, the reduced percentage of 20:3ω9 at low temperature seems to be due to another reason, since the desaturation index hardly changed at 16 to 21°C. One reason could be that fatty acid synthesis and general metabolism may be slow at low temperature. Figure 2a shows that the percentage of 16:0 plus 18:0 increased at low temperature, suggesting that a large amount of 16:0 plus 18:0 remains without being converted to 20:3ω9. We think that this is due to the slow formation of 16:0 (and 18:0) from carbon sources and that there is not enough time for the conversion of 16:0 formed at a late stage during cultivation. However, this speculation needs to be examined in more detail.

We reported previously that the maximum 20:3ω9 production by Mut48 was 0.8 g/liter of culture medium (11). Here, with the combination of a new mutant, M209-7, and optimization of the culture conditions, 20:3ω9 production reached 1.65 g/liter (Fig. 3), which is about twice that reported previously. This will lead to the practical production of 20:3ω9. Furthermore, this is the first report of a mutant with enhanced desaturation activity obtained from *M. alpina*. It was thought to be difficult to increase its desaturation activity over that of the parent strain, whose activity is very high. Although we have already achieved the production of various C_{20} polyunsaturated fatty acids, such as AA (22), EPA (16, 21), and DGLA (12, 13), with *M. alpina* 1S-4 and its desaturase-deficient mutants, this result suggests that we can raise their production further by selecting a mutant with high desaturase activity.

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