Production of an Eicosapentaenoic Acid-Containing Oil by a Δ12 Desaturase-Defective Mutant of *Mortierella alpina* 1S-4

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A Δ12 desaturase-defective mutant of an arachidonic acid (AA)-producing fungus, *Mortierella alpina* 1S-4, converted α-linolenic acid (18:3ω3) to 5(Z),8(Z),11(Z),14(Z),17(Z)-eicosapentaenoic acid (EPA). On submerged cultivation at 20°C for 10 d in a 5-L fermentor containing medium comprising 1% glucose, 1% yeast extract and 3% (vol/vol) linseed oil, EPA production amounted to *ca.* 1 g/L culture broth (64 mg/g dry mycelium), which accounted for *ca.* 20% of the total mycelial fatty acids. AA content was 26 mg/g dry mycelium (0.4 g/L), accounting for 7.8% of the total mycelial fatty acids. The other major mycelial fatty acids were palmitic acid (4.5%), oleic acid (20.4%), linoleic acid (10.0%), 18:3ω3 (20.3%) and lignoceric acid (4.3%). Most of the EPA produced (*ca.* 90 mol%) was in triglyceride form.

KEY WORDS: Δ12 Desaturation, eicosapentaenoic acid, *Mortierella alpina*, n-3 fatty acids.

5(Z),8(Z),11(Z),14(Z),17(Z)-eicosapentaenoic acid (EPA), a rare C_{20} polyunsaturated fatty acid (PUFA) of potential pharmaceutical value, has attracted much interest because of its unique biological activities (1–5). Marine fish oil is a conventional natural source for EPA but additional sources are desired. A marine alga, *Chlorella minutissima* (6), a freshwater alga, *Monodus subterraceus* (7), a moss, *Leptobryum pyriforme* (8) and *Euglena gracilis* (9) are other sources of EPA, but they have low growth rates and lipid contents.

During studies on the fermentative production of useful PUFA, we found that several members of the fungal subgenus *Mortierella* are potent producers of arachidonic acid (AA) (10,11), dihomo-γ-linolenic acid (DHGA) (12,13) and EPA (14–17). For the fermentative production of EPA, *Mortierella* fungi can use linseed oil (16,17), which is rich in α-linolenic acid (18:3ω3, *ca.* 60%). Because all fungi, including *M. alpina* 1S-4, exhibit high Δ12 desaturase activity, converting oleic acid (18:1) to linoleic acid (18:2ω6) and subsequently to AA, mycelia growing on linseed oil usually contain a considerable level of AA. From a nutritional standpoint, an EPA-containing oil with a low AA level is preferable because of the diverse biological activities of AA. Therefore, we tried to decrease the AA levels of the fungi. In a recent paper (18) we reported the isolation of mutants of *M. alpina* 1S-4 that are defective in fatty acid desaturation, one of which, Mut48, is defective in Δ12 desaturase, but not in the other enzymes in the biosynthesis of AA (Fig. 1a). The mycelial fatty acids of this mutant comprise fatty acids of the n-9 family, such as Mead acid [5(Z),8(Z),11(Z)-eicosatrienoic acid, 20:3ω9], and are completely devoid of AA, which is the most abundant fatty acid in the wild-type strain (11). Because Δ12 desaturase is not involved in the conversion of 18:3ω3 to EPA (Fig. 1b), Mut48 may convert exogenously provided 18:3ω3 to EPA without producing AA.

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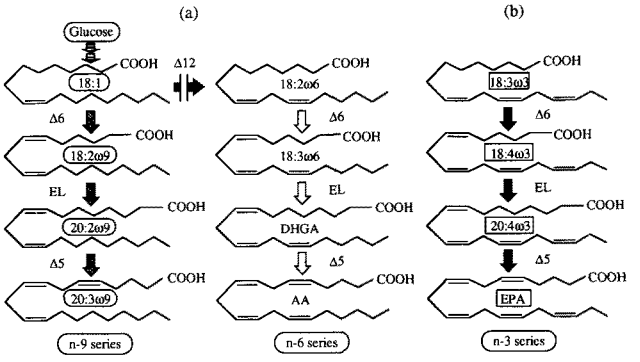

FIG. 1. Biosynthetic pathways for fatty acids. *M. alpina* 1S-4 Mut48 does not produce fatty acids of the n-6 family but produces significant amounts of n-9 fatty acids (a). The conversion route from 18:3ω3 to EPA is shown in (b). EPA, 5(Z),8Z),14(Z),17(Z)-eicosapentaenoic acid; DHGA, dihomo-γ-linolenic acid; AA, arachidonic acid; EL, elongation.

In this paper we report the conversion of 18:3ω3 to EPA by Mut48, which results in a mycelial oil which is low in AA. Several factors affecting EPA production and fatty acid profiles of major lipid classes are described.

MATERIALS AND METHODS

Chemicals. Linseed oil was purchased from Wako Pure Chemicals (Kyoto, Japan) and converted to methyl esters with methanolic sulfuric acid (19). The methyl esters were composed of palmitic acid (10% by wt), stearic acid (4%), oleic acid (10%), linoleic acid (18%) and α-linolenic acid (58%). All other reagents were of analytical grade.

Microorganism and cultivation. M. alpina 1S-4 Mut48 (18), a mutant defective in desaturation at the Δ12 position, was derived from *M. alpina* 1S-4 (10–14). The fungus was grown on medium GY (1% glucose, 1% yeast extract, pH 6.0), supplemented with 2% (vol/vol) linseed oil methyl esters at 24°C with shaking for one week, unless otherwise noted.

Fatty acid and lipid analyses. The mycelium was washed with ethanol to remove excess oil, dried at 100°C overnight and analyzed by gas-liquid chromatography (GLC) as described previously (18,20). All values in the figures and tables are the means of two independent determinations.

RESULTS

Conversion of 18:3ω3 to EPA by Mut48. Several additional fatty acids to those from glucose-grown fungi were found after growth on the medium supplemented with 18:3ω3. These fatty acids were identified as 6(Z),9(Z),12(Z),15(Z)-octadecatetraenoic acid (18:4ω3), 8(Z),11(Z),14(Z),17(Z)-eicosatetraenoic acid (20:4ω3), EPA, 11(Z),14(Z),17(Z)-eicosatrienoic acid (20:3ω3) and 5(Z),11(Z),14(Z),17(Z)-eicosatetraenoic acid (20:4Δ5). There was no AA or any

other fatty acid of the n-6 family in the resultant mycelia. The EPA percentage of the total n-3 fatty acids (including 20:4Δ5) was *ca.* 55%, which accounted for 8.1% of the total mycelial fatty acids.

Factors affecting EPA production. As described above, the production of AA-free EPA-containing oil is possible provided that pure 18:3ω3 is used. However, that would be impractical because 18:3ω3 of such high purity is presently too expensive. Thus, we selected linseed oil as an alternative starting material because of a high content of 18:3ω3, as well as its suitability for EPA production by the wild-type strain (16).

Time course of EPA formation. Mut48 efficiently incorporated linseed oil. About one day after addition of the linseed oil, the culture medium became turbid but cleared after another day or two when the oil was completely incorporated. Representative time courses for changes in the contents of some fatty acids of Mut48 on growth in the presence of the oil are shown in Figure 2. On growth at 24°C or above, the mycelial 18:3ω3 content usually reached a maximum at 3 d and then decreased gradually. However, maximal 18:3ω3 production took longer at 12°C, which could have contributed to the lower rate of EPA formation. Because the linseed oil contained about 20% 18:2ω6, which was subsequently converted to AA (Fig. 1a), the resultant mycelial oil also contained some AA. However, the level of AA was markedly lower compared with that in the wild-type, in which the EPA level never exceeded that of AA (14–17). At 24°C, the mycelial content of EPA increased markedly from 3–5 d cultivation, and then slowly to day 14. After two weeks, the mycelial EPA reached *ca.* 80 mg/g dry mycelium and AA (including 20:3ω3) reached *ca.* 40 mg/g dry mycelium.

Glucose and yeast extract concentrations. Like the wild strain (11), Mut48 grew well on a simple basal medium containing glucose and yeast extract. As shown in Figure 3a, the highest EPA production was observed at glucose concentrations of 0.5–2% when the yeast extract concentration was 0.5%. However, glucose concentrations higher than 1% resulted in marked decreases in the EPA yield in the medium containing 1% yeast extract (Fig. 3b). The optimal glucose concentrations were both in the range of 0.5–1.0%. As for the medium containing 1% glucose and 2% linseed oil, the optimal yeast extract concentration was in the range of 0.5–1% (Fig. 3c). Further increases in the yeast extract concentration resulted in increases in the mycelial mass produced, but in decreases in the EPA yield.

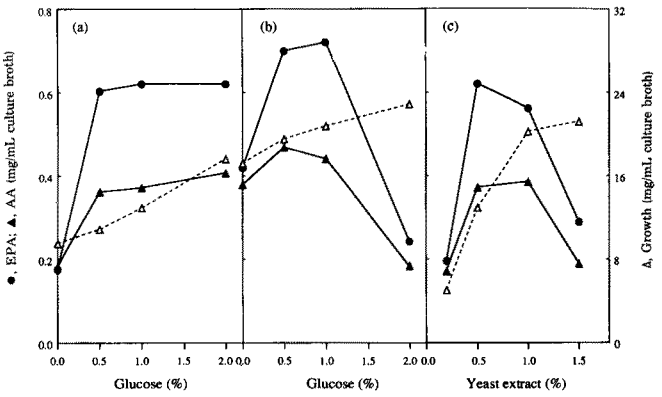

FIG. 3. Effects of glucose and yeast extract concentrations on EPA production. (a) 0.5% Yeast extract; (b) 1% yeast extract; (c) 1% glucose. The AA fraction included *ca.* 30% 20:3ω3. Abbreviations as in Figure 1.

Linseed oil concentration. For the medium containing 0.5% glucose, the highest EPA yield was at 2% (vol/vol) linseed oil; further increases in linseed oil caused marked decreases in the EPA yield (Fig. 4). However, in the medium containing 1% glucose, the highest EPA yield was obtained with 3% linseed oil and was *ca.* 1.2-fold the maximal yield in the medium containing 0.5% glucose.

Growth temperature. As shown in Figure 5, the optimal temperature for EPA production was 24°C with growth for 7 d, but it shifted to around 20°C when the cultivation time was 10 d. On growth at 20°C, the incorporation of linseed oil, as judged from the mycelial 18:3ω3 content, occurred at a slower rate than on growth at 24°C, but the change in mycelial 18:3ω3 content during the first two days was the same, as shown in Figure 2 for growth at 12°C.

Bench-scale production of EPA. Because of the above results, the fungus was cultured in a 5-L jar fermentor.

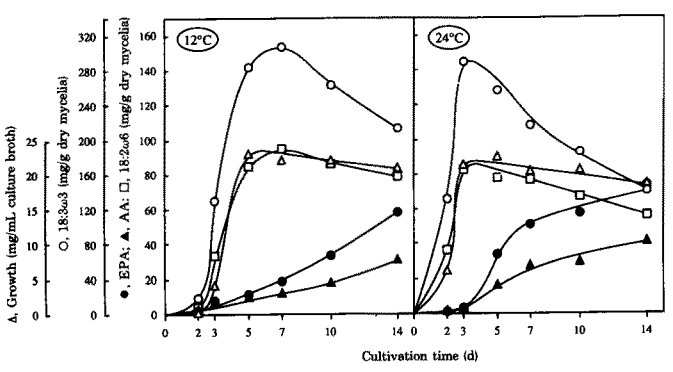

FIG. 2. Time courses of changes in the contents of some fatty acids in *M. alpina* 1S-4 Mut48. The fungus was grown at 28°C for 1 d and then at 12°C, or always at 24°C. Cultivation time indicates the total time before and after the temperature shift. The AA fraction included about 30% 20:3ω3. Abbreviations as in Figure 1.

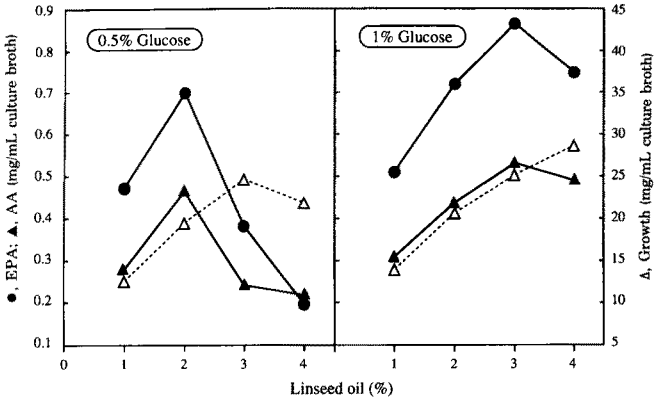

FIG. 4. Effect of the linseed oil concentration on EPA production. The AA fraction included *ca.* 30% 20:3ω3. Abbreviations as in Figure 1.

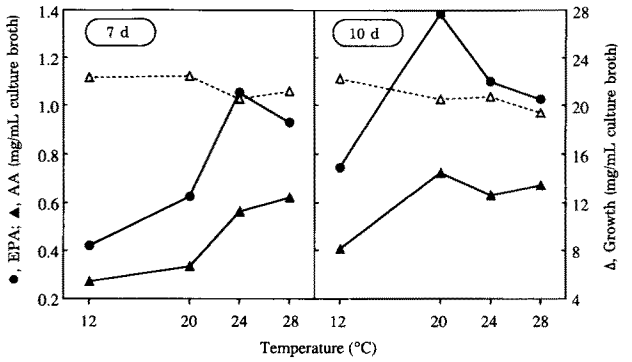

FIG. 5. Effect of the growth temperature on EPA production. Except for the cultures grown at 12°C, which were grown at 28°C on the first day before reducing the temperature to 12°C, all were grown at the temperature indicated from the first day of cultivation. The AA fraction included *ca.* 30% 20:3ω3. Abbreviations as in Figure 1.

The fungus was cultured at 24°C for 2 d before the growth temperature was reduced to 20°C to stimulate the incorporation of linseed oil, which occurred more slowly on growth at 20°C. As shown in Figure 6a, fungal growth reached a stationary phase after 5 d, but the production of EPA and AA (including 20:3ω3) increased for 10 d. After 10 d, EPA production reached *ca.* 1 g per L of culture broth (64 mg/g dry mycelium), which accounted for 19.5% of the total mycelial fatty acids. Because 20:3ω3 accounted for 30% of the sum of AA and 20:3ω3, the amount of AA produced by day 10 was *ca.* 0.4 g/L culture broth (26 mg/g dry mycelium), accounting for 7.8% of the total mycelial fatty acids. The other major fatty acids (≥1.0%) were 16:0 (4.5%), 18:0 (2.9%), 18:1 (20.4%), 18:2ω6 (10.0%), 18:3ω3 (20.3%), 18:4ω3 (1.6%), 20:3ω3 (3.4%), 20:4ω3 (1.0%), 20:4Δ5 (1.4%) and lignoceric acid (4.3%). Saturated, n-9, n-6 and n-3 fatty acids amounted to 12.5, 21.8, 18.5 and 47.2%, respectively. Mead acid and other PUFA of the n-9 family, which occur in considerable amounts on culturing in a medium without oil supplementation (21), had almost completely disappeared, the sum of these PUFA being less than 1% of the total mycelial fatty acids.

Distribution of fatty acids in the major lipid classes of Mut48 grown on linseed oil. As shown in Table 1, fatty acid methyl esters (FAME) accounted for the major part (*ca.* 80 mol/) of the extractable lipids. The FAME should be those of the added oil and not of fungal lipid, because such a large amount of this fraction has never previously been detected in fungi grown without oil supplementation (21). The fatty acid composition was comprised mainly of 18:3ω3 (*ca.* 50%), without EPA or any fatty acid other than those present in linseed oil. Irrespective of the growth temperature, most of the EPA (*ca.* 90 mol%) was contained in triglyceride (TG), and the remainder was in phospholipids, especially in phosphatidylcholine (PC, *ca.* 7%). The percentages of EPA in individual lipid classes were relatively high in PC and TG. For example, for growth at 28°C, the EPA ratios in TG, phosphatidylethanolamine (PE), PC and phosphatidylserine (PS) were 10.4, 5.0, 16.3 and 4.7%, respectively.

DISCUSSION

As suggested previously (14,16), there are two routes for EPA production by *M. alpina* 1S-4. The first, which occurs at low temperature, involves conversion of an endogenous fatty acid(s) of the n-6 family to the corresponding n-3 fatty acid and, subsequently, to EPA through the n-3 fatty acid biosynthetic pathway. The other is the conversion of exogenous 18:3ω3 to EPA through the n-3 fatty acid biosynthetic route. Due to a lack of endogenous n-6 fatty acids, the former route does not occur in this mutant unless n-6 fatty acids are provided exogenously (Jareonkitmongkol, S., and S. Shimizu, unpublished observation). Although the mutant should convert 18:2ω6 and other n-6 fatty acids to EPA on growth at 12°C, cultivation at this temperature did not lead to an increase in EPA production, as found for the wild-type strain. The optimal temperature in this study was around 20°C, which was the same as that previously found for Mead acid production (21). Δ6 Desaturation of 18:3ω3 into 18:4ω3 might be

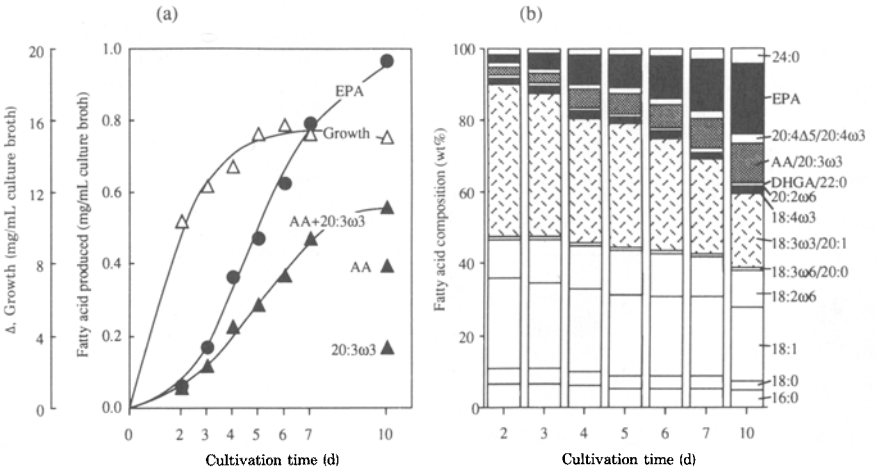

FIG. 6. Production of EPA under submerged culture conditions. The fungus was precultured at 28°C for 3 d in medium GY and then supplemented with linseed oil methyl esters in a 5-L fermentor. Cultivation was performed at 24°C for 2 d, followed by 20°C. EPA and AA contents are shown in (a), and fatty acid composition during growth is shown in (b). Abbreviations as in Figure 1.

TABLE 1

Fatty Acid Profiles of the Major Lipid Classes of *M. alpina* 1S-4 Mut48 Grown on Linseed Oil[a]

Lipid[b] composition (mol%)		Fatty acid composition (mol%)													
		16:0	18:0	18:1	18:2ω6[c] 18:2ω9	18:3ω6[c] 20:0	18:3ω3[c] 20:1	18:4ω3	20:2ω6[c] 20:2ω9	20:3ω9	DHGA[c] 22:0	AA[c] 20:3ω3	20:4Δ5[c] 20:4ω3	EPA	24:0
28°C FAME	86.0 (0)	5.6	3.4	23.2	16.7	—[d]	51.1	—	—	—	—	—	—	—	—
TG	14.0 (88)	7.9	3.4	21.8	10.2	0.8	33.4	1.6	0.2	0.2	0.9	5.4	2.0	10.4	1.8
PE	0.7 (4)	14.4	6.2	36.0	6.7	2.4	18.3	4.0	trace	0.8	—	6.3	trace	5.0	trace
PC	0.8 (5)	13.3	4.6	22.5	6.0	1.4	16.9	3.4	0.6	2.1	trace	8.1	4.2	16.3	0.6
PS	0.5 (3)	19.5	7.1	36.7	7.7	1.7	14.3	1.6	—	0.4	—	4.9	1.4	4.7	—
12°C FAME	83.2 (0)	5.0	3.2	22.7	16.8	—	52.3	—	—	—	—	—	—	—	—
TG	16.8 (86)	6.2	3.7	20.6	11.8	0.7	38.7	1.7	0.1	trace	0.5	4.3	1.5	9.4	0.8
PE	1.0 (5)	9.2	6.0	36.2	8.8	3.5	10.9	5.7	0.2	0.7	—	11.1	1.0	6.2	0.6
PC	1.4 (7)	8.0	8.2	19.0	7.9	2.7	8.1	9.5	trace	3.8	2.0	8.2	5.6	16.6	0.4
PS	0.5 (2)	19.6	9.5	38.7	7.0	3.0	6.7	2.5	—	trace	—	7.2	trace	5.9	—

[a]The fungus was grown at 28°C for 7 d (28°C), or at 28°C for 2 d, and then at 12°C for a further 8 d (12°C). Abbreviations: DHGA, dihomo-γ-linolenic acid; AA, arachidonic acid; EPA, *5(Z),8(Z),11(Z),14(Z),17(Z)*-eicosapentaenoic acid; FAME, fatty acid methyl esters; TG, triglycerides; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine.

[b]Other minor lipids, *i.e.*, sterol ester, glycolipids and diglycerides, were not included for the calculation. The values shown in brackets were calculated by assuming that FAME equals zero.

[c]These pairs of fatty acids were not separated by the gas-liquid chromatographic conditions used, the first fatty acid being the major one (more than 80%).

[d]Undetectable.

the rate-limiting step in the conversion of 18:3ω3 to EPA in the rat (22), and this also may be true for our fungus. In addition, Δ6 desaturation should also be the rate-limiting step in the formation of Mead acid, because the biosyntheses of n-3, n-6 and n-9 fatty acids involve the same enzymatic systems. According to these concepts, we suggested that the increase in EPA production on growth at 20°C is related to an increase in Δ6 desaturase activity, but not to the conversion of n-6 fatty acids to EPA.

Except for the low AA-level, there was no marked difference in the fatty acid composition between the oil produced by Mut48 and that by the wild strain. Previous studies (16,17) failed to reveal the occurrence of 20:3ω3 and 20:4Δ5 because of the use of low-resolution packed-column GLC, but these fatty acids are also present in the fungal oil obtained on incubation of the wild strain with linseed oil. 20:4Δ5 has been found by several investigators (23,24) in animal cells incubated with 18:3ω3. It is widely accepted that 20:4Δ5 is formed by carbon chain-elongation from 18:3ω3 to 20:3ω3, followed by Δ5 desaturation. It is possible that the same reactions also take place in Mut48 and in the wild type. The low AA level in the oil is promising in that it is more difficult to decrease the AA level than to increase it, which is easily performed by merely blending with AA-rich oil from the wild strain (11). As for the yield of EPA, the highest value previously reported was *ca.* 2 mg/mL culture broth, whereas the value obtained in this study was *ca.* 1 mg/mL culture broth (Table 2), but this difference should be due to differences in culture conditions, especially the cultivation time. It would be more correct to compare the production of a fatty acid in terms of production per a certain period of time. Taking the aging time as the cultivation time, the EPA production per day was calculated and is shown in Table 2. Although there were some differences in the culture conditions, it is clear that the EPA productivity of Mut48 is not so different from the highest value reported, the production of EPA per day in both cases being *ca.* 100 mg

TABLE 2

Comparison of EPA Productivities of *M. alpina* 1S-4 Mut48 and the Wild Strain[a]

Strain and culture conditions	Fatty acid								
	Yield g/L culture broth		Content mg/g dry mycelium		% in total fatty acids		Productivity mg/L/day		EPA/AA ratio
	EPA	AA	EPA	AA	EPA	AA	EPA	AA	
M. alpina 1S-4									
28°C, 2 d; 12°C, 8 d[b]	0.30	1.76	27	158	10.9	63.8	30	176	0.17
+LO,[c] 28°C, 6 d; aging 28°C, 7 d[d]	0.99	1.49	41	62	9.2	13.8	76	115	0.66
+LO, 12°C, 9 d; aging 12°C, 7 d[d]	1.88	2.44	67	88	12.0	15.7	118	153	0.77
Mut48 (present study)									
+LO, 20°C, 10 d	0.97	0.39	64	26	19.5	7.8	97	39	2.49

[a]Abbreviations as in Table 1. [b]Cited from Ref. 14. [c]LO, linseed oil. [d]Cited from Ref. 17.

per L of culture broth. The mycelial AA content was lower than half the lowest value in the case of the wild-type, and the EPA/AA ratio in the oil produced by Mut48 was *ca.* 2.5, where that found in previous studies was less than 0.8.

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