Review

Arachidonic acid production by the oleaginous fungus Mortierella alpina 1S-4: A review

Hiroshi Kikukawa a,b, Eiji Sakurada a,c, Akinori Ando a, Sakayu Shimizu a,d, Jun Ogawa a,*

a Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Kitashirakawa-oiwakecho, Sakyo-ku, Kyoto 606-8502, Japan
b Department of Chemistry and Biomolecular Science, Faculty of Engineering, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan
c Institute of Technology and Science, The University of Tokushima, 2-1 Minami-Josanjima, Tokushima 770-8506, Japan
d Department of Bioscience and Biotechnology, Faculty of Bioenvironmental Science, Kyoto Gakuen University, 1-1 Nanjo, Sagabe, Kameoka 621-8555, Japan

ABSTRACT

The filamentous fungus Mortierella alpina 1S-4 is capable of accumulating a large amount of triacylglycerol containing C20 polyunsaturated fatty acids (PUFAs). Indeed, triacylglycerol production by M. alpina 1S-4 can reach 20 g/L of culture broth, and the critical cellular signaling and structural PUFA arachidonic acid (ARA) comprises 30%–70% of the total fatty acid. The demonstrated health benefits of functional PUFAs have in turn encouraged the search for rich sources of these compounds, including fungal strains showing enhanced production of specific PUFAs. Screening for mutants and targeted gene manipulation of M. alpina 1S-4 have elucidated the functions of various enzymes involved in PUFA biosynthesis and established lines with improved PUFA productivity. In some cases, these strains have been used for industrial-scale production of PUFAs, including ARA. In this review, we described practical ARA production through mutant breeding, functional analyses of genes encoding enzymes involved in PUFA biosynthesis, and recent advances in the production of specific PUFAs through molecular breeding of M. alpina 1S-4.

Introduction

Fatty acids containing more than one carbon double bond, termed polyunsaturated fatty acids (PUFAs), are critical sources of metabolic energy, major structural components of membrane...
phospholipids, and precursors of the eicosanoid signaling molecules prostaglandins, thromboxanes, and leukotrienes. Fish oils, animal fats, and algal cells are among the most readily available lipid sources rich in 20-carbon (C20) PUFAs. Among PUFAs, ARA (ARA, C20:4n-6) is the most abundant C20 PUFA in humans, especially in the brain, muscles, and liver. ARA has multiple physiological functions and is an important nutrient for infants and the elderly [1,2]. ARA-derived lipid mediators can play various roles in establishing homeostasis for the humans [3]. However, most of the ARA in the humans is usually taken from dietary animal sources such as meat and eggs [4], and the PUFA contents of these conventional sources are insufficient for practical large-scale production. Alternatively, γ-linolenic acid (GLA, 18:3n-6)-containing oils have been produced using 

| Microorganism          | ARA productivity | Scale | Ref. |
|------------------------|------------------|-------|------|
| Mortierella alpina 1S-4| 3.6 g/L/7 days   | 5 L fermentor | [29] |
|                        | 3.0 g/L/10 days  | 2 L fermentor | [11] |
|                        | 13 g/L/10 days  | 10 L fermentor | [25] |
| M. alpina ATCC32222    | 11 g/L/16 days  | 500 L fermentor | [28] |
| M. alpina ATCC32222    | 11 g/L/11 days  | 250 mL flask | [7] |
| M. alpina DSA-12       | 18.8 g/L/12.5 days | 12 L fermentor | [26] |
| M. alpina ME-1         | 19.8 g/L/7 days  | 5 L fermentor | [27] |
| Mortierella elongata 15-5 | 1.0 g/L/4 days | 500 mL flask | [14] |
| Mortierella schmuckleri S12 | 2.3 g/L/3 days | 14 L fermentor | [24] |
| Mortierella alliacea YN-15 | 7.1 g/L/6 days | 50 L fermentor | [23] |

ARA-producing Mortierella sp.

Since the first reports of Mortierella strains producing ARA in 1987 [14,20], this genus has been studied extensively as a promising single-cell oil (SCO) source for various types of PUFAs [21,22].

Enzymes involved in ARA biosynthesis in M. alpina 1S-4

Arachidonic acid biosynthesis requires the activity of several fatty acid desaturases and elongases. The primary substrate hexadecanoic acid (16:0) is converted to ARA in sequential steps catalyzed by elongase 1 (MALCE1), Δ9 desaturase, Δ12 desaturase, Δ6 desaturase, elongase 2 (GLELO), and Δ5 desaturase, respectively (Fig. 1 and Table 2). Some of these enzymatic steps in M. alpina 1S-4 contain a NADH-cytochrome b6 reductase and cytochrome b5 as an electron transport system for fatty acid desaturation [33–35]. Cytochrome b5 is a small hemoprotein which is an integral component of the microsomal membranes and functions as an electron carrier in a number of microsomal oxidation/reduction reactions, including fatty acid desaturation, cholesterol biosynthesis and reduction of cytochrome P450.

The two Δ9 desaturase homologues (designated as Δ9-1 and Δ9-2) in M. alpina 1S-4 have a cytochrome b5-like domain linked to the carboxyl terminus, similar to yeast Δ9 desaturase [36]. The M. alpina 1S-4 Δ9-1 exhibits 45% amino acid sequence similarity with the yeast Saccharomyces cerevisiae homologue and 34% with the rat homologue, suggesting that M. alpina Δ9-1 is a conserved membrane-bound protein using acyl-CoA as substrate. Both Δ9-1 and Δ9-2 desaturate 18:0 to oleic acid (18:1n-9). Although the Δ9-2 gene is not transcribed in the wild-type, Δ9-2 protein was expressed and exhibited Δ9 desaturation activity in a Δ9-1 gene-defective mutant [37]. The M. alpina Δ12 and Ω3 desaturases, both of which lack a cytochrome b5-like domain, have been characterized by heterologous gene expression systems. The M. alpina Δ12 desaturase was confirmed to catalyze the desaturation of 18:1n-9 to 18:2n-6 in both S. cerevisiae and Aspergillus oryzae [38]. The M. alpina Ω3 desaturase shows 51% sequence identity with M. alpina Δ12 desaturase. It converts n-6 PUFAs to n-3 PUFAs with C18 and C20 chain lengths, and is particularly efficient at converting ARA to EPA [39]. Furthermore, the M. alpina Ω3 desaturase exhibits two additional activities when expressed in S. cerevisiae, insertion of C=C double bonds at the Δ12-position and Δ15-position of hexadecenoic acid (16:1n-7) [40].

The M. alpina Δ5 and Δ6 desaturases have a cytochrome b5-like domain linked to the N-terminus. A complementary DNA (cDNA) encoding Δ5 desaturase has been isolated from two M. alpina strains, CBS210.32 and ATCC32221 [41,42]. Mortierella alpina Δ5 desaturase inserts C=C double bond at the Δ5-position of PUFAs, thereby converting DGLA into ARA. Two Δ6 desaturase homologues (designated Δ6-1 and Δ6-2) are also present in M. alpina.
Expression of the full-length cDNA clone in A. oryzae resulted in greater accumulation of GLA, reaching 25.2% of the total fatty acid content. The amino acid sequence homology between Δ9-1 and Δ6-2 is very high (92%). Usually, Δ6-1 gene transcription is 2-fold to 17-fold higher than Δ6-2 gene transcription in M. alpina 15-4. However, transcription of the Δ6-2 gene was enhanced up to 2-fold to 17-fold higher than Δ6-1 when the Δ9-4 gene was confirmed to encode a fatty acid elongase that efficiently catalyzed the elongation of 16:0 to 18:0 in M. alpina 15-4. Indeed, this is its primary activity in M. alpina 15-4 [47].

**Gene manipulation in M. alpina 15-4**

A transformation system for M. alpina 15-4 has been developed using M. alpina uracil auxotrophs as the host strain and a complementary gene as a selection marker [48]. Transformation with M. alpina 15-4 spores and a vector containing the M. alpina 15-4 ura5 gene as a marker was achieved with high efficiency (transformant frequency of 0.4/mg of vector DNA) using microprojectile bombardment [49,50]. Southern blot analysis revealed that most of the integrated plasmids in stable transformants were present as multiple copies at ribosomal DNA (rDNA) positions and/or at random positions in the chromosomal DNA. An Agrobacterium tumefaciens-mediated transformation system for M. alpina 15-4 has also been developed [51] in which the ura5 gene is used as a selectable marker under control of the homologous histone H4.1 promoter in the transfer-DNA region. The frequency of transformation reached more than 400/10⁸ spores using this system, and Southern blot analysis revealed that most of the integrated transfer-DNAs appeared as a single copy at random position in the chromosomal DNA. Mortierella alpina 15-4 exhibits resistance to various antibiotics used to destroy other filamentous fungi. However, Zeocin- and Carboxin-resistance markers have been developed for selection of M. alpina 15-4 [52,53]. A high concentration of Zeocin (20 mg/mL)
completely inhibited the germination of *M. alpina* 1S-4 spores, and decreased the growth rate of fungal filaments. On the other hand, the fungicide Carboxin (100 mg/mL) completely inhibited *M. alpina* 1S-4 hyphal growth and spore germination. These genes for Zeocin and Carboxin resistance have proven useful as selective markers for the transformation of both the parental strain and mutants.

To develop a more effective gene expression system for *M. alpina* 1S-4, the transcriptional activity of each promoter was evaluated using the β-glucuronidase (GUS) reporter assay system [54]. The GUS gene was synthesized with optimized codon usage for *M. alpina* and inserted into a basic vector under control of the histone H4.1 promoter and evaluated for expression activity. Seven promoters with high-level constitutive or time-dependent expression were selected, and deletion analysis determined the promoter regions required to retain the expression activities. Furthermore, using an inducible GAL10 promoter, an approximately 50-fold increase in GUS activity was achieved by addition of galactose to the culture media at any cultivation phase [55].

The integration of exogenous DNA into chromosomes occurs through two DNA double-strand break repair pathways, homologous recombination (HR) and non-homologous end joining (NHEJ) [56]. In HR, exogenous DNA is integrated into the chromosome using homologous regions as templates for precise gene insertion. The HR method is used frequently for insertion of exogenous expression constructs to disrupt target genes (gene targeting) (Fig. 2A). However, these two pathways are independent of one another and often function competitively [57]. Gene targeting systems have also been developed by disruption of key proteins involved in NHEJ [58,59], such as Ku80 or DNA ligase IV (lig4). We identified and disrupted the ku80 and lig4 genes in *M. alpina* 1S-4 to improve gene-targeting efficiency. These gene-disrupted strains showed no defect in vegetative growth, spore formation, or fatty acid production. Importantly, the efficiency of gene-targeting through HR was improved only in the lig4-disrupted strain, where it was 21-fold (67%) greater than that of the host strain. Metabolic engineering using lig4 gene-disrupted strains as hosts is expected to produce higher levels of rare and beneficial PUFAs and contribute to basic research on fungal lipogenesis.

**PUFA production by *M. alpina* 1S-4 mutants and transformants**

Numerous desaturase-deficient and/or elongase-deficient mutants have been isolated by treating *M. alpina* 1S-4 spores with the chemical mutagen N-methyl-N′-nitro-N-nitrosoguanidine (Table 3) [60–65]. The *M. alpina* 1S-4 wild-type can accumulate n-3 PUFAs only when cultivated at low temperature (below 20 °C), while the o3 desaturase-defective mutants are unable to synthesize n-3 PUFAs even when grown at low temperature [60,66]. The wild-type usually shows the highest ARA yield at 20°C, although a portion of the accumulated ARA is further converted to EPA, so the resultant oil includes a small amount of EPA (ca. 3%). Therefore, these mutants (e.g., Y11 and Y61 strain) are superior to the wild-type for production of SCO with a relatively higher ARA content [64,66]. Additionally, rare fatty acids accumulated in *M. alpina* 1S-4 by suppression of MALCE1-mediated 16:0 elongation to 18:0 or by supplementation of exogenous fatty acids such as 16:1n-7 into the culture medium (Fig. 1b).

This practical transformation system for *M. alpina* 1S-4 allows overexpression, RNA interference (RNAi), and disruption of genes involved in PUFA biosynthesis for improved production of desired PUFAs. Several valuable *M. alpina* mutants were directly transformed with drug resistance markers, or their uracil auxotrophs were transformed with the ura5 marker. Molecular breeding of *M. alpina* 1S-4 and its mutants yielded unique fatty acid profiles and high productivities of valuable PUFAs (Table 3 and 4). Mutant JT-180 exhibits no Δ12 desaturase activity and enhanced Δ5 and tone H4.1 promoter and evaluated for expression activity. Seven promoters with high-level constitutive or time-dependent expression were selected, and deletion analysis determined the promoter regions required to retain the expression activities. Furthermore, using an inducible GAL10 promoter, an approximately 50-fold increase in GUS activity was achieved by addition of galactose to the culture media at any cultivation phase [55].

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**Table 3**

| Mutant | Deficient enzyme | Product | Productivity and characteristics | Ref. |
|--------|------------------|---------|---------------------------------|------|
| Y11    | o3 desaturase    | ARA     | 1.5 g/L, 45% of total fatty acid with no n-3 PUFAs | [64,66] |
| Y61    | o3 desaturase    | ARA     | 1.8 g/L                           | [66] |
| JT-180 | Δ12 desaturase   | MA      | 2.6 g/L, 49%                      | [65] |
| S14    | Δ5 desaturase    | DGLA    | 4.1 g/L and low ARA content (<1%) | [61] |

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*Fig. 2.* Gene-disruption through double crossing-over HR (A) and chromatograms of fatty acid methyl esters prepared from a control strain (lig4 disruptant) and Δ5 desaturase gene-disrupted strain (B).

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\(\Delta 6\) desaturase activities, resulting in the efficient production of Mead acid (MA, \(20:3n-9\)) [65]. With overexpression of the endogenous \(\Delta 12\) desaturase gene, \(JT-180\) accumulated a larger amount of ARA (2.0 g/L/7 days, 39% of total fatty acids) but little MA compared to the wild-type (1.2 g/L/7 days, 21%) due to enhanced \(\Delta 5\) and \(\Delta 6\) desaturation. Overexpression of the endogenous \(malce1\) gene in \(M.\ alpina\) 1S-4 also led to faster and greater ARA accumulation (0.76 g/L/6 days, 34%) than in the wild-type (0.68 g/L/6 days, 28%). In addition, overexpression of the gene encoding GLELO, which has been suggested to catalyze the rate limiting step in \(\Delta 5\) desaturation gene expression in the mutant \(JT-180\), led to a markedly high ARA/DGLA ratio of 0.76 g/L/6 days, 34% than in the wild-type (0.68 g/L/6 days, 38.2% of total fatty acid).

The resulting transformants yielded more ARA (3.6 g/L/10 days, 28%) than the wild-type (1.9 g/L/10 days, 19%). Overexpression of both \(malce1\) and \(glelo\) genes had substantial effects on ARA production by \(M.\ alpina\) 1S-4. The exogenous \(\Delta 5\) and \(\Delta 6\) desaturases on ARA production (\(Pav\Delta5\), \(Pav\Delta6\), \(Ost\Delta6\) from the microalgae \(Pavlova\) salina and \(Ostreococcus\) lucimarinus and the \(\Delta 12\) desaturase (\(Tri\Delta12\)) from the beetle \(Tribolium\) castaneum have desaturation activities for fatty acyl-CoA substrates. On the other hand, the homologous desaturases from \(M.\ alpina\) use phospholipids as substrates. By expressing these exogenous desaturases, higher ARA yields were obtained (unpublished data) [69]. For instance, overexpression of the \(Pav\Delta5\) gene in the wild-type led to a markedly high ARA/DGLA ratio, while overexpression of the \(Ost\Delta6\) gene in the wild-type led to higher 18:3n-6, DGLA, and ARA contents as proportions of total fatty acid compared to the wild-type. Similarly, overexpression of the \(Tri\Delta12\) gene in the wild-type led to greater proportions of 18:2n-6, 18:3n-6, DGLA, and ARA compared to the wild-type.

The RNAi method using double-strand RNA has been applied to silence gene expression in \(M.\ alpina\) 1S-4 [70]. By suppressing endogenous \(\Delta 6\)-1 gene expression by RNAi in the mutant \(JT-180\), 18:1n-9 accumulation reached 68.0% of total fatty acid content, and 18:1n-9 production in broth reached 2.76 g/L [45].

Overexpression systems using promoters that exhibit high transcriptional activities may facilitate further improvements in PUFA production. Usually, \(M.\ alpina\) can express \(\omega 3\) desaturation activity and accumulate n-3 PUFAs when cultured at low temperatures (below 20 °C), with an EPA ratio of approximately 10%, while no accumulation of n-3 PUFAs was observed at 28 °C. However, overexpression of the endogenous \(\omega 3\) desaturase gene in \(M.\ alpina\) 1S-4 at 20 °C increased EPA accumulation to 40% of total fatty acid [51]. Expression of the heterologous \(Saprolegnia\) diclina \(\Delta 17\) desaturase (\(sdd17m\)) gene in the \(\omega 3\) desaturase-defective mutant \(ST1358\) resulted in EPA content as high as 26.4% of total fatty acid or 1.8 g/L at 28 °C [72]. While wild \(M.\ alpina\) accumulates only a small amount of the n-3 eicosatetraenoic acid (EPA, 20:4n-3) at low temperature (below 20 °C), this ETA was successfully produced by molecular breeding [73]. Further, by overexpression of the heterologous \(sdd17m\) gene controlled by an \(SS\)A2 promoter showing high transcriptional activity, ETA productivity in a \(\Delta 5\) desaturase-defective mutant \(S14\) reached 24.9% of total fatty acid at 28 °C [61].

Gene targeting may also be a valuable strategy for development of \(M.\ alpina\) strains producing SCO containing rare PUFAs. DGLA-producing transformants were constructed by disruption of the \(\Delta 5\) desaturase gene, which encodes a key enzyme catalyzing the bioconversion of DGLA to \(\omega 3\), in the \(lig4\) gene-disrupted strain of \(M.\ alpina\) 1S-4 [74]. The uracil auxotroph of the \(lig4\) gene-disrupted strain was transformed for disruption of the \(\Delta 5\) desaturase gene through double crossing-over HR, and the targeting efficiency was calculated as 50%. The ratio of DGLA to total fatty acid in this disruptant reached 40.1%; however, no ARA was detected (Fig. 2). Thus, DGLA oil can be produced without ARA contamination. Such disruptants are superior to defective mutants (e.g., \(M.\ alpina\) 1S-4 mutant \(S14\) constructed by chemical mutagenesis) for practical production of DGLA. Using the same methodology, \(MA\)-producing disruptants were constructed by disruption of the \(\Delta 12\) desaturase gene (unpublished data) [75]. These disruptants showed no defects in growth, spore germination, and fatty acid production, but exhibited higher MA composition (8.4% of the total fatty acid) than the MA-producing \(\Delta 12\) desaturase-defective mutant \(JT-180\) (4.5%), with no accumulation of n-6 and n-3 PUFAs. Further application of gene targeting in \(M.\ alpina\) strains should facilitate improved PUFA productivity and help elucidate the enzyme pathways of PUFA biosynthesis.

### Conclusions and future perspectives

The present review summarizes studies on lipogenesis in \(M.\ alpina\) 1S-4, the development of efficient gene manipulation systems for this strain, and the utilization of various \(M.\ alpina\) 1S-4 mutants for the production of beneficial PUFAs, especially ARA. The \(M.\ alpina\) 1S-4 wild-type, derivative mutants, and transformants are potential sources of triacylglycerols containing various

| Fatty acid | Target gene | Parent | Method | Productivity |
|-----------|-------------|--------|--------|--------------|
| ARA       | \(\Delta 12\) | JT-180 | OE     | Higher production (2.0 g/L/7 days, 39% of total fatty acids) than the \(M.\ alpina\) 1S-4 wild-type (1.2 g/L/7 days, 21%) |
| \(malce1\) | 1S-4 | OE     | \(OE\) | Higher ARA production (0.76 g/L/6 days, 34%) than the wild-type (0.68 g/L/6 days, 28%) |
| \(glelo\) | 1S-4 | OE     | \(OE\) | Higher ARA production (3.6 g/L/10 days, 28%) than the wild-type (1.9 g/L/10 days, 19%) |
| \(Pav\Delta5\) | 1S-4 | OE     | \(OE\) | Higher ARA composition (39%) and lower DGLA composition in the transformant than the wild-type (19% and 4%, respectively) |
| \(Ost\Delta6\) | 1S-4 | OE     | \(OE\) | Higher ARA composition (37%) in the transformant than the wild-type (19%) |
| \(Tri\Delta12\) | 1S-4 | OE     | \(OE\) | Higher ARA composition (36%) in the transformant than the wild-type (19%) |
| 18:1n-9   | \(\Delta 6\)-1 | JT-180 | Ri     | 2.76 g/L/6 days, 68% of total fatty acid |
| EPA       | \(\omega 3\) | 1S-4 | OE     | 0.68 g/L, 38.2% of total fatty acid |
| \(sdd17m\) | ST1358 | OE     | \(OE\) | 1.8 g/L, 26.4% of total fatty acid |
| ETA       | \(sdd17m\) | S14   | OE     | 2.76 g/L/6 days, 68% of total fatty acid |
| DGLA      | \(\Delta 5\) | 1S-4 | GT     | Higher DGLA composition (40%) than the mutant \(S14\) strain (27%), with no ARA accumulation versus 0.2% in the mutant \(S14\) |
| MA        | \(\Delta 12\) | 1S-4 | GT     | Higher MA composition (8.4%) than the mutant \(JT-180\) (4.5%), with no n-6 and n-3 PUFAs |
PUFAs, including n-1, n-3, n-4, n-6, n-7, and n-9 PUFAs. By selective breeding of *M. alpina* and its mutants, it is possible to regulate the flow of both endogenous and exogenous fatty acids, thereby modifying the fatty acid profile and enhancing the production of desired (i.e., beneficial) PUFAs. Recent studies on *M. alpina* and its mutants have focused on molecular engineering of genes involved in PUFAs biosynthesis and yielded strains with improved PUFAs productivity. The molecular breeding of mutants and transgenic strains may make it possible to produce desired PUFAs efficiently. However, more efficient expression systems for enzymes involved in lipid synthesis, PUFAs synthesis, and lipid conversion, as well as improved gene-silencing and targeted gene-disruption systems are needed to facilitate the breeding of *M. alpina* strains for large-scale production of functional lipids with industrial applications.

Conflict of interest

The authors declare no conflict of interest.

Compliance with Ethics Requirements

This article does not contain any studies with human or animal subjects.

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Sakayu Shimizu is an Emeritus Professor at Kyoto University. He was a Professor in the Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University from 1992 to 2009, and a Professor in the Department of Bioscience and Biotechnology, Faculty of Bioenvironmental Science, Kyoto Gakuen University from 2009 to 2016. He completed his doctorate on fermentation physiology and applied microbiology in 1973 at Kyoto University. He was awarded a prize of the Vitamin Society of Japan in 2002, a prize of the Japan Society for Bioscience, Biotechnology and Agrochemistry in 2003, and an International Enzyme Engineering Award in 2009. He is now serving as Chairman of the Board of Directors of the Japan Bioindustry Association. He is one of the pioneers of Single Cell Oil development and arachidonic acid rich-oil fermentation. He is also widely regarded for enzyme engineering research and has established several industrial processes for chiral chemical synthesis using microbial enzymes.

Jun Ogawa is a Professor at the Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University since 2009. He studied fermentation physiology and applied microbiology and completed his doctorate in 1995 at Kyoto University. In 2004, he was awarded a prize for Encouragement of Young Scientists from the Japan Society for Bioscience, Biotechnology, and Agrochemistry. In 2015, he was awarded the “Oleoscience Award” by the Japan Oil Chemists’ Society. He is serving as a Director of the Japan Society for Bioscience, Biotechnology, and Agrochemistry and is Chair of the Biotechnology Division of the American Oil Chemists’ Society (AOCS). His current research interests are screening and development of novel microbial functions useful in life sciences, food sciences, environmental sciences, and green chemistry, especially, fermentation physiology relating to functional lipid production.