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Production of Dihomo-γ-Linolenic Acid by a Δ5-Desaturase-Defective Mutant of *Mortierella alpina* 1S-4

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A mutant, which has low Δ5-desaturase activity, of an arachidonic acid-producing fungus, *Mortierella alpina* 1S-4, was shown to be a novel potent producer of dihomo-γ-linolenic acid (DHGA). On submerged culture under optimal conditions for 6 days at 28°C in a 10-liter fermentor, the mutant produced 3.2 g of DHGA per liter of culture broth (123 mg/g of dry mycelia), which accounted for 23.4% of the total mycelial fatty acids. Mycelial arachidonic acid amounted to only 19 mg/g of dry mycelia (0.5 g/liter of culture broth), which accounted for 3.7% of the total mycelial fatty acids. The other major mycelial fatty acids were palmitic acid (11.0%), stearic acid (12.8%), oleic acid (22.7%), linoleic acid (8.9%), γ-linolenic acid (6.5%), and lignoceric acid (7.8%). More than 97 mol% of the DHGA produced was found in the triglyceride fraction irrespective of the growth temperature employed (12 to 28°C).

Dihomo-γ-linolenic acid [8(*Z*),11(*Z*),14(*Z*)-eicosatrienoic acid, DHGA], a C_{20} polyunsaturated fatty acid with three double bonds, has attracted great interest recently because of its unique biological activities (4, 8). It is an important precursor of a large family of structurally related C_{20} compounds, including group 1 prostaglandins and thromboxanes. This fatty acid has been detected, in small amounts, as a component of cellular lipids in fungi (15), algae (3), protozoa (5), and animals (7). During a series of studies of the fermentative production of useful polyunsaturated fatty acids by microorganisms, we found that several *Mortierella* fungi accumulate DHGA and arachidonic acid (AA) and under certain conditions also produce 5(*Z*),8(*Z*),11(*Z*),14(*Z*),17(*Z*)-eicosapentaenoic acid (13–16, 18, 19). The mycelial content of DHGA is usually low unless the mycelia are cultured in the presence of some kinds of Δ5-desaturase inhibitors. In a previous article, we reported that the AA-producing filamentous fungus, *Mortierella alpina* 1S-4, accumulated a relatively large amount of DHGA when cultivated in a medium supplemented with sesame oil (10); this phenomenon is considered to be due to the inhibitory effects of the Δ5-desaturase inhibitors present in sesame oil. The inhibitors were isolated and identified as sesamin and some structurally related compounds (11). However, there were several problems with respect to the production of DHGA with supplementation of inhibitors to the culture medium. For example, a large amount of oil remained during cultivation in a medium supplemented with sesame oil, which resulted in high percentage contents of oleic acid (18:1) and linoleic acid (18:2) and consequently a low concentration of DHGA in the fungal mycelia. This problem can be overcome by supplementing a mixture of inhibitors instead of oil, but the preparation of such inhibitors is tedious and feeding of the inhibitors during cultivation is necessary to repress the conversion of DHGA to AA.

In order to overcome these problems, we focused on the isolation of mutants defective in fatty acid desaturation. In a recent article (6), we reported the isolation of some mutants which were defective in fatty acid desaturation, one of which (termed Mut44) was found to exhibit low Δ5-desaturation activity and therefore to have a markedly high mycelial DHGA content. We report here the DHGA productivity of the Mut44 strain. Various factors affecting the DHGA production and distribution of DHGA among lipid classes are discussed.

MATERIALS AND METHODS

Chemicals. All chemicals used were commercially available and as described previously (6).

Microorganisms and cultivation. *M. alpina* 1S-4 Mut44 (6) is a mutant defective in Δ5-desaturation derived from *M. alpina* 1S-4 (15, 18). The fungus was inoculated into a 10-ml Erlenmeyer flask containing 2 ml of medium GY (2% glucose and 1% yeast extract, pH 6.0) and then incubated with reciprocal shaking (120 strokes per min) at 28°C for 1 week, unless otherwise noted.

Analysis of fatty acids 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 fatty acid methyl esters were extracted with *n*-hexane and then analyzed by gas-liquid chromatography. The column used was a glass column (3 mm by 2 m) packed with Advanced DS (Nishio Kogyo, Tokyo, Japan). The column temperature was 190°C and the other conditions were as given in detail previously (12). Fungal lipids were extracted with $CHCl_3$-CH_3OH (2:1, vol/vol) according to the method of Folch et al. (2). The lipids were separated into individual lipid classes by thin-layer chromatography, and the fatty acid compositions of each of the lipid classes were analyzed by gas-liquid chromatography as described previously (12). Lipid compositions were calculated from the amounts of total fatty acids of each lipid class as recommended by Christie et al. (1). Heptadecanoate was usually added before transmethylation as an internal standard.

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

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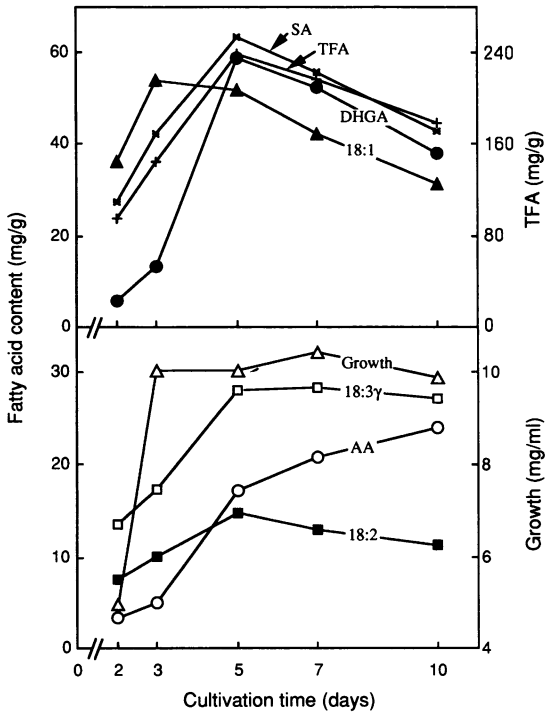

FIG. 1. Time courses of changes in the contents of mycelial fatty acids in *M. alpina* 1S-4 Mut44. The fungus was grown in medium GY at 28°C for the periods indicated. SA, sum of 16:0, 18:0, and 24:0. TFA, total fatty acids. A small amount (less than 5%) of 22:0 was also included in DHGA.

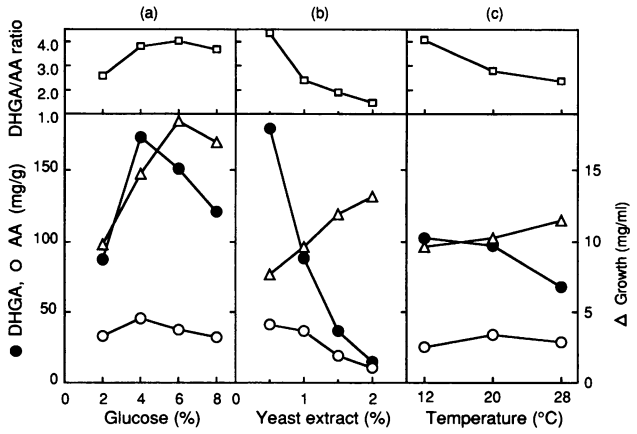

FIG. 2. Effects of the concentrations of glucose and yeast extract and the growth temperature on DHGA production. The fungus was grown at 28°C for 7 days in a medium containing 1% yeast extract and the indicated concentrations of glucose (a) or in a medium containing 2% glucose and the indicated concentrations of yeast extract (b). For panel c, the fungus was grown in medium GY for 7 days at the temperatures indicated.

with a commercial kit (Blood Sugar-GOD-Period-Test; Boehringer Mannheim, Indianapolis, Ind.) essentially according to the method of Werner et al. (17). All values shown in the figures and the table are the means of two independent determinations.

RESULTS

Accumulation of various fatty acids in *M. alpina* 1S-4 Mut44. Representative time courses of the changes in the contents of mycelial fatty acids of *M. alpina* 1S-4 Mut44 are presented in Fig. 1. The fungal growth in terms of dry mycelial mass reached a plateau after 3 days of cultivation and remained almost unchanged thereafter. The amount of 18:1 reached the maximal value after 3 days and decreased gradually thereafter. Except for AA, which continued to increase up to the 10th day of cultivation, the amounts of the other fatty acids as well as that of the total mycelial fatty acids reached the respective maximal values after 5 days and then decreased gradually. During cultivation for 5 days, the mycelial DHGA content reached 59 mg/g of dry mycelia, but it decreased to 38 mg/g of dry mycelia during cultivation for a further 5 days, the corresponding values for AA being 17 and 24 mg/g of dry mycelia, respectively.

Effects of various factors affecting DHGA production. (i) Glucose and yeast extract concentrations. Like the wild-type strain, this mutant grew well on a simple medium containing glucose and yeast extract as basal constituents. As shown in Fig. 2a, the mycelial production increased in parallel with the medium glucose concentration. The highest content of mycelial DHGA (ca. 170 mg/g of dry mycelia) was observed at a concentration of 4%, but the highest DHGA yield (2.8 mg/ml of culture broth) was observed with a medium containing 6% glucose, at which concentration the mycelial DHGA/AA ratio reached 4.0. A further increase in the glucose concentration repressed the production of mycelia and the mycelial content of DHGA, which resulted in decreases in the production of DHGA. Figure 2b shows the effect of the yeast extract concentration. The mycelial contents of DHGA and AA decreased markedly upon increase of the medium yeast extract concentration. The maximal mycelial DHGA content (ca. 180 mg/g of dry mycelia) as well as the yield (1.4 mg/ml of culture broth) with the medium containing 2% glucose occurred at a concentration of 0.5%, the DHGA/AA ratio being 4.3. A further increase in the yeast extract concentration caused an increase in the production of mycelia, but the mycelial content of DHGA, the total DHGA yield, and the DHGA/AA ratio decreased markedly, reaching 15 mg/g of dry mycelia, 0.2 mg/ml of culture broth, and 1.5, respectively, with 2% yeast extract. Similarly, a further decrease in the yeast extract concentration resulted in a decrease in the production of mycelia and consequently in the production of DHGA (data not shown).

(ii) Growth temperature. As shown in Fig. 2c, the production of fungal mycelia increased with the growth temperature. The mycelial DHGA content decreased markedly when the growth temperature was increased to 28°C, while that of AA remained almost unchanged. The mycelial DHGA/AA ratio also decreased upon an increase in temperature, the value for growth at 12°C being 4.1 and that for growth at 28°C being 2.4.

(iii) Oil addition. The various oils listed in Fig. 3 were examined for their effects on the production of DHGA. Except for cedar oil, which was not completely incorporated into the fungal cells, most oils added to the culture medium were efficiently incorporated, completely disappearing from the culture medium within 3 days. Addition of most of the oils caused increases in the mycelial mass production and the total yields of DHGA but decreases in the mycelial DHGA contents. For example, in a medium supplemented with 2% soybean oil, the mycelial mass production increased from 11.1 to 23.1 mg/ml of culture broth; the production of DHGA amounted to 1.2 mg/ml of culture broth, which

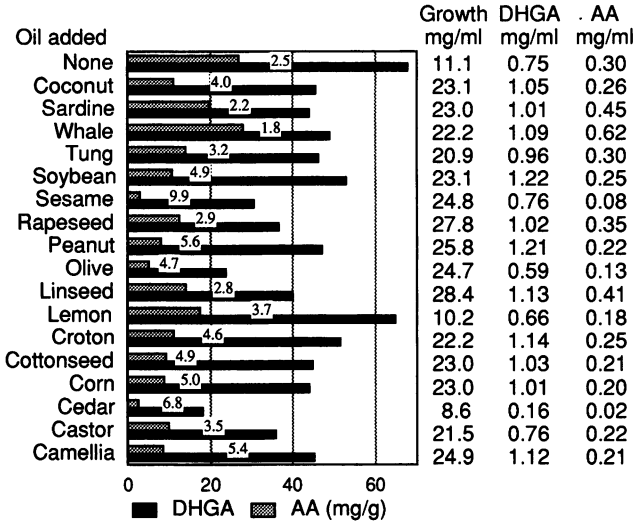

FIG. 3. Effects of various oils on the DHGA production by *M. alpina* 1S-4 Mut44. The fungus was grown in medium GY supplemented with 2% (vol/vol) of one of the oils indicated at 28°C for 7 days. The values at the tops of the bars are the mycelial DHGA/AA ratios.

accounted for ca. 1.5-fold that of growth without oil supplementation, but the mycelial DHGA content decreased from 68 to 53 mg/g of dry mycelia. It is possible that only a small amounts of the oils incorporated into the fungal cells were converted to DHGA, resulting in the increases in the total mycelial mass but decreases in the mycelial contents of DHGA. The mycelial DHGA/AA ratio also increased in the presence of most of the oils, the maximal value (9.9) being reached with the addition of sesame oil, in which sesamin, an inhibitor of Δ5-desaturase, was found (11).

(iv) Aging effects. It was previously found that the mycelial AA in the wild-type strain increased when the latter was allowed to stand for a period of time after cultivation (16). However, in the present study, aging of the harvested mycelia did not result in a marked increase in the amount of DHGA compared with the increase in the AA level. During cultivation at 28°C for 5 days, the amount of DHGA was 51 mg/g of dry mycelia, but it increased to 58 mg/g of dry mycelia after standing at 28°C for a further 5 days, while the mycelial AA content increased from 13 to 23 mg/g of dry mycelia (data not shown).

Bench scale production of DHGA. According to the results for factors affecting DHGA production, the optimal conditions were determined to be as given in the legend to Fig. 4. As shown in Fig. 4a, the feeding of glucose was necessary for bench-scale fermentor production. The mycelial DHGA content increased markedly from 2 to 4 days of cultivation, reaching 123 mg/g of dry mycelia (3.2 g/liter of culture broth) after 6 days of cultivation. The percentage of DHGA in the total mycelial fatty acids increased markedly from 11.1% on the second day to 20.7% on the third day, the value on the sixth day being ca. 23% (Fig. 4b). Under these culture conditions, the mycelial AA content also increased with growth, reaching 19 mg/g of dry mycelia (0.5 g/liter of culture broth) on the sixth day, which accounted for 3.7% of the total mycelial fatty acids. The other major fatty acids were palmitic acid (11%), stearic acid (12.8%), oleic acid (22.7%), linoleic acid (8.9%), γ-linolenic acid (6.5%), and lignoceric acid (7.8%). The DHGA/AA ratio reached ca. 6 after 3 days and remained almost constant throughout the remaining period of cultivation.

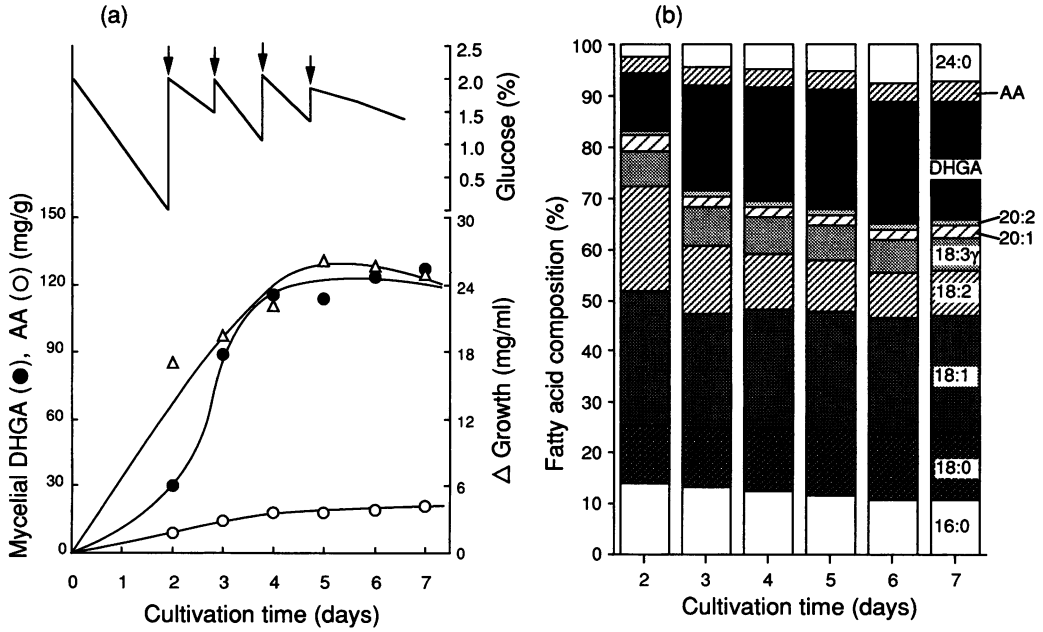

FIG. 4. Production of DHGA by *M. alpina* 1S-4 Mut44 under optimal culture conditions. The fungus was precultured at 28°C for 3 days in 100 ml of medium GY and then the resultant culture was inoculated into 4 liters of medium GY containing 0.05% (vol/vol) soybean oil and 0.05% Adekanol (Asahi Denka Industries, Tokyo, Japan) in a 10-liter jar fermentor (Able Ltd., Tokyo, Japan). Cultivation was performed at 28°C, with aeration at 1 vol/vol/min and agitation at 300 rpm. Glucose was added at the times indicated by arrows (a). The changes in the mycelial fatty acid composition during growth are shown in panel b.

TABLE 1. Fatty acid profiles of major lipid classes in *M. alpina* 1S-4 Mut44 and the wild-type strain[a]

Fraction	Lipid[b] composition (mol%)	Fatty acid composition (mol%)												
		16:0	18:0	18:1	18:2	18:3γ[c] 20:0	20:1[c] 18:3α	18:4	20:2	DHGA[c] 22:0	AA[c] 20:3ω3	20:4ω3	EPA	24:0
28°C														
TG	84	17.5	8.0	30.7	5.2	5.8	1.9	—[d]	0.7	18.3	8.0	—	—	3.9
PE	6	14.9	4.4	28.4	8.8	24.5	2.2	—	0.8	3.8	12.1	—	—	—
PC	7	16.3	1.7	7.5	8.6	53.5	3.5	—	0.9	0.3	7.8	—	—	—
PS	3	24.8	2.3	27.3	15.3	13.7	0.8	—	0.6	7.2	8.1	—	—	—
12°C														
TG	80	8.0	8.8	25.9	5.6	6.4	2.2	—	1.0	28.5	6.3	4.5	1.2	1.7
PE	8	10.1	5.0	19.2	8.7	27.4	4.5	—	1.1	5.4	15.6	1.4	1.6	—
PC	9	6.5	3.7	6.8	7.0	42.3	—	8.4	1.5	1.5	17.2	—	5.2	—
PS	3	21.5	5.5	17.5	10.0	9.0	1.6	—	4.0	13.3	15.4	1.2	1.1	—
Wild type														
TG	82	17.3	6.2	15.7	6.9	3.3	1.0	—	0.7	4.3	39.7	—	—	5.0
PE	7	16.3	4.3	25.2	12.5	8.3	1.0	—	0.6	2.4	29.5	—	—	—
PC	8	16.2	1.6	8.3	17.5	4.2	0.3	—	0.8	2.7	48.5	—	—	—
PS	3	25.5	3.0	29.2	21.0	4.4	—	—	0.5	1.7	14.7	—	—	—

[a] Mut44 was grown in medium GY at 28°C for 7 days (28°C) or at 28°C for 2 days and then at 12°C for a further 8 days (12°C). The wild-type strain was grown in the same medium at 28°C for 7 days (Wild type). EPA, 5 (*Z*), 8 (*Z*), 11 (*Z*), 14 (*Z*), 17 (*Z*)-eicosapentaenoic acid.

[b] Other minor lipids, such as sterols, sterol esters, and glycolipids, were not included for the calculation.

[c] These pairs of fatty acids are not separated under the gas-liquid chromatography conditions employed. The first fatty acids in each pair are the major ones (more than 95%), 18:3α and 20:3ω3 being undetectable during growth at 28°C.

[d] —, Undetectable.

Distribution of fatty acids among major lipids. As shown in Table 1, the mycelial DHGA was found in all lipid classes. Irrespective of the growth temperature, the major part (more than 97 mol%) of it was present in the triglyceride (TG) fraction, the remainder being in the phosphatidylethanolamine (PE), phosphatidylcholine (PC), and phosphatidylserine (PS) fractions in the ratio of 45:5:50. It is notable that although the amount of DHGA was much larger than that of AA in the TG fraction, the levels of DHGA were lower than those of AA in individual phospholipids. For example, in the case of growth at 28°C, the percentage of DHGA in TG in the total fatty acids in this fraction was 18.3% and that of AA was 8%. However, the values for DHGA in PE, PC, and PS were 3.8, 0.3, and 7.2%, respectively, and those for AA were 12.0, 7.8, and 8.1, respectively. It was also striking that the percentages of 18:3γ in the total fatty acids in individual lipid classes were very high in PC compared with those in the case of the wild type. In the case of growth at 28°C, 18:3γ accounted for 53.5% in PC, while the values for TG, PE, and PS were 5.8, 24.5, and 13.7%, respectively. The distribution ratio of this fatty acid in TG, PE, PC, and PS was calculated to be 58:11:28:3, and that for the wild type was 80:11:7:2.

Isolation and identification of DHGA. DHGA was isolated as its methyl ester by reversed-phase high-performance liquid chromatography on a Cosmosil column ($5C_{18}$-AR, 20 by 250 mm; Nacalai Tesque, Kyoto, Japan). The mobile phase was CH_3CN-H_2O (90:10, vol/vol) and the flow rate was 9 ml/min, and the effluent was monitored by means of UV detection (210 nm). The elution time under these conditions was 40 min. The DHGA was identified on the basis of the results of mass spectrometry and proton nuclear magnetic resonance measurement. Mass spectrometry and nuclear magnetic resonance were measured with a Hitachi M-80B at 70 eV and a Nicolet NT-360 with tetramethylsilane as an internal standard. The analytical data for the methyl ester derivative were as follows. Nuclear magnetic resonance δ ($CDCl_3$): 0.90 ppm (*t*, 3H), 1.33 (*m*, 14H), 2.06 (*m*, 4H), 2.31 (*t*, 2H), 2.81 (*m*, 4H), 3.67 (*s*, 3H), and 5.36 (*m*, 6H). Mass spectrometry (70 eV) *m/z* (relative intensity, %): 320 (M^+, 24%), 222 (23), 150 (43), 135 (22), 121 (25), 93 (60), 79 (95), 67 (100), 55 (55), 41 (54), and 28 (41). These data agreed well with those of the authentic standard.

DISCUSSION

The present study showed that a mutant, Mut44, which was considered to exhibit low Δ5-desaturation activity of a potent AA-producing fungus, *M. alpina* 1S-4, is a novel promising producer of DHGA. Under the optimal conditions, it could produce more than 3 g of DHGA per liter of culture broth. This value is larger than that (2.2 g/liter of culture broth) obtained previously by means of cultivation in the presence of Δ5-desaturase inhibitors (10). The mycelial DHGA content (123 mg/g of dry mycelia) was also higher than that obtained by the previous method (107 mg/g of dry mycelia). However, the level of AA in the mycelia of Mut44 (19 mg/g of dry mycelia) was very low compared with that (54 mg/g of dry mycelia) obtained by the previous method (10), and the percentage of AA in the total mycelial fatty acids in this study (ca. 4%) was lower than that previously found (ca. 10%).

Studies of the distribution of fatty acids showed that most of the mycelial DHGA was present in the TG fraction. It is striking that although the proportion of it in TG was much higher than that of AA, the proportion of it was lower than that of AA in phospholipids, especially in PC (Table 1). According to Pugh and Kates (9), who showed that PC-linked DHGA could be directly converted to AA, this observation could be explained by the hypothesis that there is more than one pathway for the conversion of DHGA to AA, e.g., one requiring acyl coenzyme A and another requiring (phospho)lipids as substrates, and thus one of these pathways, possibly that requiring acyl coenzyme A as a substrate, would be defective in this mutant.

In conclusion, the fermentative production of DHGA involving Mut44 is promising in that this mutant can grow in a simple medium, producing a significant amount of DHGA, and there were no problems with the inhibitors mentioned in the introduction.

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