Biochemical analysis and molecular breeding of oleaginous microorganisms for ω3 polyunsaturated fatty acid production

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CONTENTS

ABBREVIATIONS

16:0	Palmitic acid
16:1	Palmitoleic acid
18:0	Stearic acid
18:1	Oleic acid
18:2	Linoleic acid
ALA	α-Linolenic acid
ARA	Arachidonic acid
DGLA	Dihomo-γ-linolenic acid
DHA	ω3 Docosahexaenoic acid
DPA	ω3 Docosapentaenoic acid
DTA	ω6 Docosatetraenoic acid
EPA	ω3 Eicosapentaenoic acid
ETA	ω3 Eicosatetraenoic acid
GLA	γ-Linolenic acid
LA	Linoleic acid
OA	Oleic acid
SDA	Stearidonic acid
5-FOA	5-Fluoroorotic acid
bp	Base pair(s)
cDNA	Complementary DNA
DNA	Deoxyribonucleic acid
EST	Expression sequence tag
GLC	Gas-liquid chromatography
GUS	*β*-Glucuronidase
kb	Kilobase(s)
LB medium	Luria-Bertani medium
mRNA	Messenger RNA
ORF	Open reading frame
PCR	Polymerase chain reaction
PUFA	Polyunsaturated fatty acid
rDNA	Ribosomal DNA
rRNA	Ribosomal RNA
RNA	Ribonucleic acid
SdhB	Succinate dehydrogenase subunit B
TrpC	*N*-(5-phosphoribosil) anthranilate isomerase

INTRODUCTION

Omega-3 polyunsaturated fatty acid (ω3-PUFA) is a general term for polyunsaturated fatty acids with a double bond (C=C) at the third-carbon bond from the methyl end of the carbon chain. Eicosapentaenoic acid (EPA, C20:5ω3), docosahexaenoic acid (DHA, C20:6ω3) and α-linolenic acid (ALA, C18:3ω3) are known as major ω3-PUFAs which abuntandly exists in nature. Stearidonic acid (SDA, C18:4ω3) and docosapentaenoic acid (DPA, C22:5ω3) can be found in oils accumulated in some kinds of plants and marine life. Eicosatetraenoic acid (ETA C20:4ω3) is hardly obtained from natural sources.

Omega-3 PUFAs such as EPA and DHA are known to be important structural components of membrane phospholipids, as well as precursors of signaling molecule eicosanoids [1]. Omega-3 PUFAs have attracted much attention for their beneficial effects on human health in reducing cardiac diseases such as arrhythmia, stroke and high blood pressure [2-4]. In addition, some reports suggest that ω3-PUFAs could prevent rheumatoid arthritis and asthma [5-7]. An ω3-PUFA derivative has also been identified as an important anti-inflammatory lipid mediator and possible anti-influenza agent [8, 9]. Therefore, the demand for ω3-PUFAs is rapidly increasing in the pharmaceutical, medical and nutritional fields.

Currently, ω3-PUFAs for human consumption are typically derived from a natural source, such as fish oils, sea animal oils and plant oils. However, these sources have some disadvantages, including unstable and limited supply, lower ω3-PUFA content, and undesirable contaminations. Recent investigations have focused on ω3-PUFA production by altenative source such as oleaginous bacteria, fungi, plants and microalgae [10, 11]. In particular, oleaginous microorganisms are more suitable as an alternative source for ω3-PUFA production than conventional sources, because these microorganisms can be cultivated easily and rapidly on a large scale and produce considerable amounts of high-quality ω3-PUFAs.

Thus, the author focused on oleaginous microorganisms as ω3-PUFA producers and carried out their biochemical analysis and molecular breeding for the production of ω3-PUFAs.

Chapter I describes the selection and characterization of promoters based on genomic approach for the molecular breeding of oleaginous fungus *Mortierella alpina* 1S-4. Chapter II describes the characterization of galactose-dependent promoters from *M. alpina* 1S-4. Chapter III describes ω3-eicosatetraenoic acid (ETA) production by molecular breeding of the mutant

strain S14 derived from *M. alpina* 1S-4. Chapter IV describes EPA production by molecular breeding of the mutant strain ST1358 derived from *M. alpina* 1S-4. Chapter V describes the screening, isolation and characterization of docosahexaenoic acid (DHA)-producing microorganisms.

CHAPTER I
Selection and characterization of promoters based on genomic approach for the molecular breeding of oleaginous fungus *Mortierella alpina* 1S-4

As mentioned in general introduction to this thesis, lipid fermentation by microorganisms is noticed as an alternative method supplying PUFA more stably than conventional production [12], therefore the development of gene manipulation tools for lipid-producing microorganisms is important. Various lipids have been produced by means of molecular breeding of microorganisms in some studies [13-16]. *Mortierella alpina* 1S-4, an oleaginous fungus, is a lipid-producing microbe [17]. To date, the production of various kinds of PUFAs has been achieved by molecular breeding of *M. alpina* [14, 18-20]. Basic molecular breeding tools such as gene delivery systems, host-vector systems and transformation systems using auxotrophy or antibiotic resistance have been established in *M. alpina* 1S-4 [21-23]. However, the gene modifiability of *M. alpina* is still limited due to lack of identification of variations in promoters [24]. The properties of promoters strongly influence the expression level and duration of target genes [25-27]. The application of highly expressing and/or regulated promoters is one of most important factors in a valuable expression system [28-35]. In *M. alpina*, enrichment of promoter types would contribute to improving PUFA productivity and modifying PUFA composition, and may help elucidate the mechanisms regulating gene expression in this strain.

In general, promoter discovery in fungal biotechnology has been mainly based on the information of highly- or constitutively-expressed proteins [36, 37]. Recently, expression sequence tag (EST) analysis has been used as a powerful tool for investigating expressed genes. EST abundance data can present directly gene transcriptional levels, and make possible widespread approaches to find desired promoters in combination with the genomic information [38, 39].

In this chapter, the author describe selection and cloning of promoter regions of various genes of *M. alpina* 1S-4 on the basis of EST abundance data, and characterized these promoter regions by fusing β-glucuronidase (GUS) reporter assays.

MATERIALS AND METHODS

Strains, media, and growth conditions

A uracil auxotroph (*ura5*$^-$ strain), previously isolated from *M. alpina* 1S-4 deposited at the Graduate School of Agriculture of Kyoto University [40], was used as a recipient host strain for transformation. Czapek-Dox agar medium, supplemented with 0.05 mg/ml uracil, was used for sporulation of the *ura5*$^-$strain, as described previously [40]. SC agar medium [40] was used as a uracil-free synthetic medium for cultivation of the transformants derived from *M. alpina* 1S-4 *ura5*$^-$ strain at 28°C. GY medium (2% [wt/vol] glucose and 1% yeast extract) was used for reporter assays and extracting genomic DNA. GS medium (5% [wt/wt] soy flour, 0.3% K_2HPO_4, 0.05% $MgCl_2 \cdot 6H_2O$ and 0.05% $CaCl_2 \cdot 2H_2O$) was used for large-scale cultivation. Liquid cultivations were performed at 28°C with shaking (300 rpm), except for large-scale cultivation when a jar-fermentor was used.

Escherichia coli strain DH5α was used for DNA manipulation and grown on LB agar plates containing 50 μg/ml kanamycin.

Agrobacterium tumefaciens C58C1 was used for the transformation of *M. alpina* 1S-4 *ura5*$^-$ strain. LB-Mg agar medium, minimal medium (MM) and induction medium (IM) were used for the transformation, cultivation and infection of *A. tumefaciens*, respectively. The compositions of LB-Mg agar medium, MM, and IM have been described previously [40].

Genomic DNA preparation

M. alpina 1S-4 was cultivated in 10 ml of GY medium at 28°C for 4 d with shaking (300 rpm). Fungal mycelia were harvested by suction filtration and washed twice with sterile water. Preparation of genomic DNA was performed using a method described previously [41].

Construction of cDNA libraries of *M. alpina* 1S-4 and EST analysis

For large-scale cultivation, an inoculum was prepared in a 50-L jar fermentor containing 30 L of GY medium supplemented with 0.1% soybean oil, followed by cultivation for 2 d at 28°C. The main cultivation was carried out in a 10-kL fermentor (Kansai Chemical Engineering Co., Hyogo, Japan) with 4 kL of GS medium at 26°C with stirring. At 18, 42, 66, 90 and 114 h after starting cultivation, 5.33% or 4% glucose was added. For extracting the total RNA of *M. alpina*,

fungal mycelia were sampled after 17, 25, 42, 114, 209 and 281 h of cultivation. Total RNA was extracted from each sample by using RNeasy Mini Kit (QIAGEN).

First strand cDNA was synthesized by using SOLiD^{TM} Total RNA-Seq for Whole Transcriptome Libraries (Applied Biosystems, Inc., California, USA). For EST and transcriptome analysis, a research contract service (Genaris, Inc., Kanagawa, Japan) was used.

Cloning of *M. alpina* promoters

Information regarding selected promoters analyzed in this chapter is shown in Table 1. Selected promoter regions were cloned from the genome of *M. alpina* 1S-4 by PCR performed using specific primers (Table 1-1) designed on the basis of the information available in the genomic database for this strain. For deletion constructs, the anti-sense primers used for PCR are shown in Table 1-1 and forward primers are shown in Table 1-2. *Xba*I and *Spe*I restriction enzyme sites were created at the 5′ end of each forward primer and at the 3′ end of each reverse primer, respectively. When an *Xba*I site was present in the promoter region, an *Spe*I site was created instead of the *Xba*I site at the 5′ end of the forward primer. When an *Spe*I site was present in the promoter region, an *Xba*I site was created instead of the *Spe*I site at the 3′ end of the reverse primer.

Construction of *GUS* reporter gene-carrying vectors for promoter analysis

The reporter gene vectors were constructed on the backbone of pBIG3ura5s [42]. The histone promoter (the histone H4.1 promoter short fragment [42]), succinate dehydrogenase subunit B (*SdhB*) terminator [22] and the *ura5* marker gene [21] were amplified from the genomic DNA of *M. alpina* 1S-4. The *ura5* expression cassette controlled by a histone promoter and *SdhB* terminator was generated by fusion PCR with additional *Eco*RI and *Xba*I restriction enzyme sites at the 5′ and 3′ ends, respectively, of this cassette. The *ura5* expression cassette, digested with *Eco*RI and *Xba*I, was ligated to pBIG3ura5s [42] digested with the same restriction enzymes and designated as pBIG35Zh.

The *β*-Glucuronidase (*GUS*) gene was synthesized with optimized codon usage to reflect the codon bias of *M. alpina* 1S-4 obtained from the Kazusa database (http://www.kazusa.or.jp/codon/), with additional SpeI and *Bam*HI restriction enzyme sites at the 5′ and 3′ flanking ORFs, respectively. The *GUS* expression cassette, controlled by a histone promoter and *SdhB*

terminator, was generated by fusion PCR with additional *Xba*I and *Nhe*I restriction sites at the 5′ and 3′ ends of the cassette, respectively. This *GUS* expression cassette was digested with *Xba*I and *Nhe*I and ligated to pBIG35Zh digested with same restriction enzymes and designated pBIG35ZhGUSm (Fig. 1-1). In this vector, the histone promoter region, located upstream of the *GUS* gene, can be removed by digestion with *Xba*I and *Spe*I, and replaced by another promoter fragment digested with *Xba*I and/or *Spe*I for promoter assays.

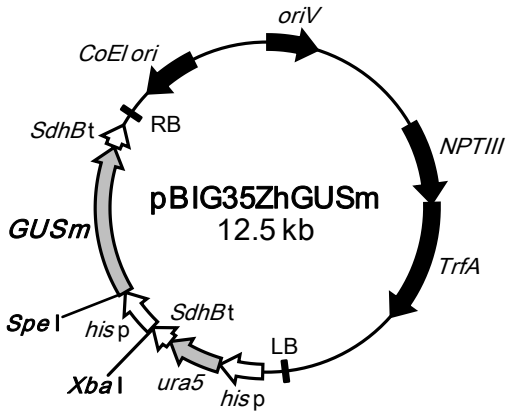


Fig. 1-1. Vector construct used in *M. alpina* 1S-4 promoter assays.
GUSm, codon-optimized *β*-glucuronidase gene for *M. alpina*; *his* p, *M. alpina* histone H4.1 promoter short fragment; *SdhB* t, *M. alpina* SdhB transcription terminator; *ura5,* orotate phosphoribosyl transferase gene of *M. alpina* 1S-4; *NPTIII*, neomycin phosphotransferase III gene; *TrfA,* TrfA locus, which produces 2 proteins that promote replication of the plasmid; *ColEI ori*, ColEI origin of replication; *oriV,* pRK2 origin of replication; RB, right border; LB, left border.

Transformation of *M. alpina* 1S-4 *ura5*⁻ strain

A spore suspension of *M. alpina* 1S-4 *ura5*⁻ strain was freshly prepared by harvesting from cultures grown on Czapek-Dox agar medium supplemented with 0.05 mg/ml uracil and then filtering the suspension through Miracloth (Calbiochem) [40].

Transformation of *M. alpina* 1S-4 *ura5*⁻ strain was performed using the *Agrobacterium tumefaciens*-mediated transformation (ATMT) method described previously [42] with slight modification. Briefly, *Agrobacterium tumefaciens* C58C1 was transformed with each vector via electroporation as described previously [43] and its transformants were isolated on LB-Mg agar plates supplemented with kanamycin (20 μg/ml), ampicillin (50 μg/ml) and rifampicin (50 μg/ml). *Agrobacterium tumefaciens* transformants were cultivated in 100 ml of MM supplemented with kanamycin (20 μg/ml) and ampicillin (50 μg/ml) at 28°C for 48 h with shaking (120 rpm). Bacterial cells were harvested by centrifugation at 8,000 × *g*, washed once

with fresh IM, and then diluted to an optical density of 660 nm (OD_{660}) of 0.1–0.2 in 10 ml of fresh IM. After pre-incubation for 12–16 h at 28°C with shaking (300 rpm) to an OD_{660} of 1.5–2.0, 100 µl of the bacterial cell suspension was mixed with an equal volume of a spore suspension (10^8 spores/ml) of *M. alpina* 1S-4 *ura5*$^-$ strain, and then spread on membranes (Whatman #50 Hardened Circles, 70 mm, Whatman International Ltd. UK) kept on cocultivation media (IM with 1.5% agar) and incubated at 23°C for 5 d. After cocultivation, the membranes were transferred to uracil-free SC agar plates that contained 0.03% Nile blue A (Sigma-Aldrich Japan) to distinguish between fungal colonies and the white color of the membrane. After 2 d of incubation at 28°C, hyphae from visible fungal colonies were transferred to fresh uracil-free SC agar plates, this was repeated 3 times to obtain candidates. Integration of the vector into the chromosome of the host strain was verified by PCR, as described previously [40].

GUS assay

Cell-free extracts of *M. alpina* were prepared by a slight modification of a method described previously [21]. All transformants and the wild-type strain of *M. alpina* 1S-4 were cultivated in 10 ml of GY medium for 2–14 d at 28°C with shaking (300 rpm), harvested by suction filtration, and washed twice with sterile water. Fungal mycelia were suspended in 2 volumes of 100 mM Tris-HCl containing 5 mM 2-mercaptoethanol (pH 7.5) and then disrupted by using a bead shocker (Wakenyaku Co. Ltd., Kyoto, Japan) at 5,000 rpm for 30 s twice with glass beads (φ 1.0 mm, Waken B Tech Co. Ltd., Kyoto, Japan). The extract was centrifuged at 15,000 × *g* for 10 min to remove cell debris and intact cells. The supernatant was used for the GUS assay as cell-free extract. All steps were performed at 4 °C.

β-Glucuronidase (GUS) assays were performed as described previously [44]. Enzyme activity was calculated in terms of nanomoles of *p*-nitrophenol production per milligram of protein per minute at 37°C. Protein concentration was measured according to the Bradford method, using bovine serum albumin as a standard [45].

Table 1-1. PCR primers for selected promoters.

Promoter	Primer F sequence (5′- 3′)	Primer R sequence (5′- 3′)
PP1 p	AATCTCTAGA[a]GCGCAGTCGGAATGCC	AGTAACTAGTCGTGTTTTCTTTTGAAATGGG
PP2 p	AAGCTCTAGAGACTGTAAAGACGGAGGGG	AGTAACTAGTTGTGGATAGTGGGTAGTGG
PP3 p	AACGTCTAGACGTGTTATCTTGCGCTGC	TCATACTAGTGATGATTTAGAGGTGTTGG
SSA2 p	TTAGTCTAGAAAAGTGCTGCTTCGGAACC	AGATACTAGTGATGTAGATGTGAGTGTGAG
PP7 p	AATATCTAGATGACCGTGCGCTTTTTGAGAC	AGCAACTAGTCGTATATTTGTTGAAAGGTG
SSA22 p	AATATCTAGAGGGTGCAGGTCCGGTCC	AGCCACTAGTTCTACTCACCTTTTCCCTCAG
PP4 p	TGAGTCTAGAAGAGTGATTTTGTGGCTGTAC	CAATACTAGTGGCTGATGTATGTGTTGATG
PP8 p	ATGCTCTAGATATGGCGACCCATTCACG	AAGAACTAGTGGTTGAACAGAGTATGTTTGC
SAH1 p	AATCTCTAGACTGGCGAATACATGCGCAC	ATAGTCTAGAGGTGGATATGAAGGGTGG
PET9 p	ACCTTCTAGAAGACGAGAAGAGTTCATGATG	AATAACTAGTGATGAGTGTATGTGGAGAGTG
HSP104 p	AATATCTAGAGTTGAAGGTGCAGACACCGG	AATAACTAGTGGTGGGGCGTTATGTGG
HSC82 p	ATCATCTAGAGAGCTCAAGATGAAGGTGCTC	AATAACTAGTGGTGTGTGTGGTTTGCGGG
UBC5 p	AACTACTAGTGTATACAGGTCTTAGAGACC	ATTCACTAGTCGTGGGTGGAGAGAGTG
CDA1 p	AACTCTAGATGAAAATAGAAATGGGTGGATGG	ATTGACTAGTCGTAGGTTTCTTTGTGTGTG
RPP0 p	AATGTCTAGACACAGTGACAAGGGTGTTAAC	ATGCACTAGTGTTGATTATTGTTCGAGGG
PP5 p	AACGTCTAGATGTTTTTTGTGCAAATTACCTCG	AAGCACTAGTTTTGGATTGGGATTGCTTGAG
PP6 p	AAAGTCTAGACTGGCAATAGTTAGTGCACG	ATCAACTAGTGATGGAGGTTTGTTTGAGAAG
RPS16B p	AATGTCTAGACCTGCAGAAAGATGATCCAAAAG	AAGCACTAGTGATGAATAATGCCTATGATCAG
EFB1 p	TTAGACTAGTCGTAGTTGACTCTTTTATG	CAGTACTAGTGGTGGGTGCTTTGTCGATTTG
TDH1 p	AACCTCTAGAAGGAAATAAATTCTCCTCGGTG	AATAACTAGTGTTGAGTGGGTGTGTGTGG
CIT1 p	ATTTTCTAGACACCTCAAAAACGTGCCTTG	AATAACTAGTGGCGGATATGTGTATGGAG
TIF2 p	AAGTTCTAGAGTCGACCTATCATCATTTTTGGC	AGCGACTAGTGTTTTTTTTTGCTTTTTTTTTTTATG
CAT2 p	AATCACTAGTAAACGGTGGAGCATTCTCAC	TATCACTAGTGAAGGCGATGGGCAGGG
ELO1 p	AATGTCTAGACTTGCCCAGCATTACTCC	TCATACTAGTCTTTGAGGGGAGGAATTGC
IPP1 p	ACAATCTAGAGGCTGCGTTGCCGGGAG	ATAGACTAGTGGTGGTGGTGAAGAGTAG
OLE1 p	AGCATCTAGAGGGTTCTCACATTGAATTTG	AATAACTAGTCGCTGTGCGTCCTGCGTTG
PGK1 p	TGAATCTAGACACCGTCGCTATGTGAAG	TTGCTCTAGAGCAGAAACACACTGGCAG

[a] The underlined sequences show synthesized *Xba*I (TCTAGA) and *Spe*I (ACTAGT) sites.

Table 1-2. PCR primers used for deletion clones.

Promoter	Length of deletion clone (bp)	Primer F sequence (5′- 3′)
PP2 p	1199	ATTTCTAGA[a]TGCATTTACAGGTGAATATTAC
	820	TTATCTAGACATAAAAGTGTCTGGAGCG
	399	TTATCTAGAACTAAGTGGTGTCTACTTTGG
	202	AATTCTAGAGGATACTCCATCCCCACCC
PP3 p	1651	AATATCTAGAGATCCTGGTCGAAAAAGACAG
	1201	AATGTCTAGATGAGTTTCTGTTTTTTCCTTTTTGC
	801	AATATCTAGATGAACAATTCATGCAGCTTCACG
	401	AATATCTAGACGTCTAAGCGTTTACGTGCC
	201	AATATCTAGACTCGTTTTGATGGAGTTCTC
SSA2 p	843	AGTATCTAGATGACGGCGTGTATATGTCAG
	599	AGGTTCTAGACCATTGTATCGATTTCTGAT
	399	AGTATCTAGAGCTATGCGAACGGTTCATTTTG
	199	AGGTTCTAGATTTTTTCTCTCTGGTGTGAACG
PP7 p	1079	AGCATCTAGAAAAACTATTCAATAATGGGCG
	785	ATTTCTAGAATGGCGAGACGCAGGGGGTAG
	500	AATATCTAGAGAGTGGGCACTGAACTAAAAAG
	250	AATATCTAGAGACACTGCATGACGCGAAATC
HSC82 p	800	AATTCTAGATTTTACTACCGCATTCCCTTTTC
	599	ACGTCTAGACCTTTTCAGTAAACAATTTC
	400	ATTTCTAGACACAAAGAAGAAGGGTGTGTC
	200	ACGTCTAGAACTGTTTTCTTGAAACTTC
PP6 p	1000	AATTCTAGACAGTTACCGTGCGCCCACTG
	750	AATTCTAGACTTTCACAAATAGGCATCCTATC
	500	AATTCTAGAGGCTTTTTCGTTTATTGGATTG
	93	ACGTCTAGATATCCAATTCTCACCACTTC
CIT1 p	1263	AAGTCTAGATGTCAATCATCTTTGCTGCTG
	963	TGCGTCTAGAATTATAATTATAATGAGGAAGTG
	663	TTATCTAGAGGCGAGTGGCGGACTGC
	363	TTGTCTAGACAATTGGCAAGGCTGGGTTG

[a] The underlined sequences show synthesized *Xba*I (TCTAGA) site.

RESULTS

Selection, cloning and evaluation of various promoters of *M. alpina* 1S-4

The cDNA libraries were prepared by using RNA extracted from the mycelia of *M. alpina* 1S-4 on during different cultivation stages (see Materials & Methods). EST analysis were performed for each cDNA sample, and the abundances of each EST clone during all cultivation stages were summed (data not shown). These totals were sorted in descending order. On the basis of these EST abundance data and previous reports regarding conventional promoters of other organisms [27, 46-48], putative promoter regions of 28 genes of *M. alpina* 1S-4 were selected as candidates of highly-expressing and/or temporally-regulated promoters (Table 1-3). Considering of the positions of putative transcriptional factor-binding sites in each selected promoter region, approximately 1000–2500 bp of the 5′ flanking region of individual ORFs were cloned as putative promoter regions from the genomic DNA of *M. alpina* 1S-4. To evaluate the activity of these putative promoters in *M. alpina*, pBIG35ZhGUSm plasmids carrying each putative promoter region, instead of the histone promoter, located upstream of the *β*-glucuronidase (*GUS*) gene were constructed (Fig. 1-1) and transformed into *M. alpina* 1S-4 using the ATMT method. For each construct, 30 transformants were randomly selected and cultivated for 5 d in GY liquid medium, and then their GUS activities were measured. Due to the variety in GUS activity in individual *M. alpina* transformant lines with each promoter construct (a representative pattern is shown in Fig. 1-2), the average value of GUS activities in the 10 moderately expressing lines was used for comparison with different promoter activities (Fig. 1-3). As shown in Fig. 1-3, *PP1*, *PP3, SSA2*, *PP7*, *HSC82*, *PP6*, *TDH1* and *CIT1* promoters led to increased GUS activity compared with a conventional histone promoter. In particular, *PP3* and *PP6* promoters showed approximately 5-fold higher activity than the histone promoter.

The author also carried out the same experiments with GS medium, which was used for large-scale cultivation (see Materials and Methods). There were no apparent differences in the GUS activity levels between GY and GS media (data not shown). Therefore, GY medium was used to cultivate transformants in all subsequent GUS assays.

Table 1-3. Information regarding genes for selected promoters.

Gene	Annotation	Relative EST transcript abundance[a]
PP1	Predicted protein	35.7
PP2	Predicted protein	29.0
PP3	Predicted protein	11.7
SSA2	ATP binding protein (member of HSP70 family)	8.9
PP7	Predicted protein	7.9
SSA22	ATP binding protein (member of HSP70 family)	7.6
PP4	Predicted protein	7.3
PP8	Predicted protein	6.6
SAH1	*S*-Adenosyl-L-homocysteine hydrolase	6.6
PET9	ADP/ATP carrier of the mitochondrial inner membrane	6.0
HSP104	Hsp that cooperates with Hsp40 and Hsp70	5.9
HSC82	Cytoplasmic chaperone of the Hsp90 family	5.6
UBC5	Ubiquitin-conjugating enzyme	4.7
CDA1	Chitin deacetylase	4.5
RPP0	Ribosomal protein P0	4.0
PP5	Predicted protein	4.0
PP6	Predicted protein	3.8
RPS16B	Protein component of 40S ribosormal subunit	3.2
EFB1	Translation elongation factor 1 beta	2.6
TDH1	Glyceraldehyde-3-phosphate dehydrogenase	2.4
CIT1	Citrate synthase	2.0
TIF2	Translation initiation factor eIF4A	1.9
CAT2	Carnitine acyl-CoA transferase	0.9
ELO1	Fatty acid elongase I	0.7
IPP1	Cytoplasmic inorganic pyrophosphatase	0.7
OLE1	Delta-9 fatty acid desaturase	0.6
PGK1	3-Phosphoglycerate kinase	0.4

[a]EST abundance data show the total for EST transcriptional abundance at different cultivation stages, by using relative values for histone H4.1.

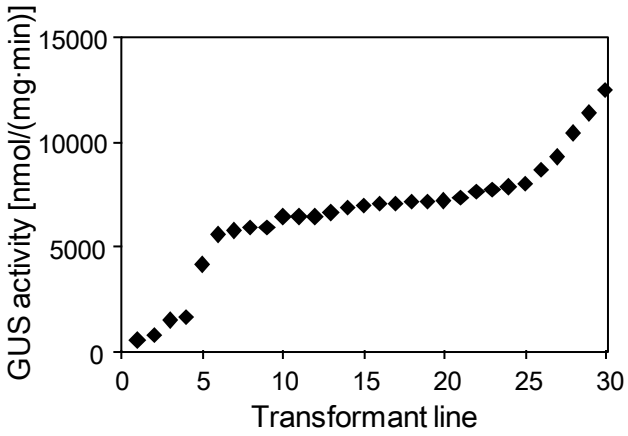


Fig. 1-2. Distribution of GUS activity levels driven by the *HSC82* promoter in *M. alpina* transformants cultivated for 5 d in GY liquid medium.
Each plot denotes individual transformants, and all plots are sorted in ascending order of GUS activity. GUS activity is expressed in nanomoles of *p*-nitrophenol produced per minute per milligram of protein.

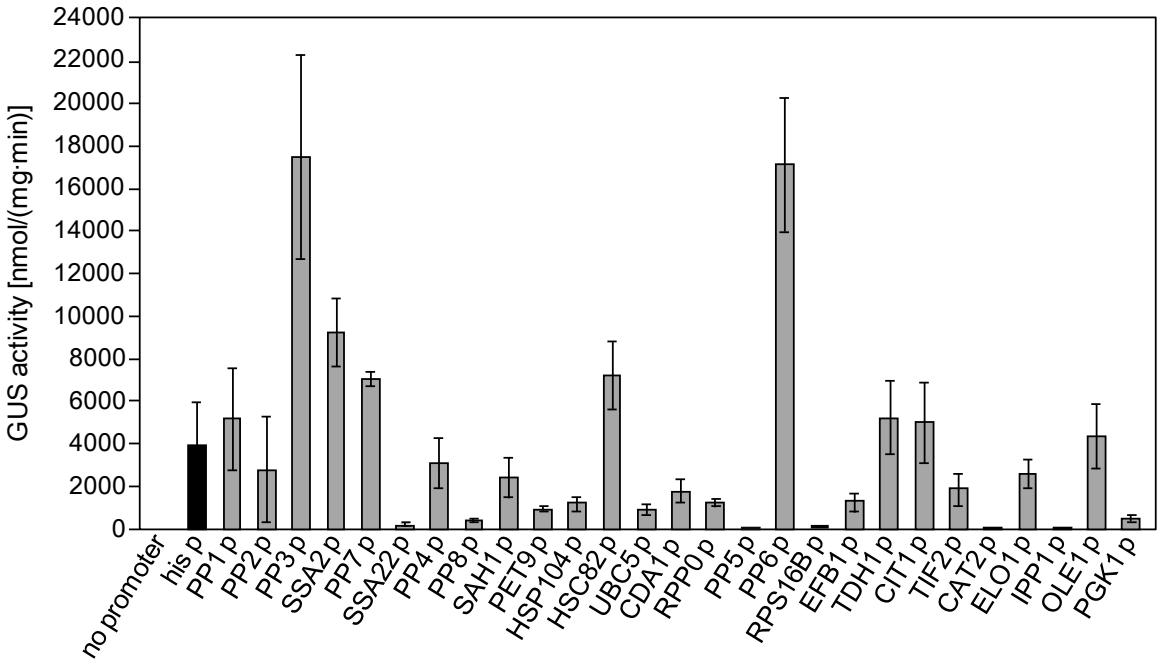


Fig. 1-3. GUS activity driven by various promoters in *M. alpina* transformants cultivated for 5 d in GY liquid medium.
GUS activity is expressed in nanomoles of *p*-nitrophenol produced per minute per milligram of protein. The Bars represent the mean values with standard deviations of GUS activity in 10 individual transformant lines for each promoter construct.

Time course measurements of promoter activity during cultivation of *M. alpina* 1S-4

Transformants with each promoter construct were cultivated in GY medium for 2–14 d and then GUS activity was evaluated in order to investigate the effect of cultivation time on GUS activity with different promoters (Fig. 1-4). Based on the pattern of time-dependent changes in GUS activity, promoters could be categorized into the following 4 groups; GUS activity levels controlled by the *HSC82*, *PP7*, *SSA2*, *HSP104*, *UBC5* or *PET9* promoter were almost constant throughout the cultivation period (Fig. 1-4A). With the *CIT1*, *PP8*, *SAH1*, *EFB1*, *OLE1*, *HSC82*, *CDA1*, *RPP0*, *RPS16B* or *CAT2* promoter, GUS activity levels were higher in the early stage of cultivation and then decreased (Fig. 1-4B). GUS activity controlled by the *PP6*, *ELO1* or *TDH1* promoter peaked at the middle stage of cultivation (Fig. 1-4C). With the *PP3*, *PP2*, *PP4*, *PP5*, *SSA22*, *IPP1* or *PGK1* promoter, GUS activity levels were low in the early stage, and then increased with cultivation time (Fig. 1-4D).

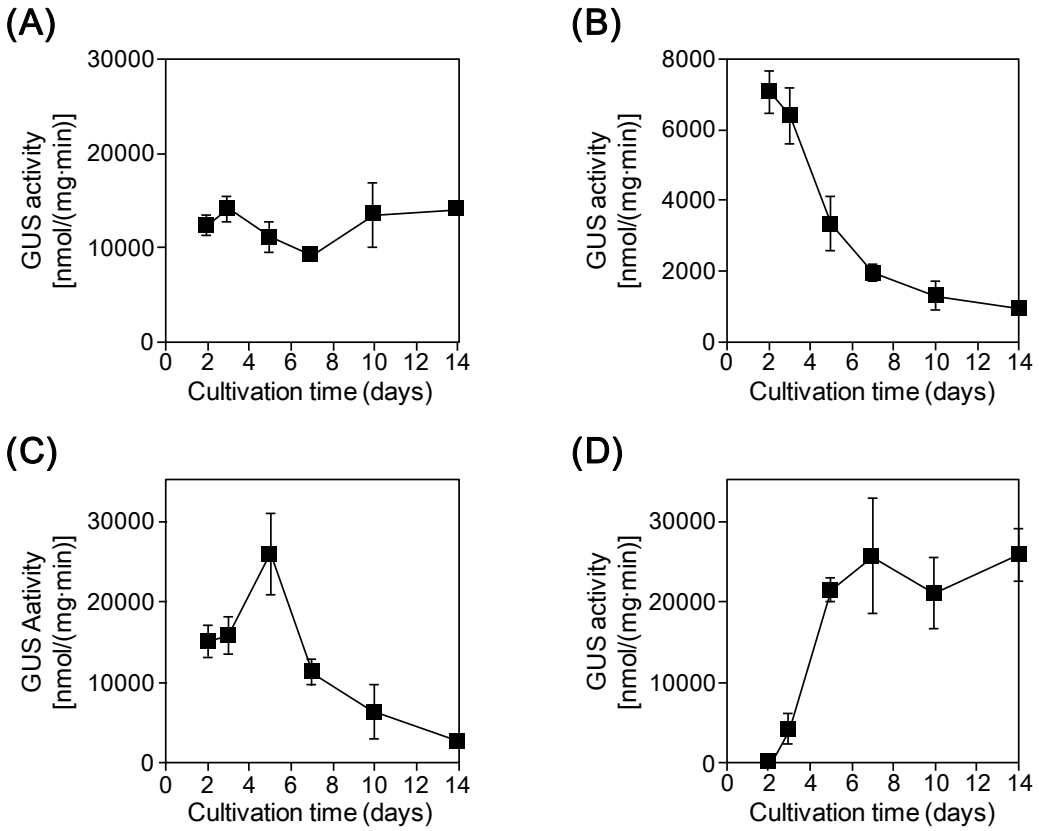


Fig. 1-4. Representative patterns of time-depend changes in GUS activity with different promoters. Results with (A) *HSC82*, (B) *CIT1*, (C) *PP6* and (D) *PP3* promoters are shown as representative.
All transformants for each promoter construct were cultivated in GY medium for 2–14 d. GUS activity is expressed in nanomoles of *p*-nitrophenol produced per minute per milligram of protein. Plots represent the mean values with standard deviations of GUS activity in 3 individual transformant lines for each construct.

PP2, *PP3*, *PP6*, *PP7*, *SSA2*, *HSC82* and *CIT1* promoters with constitutive or time-dependent high-level activity were selected and used for subsequent studies.

Deletion analysis

In order to investigate the length of the promoter regions required to maintain high expression activity, a series of 5′ deletion constructs of the 7 selected promoters were generated (Fig. 1-5, left column) and introduced into *M. alpina* 1S-4. For each deletion construct, 30 randomly selected transformants were cultivated in GY medium for the appropriate number of days based on the above results, and then GUS activity was evaluated. For comparison, the GUS activity levels of 10 moderately expressing lines were averaged and represented as a value relative to each full-length promoter, which was set as 100% (Fig. 1-5, right column). In the *PP2*, *PP3* and *PP6* promoters, relatively long lengths of the promoter regions (over 1,000 bp) were required for high GUS expression, and the GUS activity levels dramatically diminished with deletion of the 5′ regions. In contrast, the other promoters maintained high activity even in relatively short regions (approximately 400–800 bp).

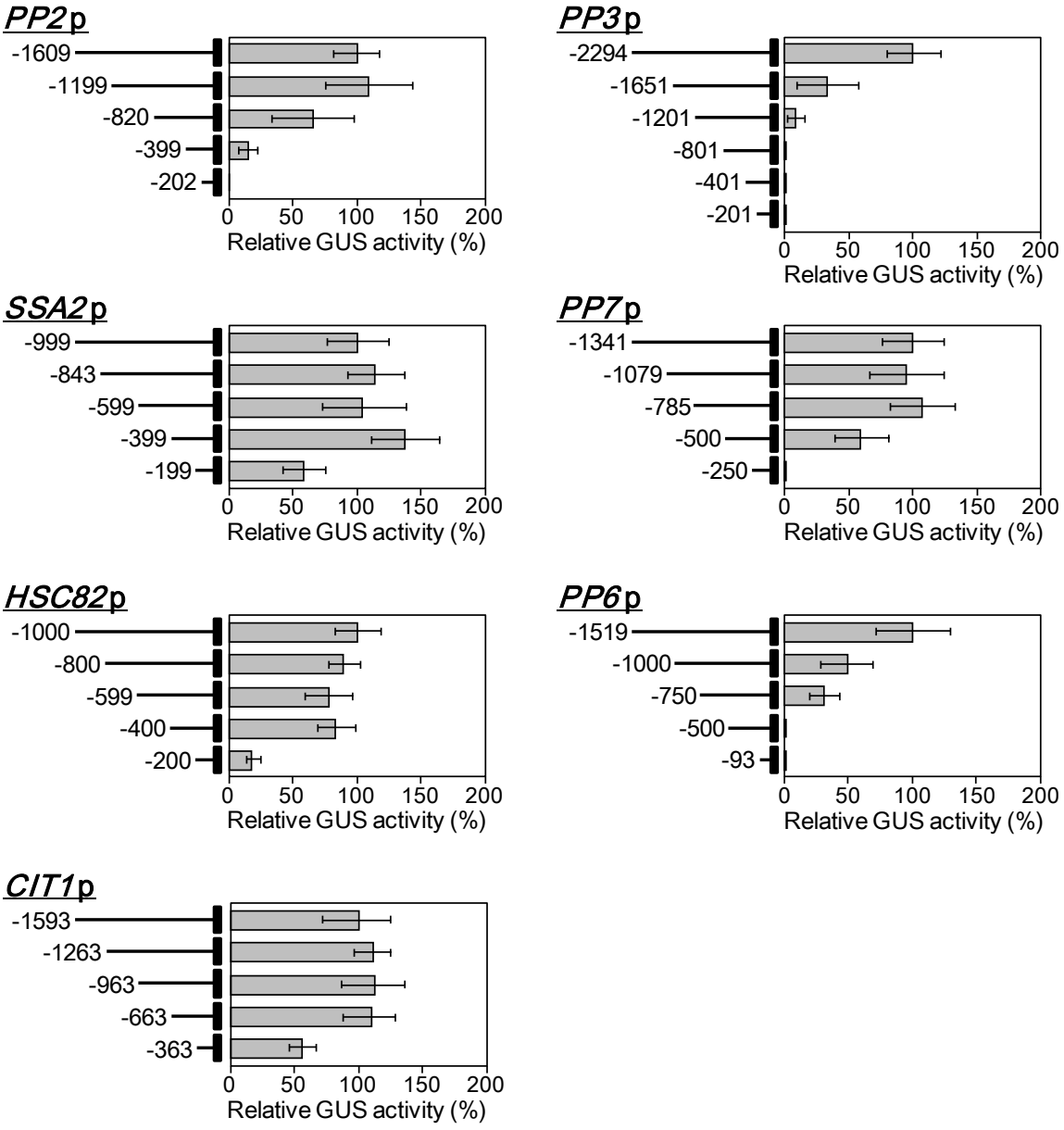


Fig. 1-5. 5′-deletion analysis of 7 different promoters.
In the left column, constructs with different 5′ upstream deletions of individual promoters are shown. For each construct, the length of the fragment upstream from the transcription start site is shown on the left end. In the right column, GUS activity levels with the deleted constructs in *M. alpina* transformants are shown. All transformants were cultivated in GY liquid medium. Cultivation times were 5 d for the *SSA2*, *HSC82*, *PP7* and *PP6* promoters, 3 d for the *CIT1* promoter, and 14 d for the *PP2* and *PP3* promoters. The average GUS activity of each full-length construct is set at 100% and has been used to define the relative GUS activity of individual deletion constructs. Bars represent the mean values with standard deviations of GUS activity in 10 individual transformant lines for each construct.

DISCUSSION

In general, promoters that are useful for gene manipulation systems exhibit either constitutively high, time-dependent and/or conditionally inducible expression. Thus, screening and investigation of beneficial promoters for *M. alpina* gene manipulation was performed by using an EST-based approach in this view point. EST abundance data can provide gene expression levels without post-translational influence. Therefore, by using an EST-based approach, the desired promoters can be identified more directly and efficiently than by using conventional approaches based on information on protein expression.

In many cases, EST analysis is employed to obtain transcriptional information at a certain point in the cultivation period. Because the transcriptional level of each gene generally changes depending on the cultivation stage, in this chapter, EST analysis with *M. alpina* was carried out at different cultivation stages. On the basis of the EST data and previous reports on conventional promoters of other organisms, 28 promoters of *M. alpina* 1S-4 were selected as candidates for highly expressing and/or regulated promoters (see Table 1-3).

The *GUS* reporter gene was used to monitor the promoter activity in this chapter because the *GUS* gene has been commonly used as a reporter gene for promoter assays for various organisms [25, 36]. In addition, the author considered that this study also means investigation of heterologous gene expression in *M. alpina*, because the *GUS* gene is a heterologous gene for this strain.

The GUS activity in *M. alpina* transformant lines with each promoter construct was distributed across a wide range (Fig. 1-2). This dispersion might be attributable to the differing locations of the GUS gene in chromosomal DNA, *i.e.*, the position effect. It has previously been reported that *M. alpina* transformants generated by the ATMT method have a single copy of T-DNA at a random location in chromosomal DNA [42].

The comprehensive analysis showed that the *PP3* and *PP6* promoters were demonstrate remarkably higher GUS activity than the conventional histone promoter in *M. alpina*. The functions of the proteins coded by the *PP3* and *PP6* genes are unknown. Investigation of the function of these proteins functions might lead to new findings, which may in turn lead to new insights on *M. alpina* physiology. Interestingly, the GUS expression levels were not necessarily proportional to the EST abundance values (compare Fig. 1-3 with Table 1-3). There were some

cases where the GUS expression levels were much lower than expected from the EST abundance data, *e.g.* the *SSA22* and *PP8* promoters. In such cases, other factors besides promoters, such as the terminator and post-transcriptional processing might lead to high-transcriptional levels of the original gene, unlike the findings seen for heterologous GUS gene expression.

Time-course measurements of GUS activity levels with various promoters showed several temporally-different patterns of expression (Fig. 1-4). These promoters allow for phase-specific expression in *M. alpina*, unlike the conventional histone promoter expressing constitutively during cultivation time (data not shown). These time-dependent promoters could contribute to more efficient production of PUFAs in *M. alpina* by means of temporal coordination of enzyme expression with PUFA biosynthesis.

For the 5′ deletion analysis of promoter regions, 7 promoters were selected because of their characteristic expression patterns, such as high-level expression and/or time-dependent expression. A relatively long length (over 1,000 bp) was required to maintain high activity in the *PP2*, *PP3* and *PP6* promoters. This finding suggests that transcription factor binding sites or enhancer elements of these promoters are located considerably upstream. In contrast, the *SSA2*, *PP7*, *HSC82* and *CIT1* promoters retained sufficient activity even in the truncated form (400–800 bp). These short promoters with high activity will be advantageous in applications involving *M. alpina* gene manipulation because they will be useful for convenient vector construction.

More detailed deletion analysis and consensus sequence analysis of highly-expressing and/or regulated promoters will help identify functionally essential elements for transcriptional regulation. This in turn could help elucidate the transcriptional regulatory mechanisms of *M. alpina*. The information of transcriptional regulatory elements of promoters for high-level expression and time-dependent expression is also useful for applications. For example, in *Aspergillus oryzae*, the introduction of multiple copies of the consensus sequence found in the high-expression promoters has been reported to improve promoter activity [49].

SUMMARY

The promoter regions of 28 genes in *M. alpina* 1S-4 were selected and cloned on the basis

of EST abundance data. The activity of each promoter was evaluated by using the *GUS* reporter gene. Eight of these promoters were shown to enhance GUS expression more efficiently than the conventional histone promoter. Especially, the predicted protein 3 (*PP3*) and the predicted protein 6 (*PP6*) promoters demonstrated approximately 5-fold higher activity than the histone promoter. The activity of some promoters changed along with the cultivation phase of *M. alpina* 1S-4. Seven promoters with constitutive or time-dependent, high-level expression activity were selected, and deletion analysis was carried out to determine the promoter regions required to retain activity. The promoters described in this chapter will be useful tools for gene manipulation in this strain.

CHAPTER II
Characterization of galactose-dependent promoters from an oleaginous fungus *Mortierella alpina* 1S-4

An inducible expression system is an significantly important tool for the control of gene expressions. It is necessary for the expression analysis of given genes, especially lethal and essential genes. Many investigations of inducible expression system have been carried out in various microorganisms [29, 35, 50]. Some of the most widely used regulatory systems are based on promoters that can be activated or repressed by the presence/absence of the inducer such as a carbon source in the medium [29-35, 51, 52]. These inducible expression systems have contributed to the functional analysis of genes of interest as well as for the efficient production of the heterologous proteins in these microorganisms.

As mentioned in chapter I, in *Mortierella alpina* 1S-4, a few constitutive expression promoters have been identified and applied to the gene expression system at present. The lack of an inducible expression system in *M. alpina* limits of detailed study of genes of interest, especially essential or lethal genes. To increase knowledge of *M. alpina*, it is essential to establish an inducible expression system.

In this chapter, the author describe the cloning and initial characterization of endogenous galactose inducible promoters for use in *M. alpina* 1S-4.

MATERIALS AND METHODS

Strains, media, and growth conditions

The strains and media described in chapter I were used. For galactose induction in the submerged cultivation, 500 mg/ml sterile galactose solution was added to the medium at 2% final concentration. All cultivations were performed as described in chapter I otherwise mentioned.

Genomic DNA preparation

Genomic DNA of *M. alpina* 1S-4 was prepared as described in chapter I.

Construction of *GUS* reporter gene-carrying vectors for promoter analysis

For GUS reporter assay, the vector pBIG35ZhGUSm (see chapter I) was used. The *GAL1* and *GAL10* promoter regions were amplified from the genome of *M. alpina* 1S-4 by PCR with specific primers (Table 2-1) designed based on the genomic database of this strain. For deletion constructs of the *GAL10* promoter, GAL10pR was used as the anti-sense primer, and GAL10p2000F, GAL10p1600F, GAL10p1200F, GAL10p800F and GAL10p400F were used as the sense primers (Table 2-1). All cloned fragments were treated with *Xba*I and/or *Spe*I and inserted in front of the GUS ORF from pBIG35ZhGUSm digested with *Xba*I and *Spe*I.

Table 2-1. PCR primers used to clone the *GAL1* and *GAL10* promoter regions.

Primer name	Sequence (5′ to 3′)
GAL1pF	AATATCTAGA[a]ACCACGCATGACAATGCCAC
GAL1pR	AAGAACTAGTTGTAAAAGGGGCTGACAGTG
GAL10pF	AATATCTAGAGGTTCCGAGAGGTGGATTTG
GAL10pR	ATAATCTAGATGGCTCCTGAAAGGACGAG
GAL10p2000F	AATTCTAGACGCAGAGTGATGGTCATTACC
GAL10p1600F	AATTCTAGACTCTATGGCAAGATTACGAG
GAL10p1200F	AATTCTAGATGCTCGTGAAGAGGGGCAC
GAL10p800F	ACGTCTAGACATTTTTTGCCGCCAATTCTG
GAL10p400F	ATTTCTAGACCCCCGCCTATTTTTTTTTTC

[a] The underlined sequences indicate inserted XbaI (TCTAGA) and SpeI (ACTAGT) sites.

Transformation of the *M. alpina* 1S-4 ura5⁻ strain

The transformation of the *M. alpina* 1S-4 ura5⁻ strain was performed by the ATMT method as described in chapter I.

GUS assay

Preparation of cell-free extracts of *M. alpina*, GUS assays and measurement of protein concentrations were performed as described in chapter I.

RESULTS

Cloning and basic evaluation of two *GAL* promoters in *M. alpina* 1S-4

The putative promoter region of the *GAL1* and*GAL10* genes of *M. alpina* 1S-4 were cloned as candidates for galactose-dependent promoters based on the *M. alpina* genome database. The lengths of cloned *GAL1* and *GAL10* promoter regions were 962 bp and 2331 bp, respectively.

To ascertain if these promoters were regulated by galactose in this strain, plasmids carrying the predicted *GAL1* and *GAL10* promoters fused to the *GUS* reporter gene were constructed and transformed into *M. alpina* 1S-4 by the ATMT method. All transformants had a single copy of T-DNA at a random location in the chromosomal DNA (data not shown). At least 30 independent transformants for each construct were randomly selected, evaluated for GUS activity, and cultivated on SC medium containing 2% galactose substituted for glucose. All transformants exhibited detectable levels of GUS activity (data not shown), and three individual transformants that showed moderate levels of GUS activity were used in subsequent studies.

The transformants carrying *GAL1* or *GAL10* promoter-*GUS* genes were cultivated on SC agar medium containing 2% of sugars substituted for glucose (Table 2-2). As shown in Table 2-2, the expression of *GUS* regulated by the *GAL* promoters was clearly dependent on the presence of galactose in the medium. The GUS activity of fungi with *GAL1* or *GAL10* promoters grown on galactose medium was approximately 7-fold or 100-fold higher than those grown on glucose medium, respectively. With the *GAL1* promoter, GUS expression was induced by galactose, lactose and raffinose; furthermore, the not-negligible level of GUS activity was detected even when grown on the medium without a carbon source. On the other hand, GUS expression with the *GAL10* promoter was fairly repressed wh en fungi were grown on media lacking galactose with/without other kinds of sugars.

Because *GUS* expression with the *GAL10* promoter were more sensitively induced/repressed by the presence/absence of galactose, the author focused on the *GAL10* promoter for further investigation.

Table 2-2. GUS activity resulting from the β-glucuronidase gene fused to *GAL1* and *GAL10* promoters in transformants cultivated on solid media containing different carbon sources.

	GUS activity [nmol/(mg·min)]	
Carbon source	*GAL1* p	*GAL10* p
no carbon source	593.8 ± 43.4	72.1 ± 14.9
glucose	440.1 ± 46.4	19.5 ± 2.3
galactose	3360.1 ± 780.7	1890.8 ± 372.1
lactose	1653.6 ± 84.2	282.6 ± 67.9
raffinose	916.7 ± 63.0	63.3 ± 14.6
glucose + galactose	3407.3 ± 253.6	1562.6 ± 137.2
lactose + galactose	3543.4 ± 526.7	1876.8 ± 299.2
raffinose + galactose	3152.3 ± 187.9	2223.9 ± 256.9

All transformants were cultivated in SC medium containing 2% of each sugar substituted for glucose for 3 days at 28°C. The values represent mean GUS activity of three transformant lines (± standard deviation).

Induction-response of the *GAL10* promoter by galactose addition

Time course measurements of *GAL10* promoter activity after addition of galactose to the medium were carried out. The transformants were cultivated for 4 days in synthetic SC liquid medium containing 2% raffinose substituted for glucose as a sole carbon source, and then galactose (2% final concentration) was added to the medium. The GUS activity was monitored over a 48-h time course (Fig. 2-1). An increase in GUS expression was detected at 10 h after the addition of galactose, and then the GUS expression level reached a peak at 36 h.

As shown in Fig. 2-2, GUS expression was induced by the addition of galactose, regardless of cultivation phase of mycelia. In all cases, the induction of GUS expression was maintained for 2–3 days, and then GUS activity declined.

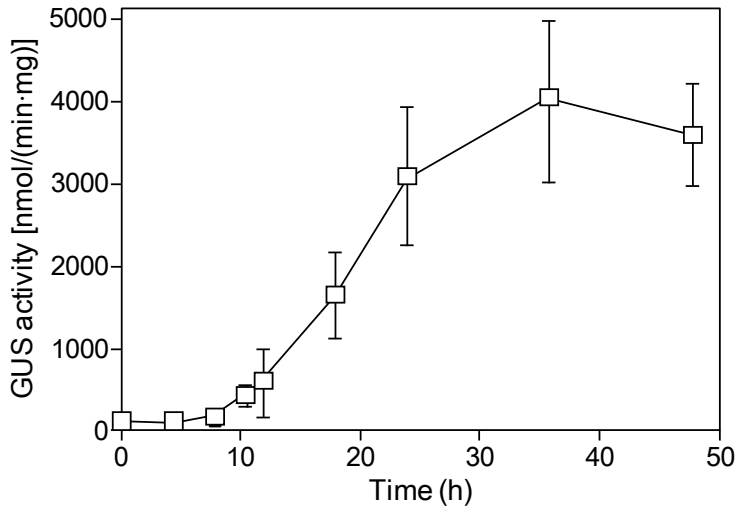


Fig. 2-1. GUS activity in response to *GAL10* promoter induction by galactose addition in submerged cultivation. Transformants were pre-cultivated in SC liquid medium containing raffinose substituted for glucose for 4 days, and then galactose was added at t=0. GUS activity was monitored over a 48 h time course. The values represent mean GUS activity of three transformant lines (± standard deviation).

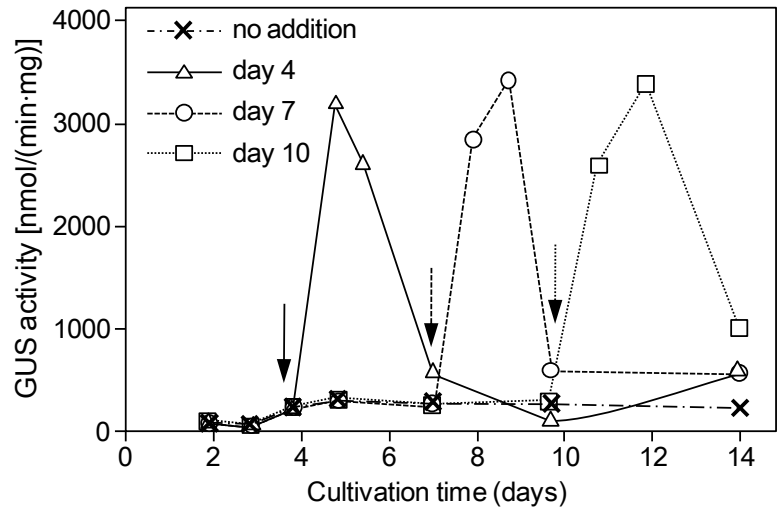


Fig. 2-2. GUS activity in response to *GAL10* promoter induction by galactose addition in different cultivation phase in synthetic medium.
Transformants were cultivated in SC liquid medium containing raffinose substituted for glucose, and then galactose was added on day 4, 7 or 10 (arrows). The values represent mean GUS activity of three transformant lines.

Induction-response of the *GAL10* promoter in complex medium

The induction of expression by the *GAL10* promoter in the nutrient rich medium was also investigated. The transformants were cultivated in GY liquid medium (2% glucose and 1% yeast extract), and then galactose was added at day 4, 7 or 10 during the cultivation (Fig. 2-3). As shown in Fig. 2-3, GUS activity was induced by the addition of galactose in the same manner as that observed with synthetic medium. In all cases, the induction of GUS expression was maintained for approximately 3 days, and then GUS activity declined.

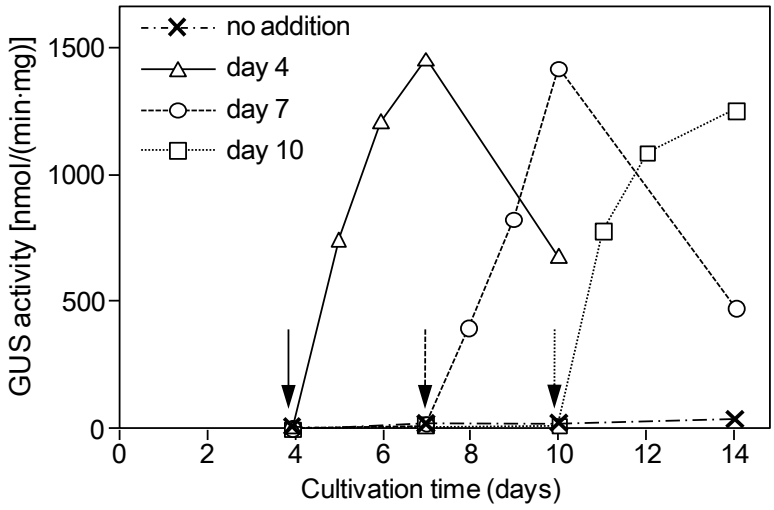


Fig. 2-3. GUS activity in response to *GAL10* promoter induction by galactose addition in different cultivation phases in complex medium.
Transformants were cultivated in GY liquid medium, and galactose was added on day 4, 7 or 10 (arrows). The values represent mean GUS activity of three transformant lines.

Deletion analysis of the *GAL10* promoter

In order to investigate the length of promoter regions required to induce high expression, a series of 5′ *GAL10* promoter deletion constructs was generated (Fig. 2-4, left) and introduced into *M. alpina* 1S-4. For each deletion constructs, 30 transformants were randomly selected and were cultivated for 3 days in SC agar medium containing galactose substituted for glucose, and then GUS activity was evaluated. For comparison, the GUS activity levels of each 10 moderately expressing lines were averaged and represented as relative values normalized to activity of the undeleted promoter, with 100% activity (Fig. 2-4, right). As shown in Fig. 2-4, a

relatively long promoter region (over 2,000 bp) was required to induce sufficient GUS expression. GUS activity dramatically diminished with the deletion of 5′ regions. The deleted promoter (2,000 bp) showed the same tendency of response to sugars as the undeleted *GAL10* promoter (data not shown), although GUS activity was lower.

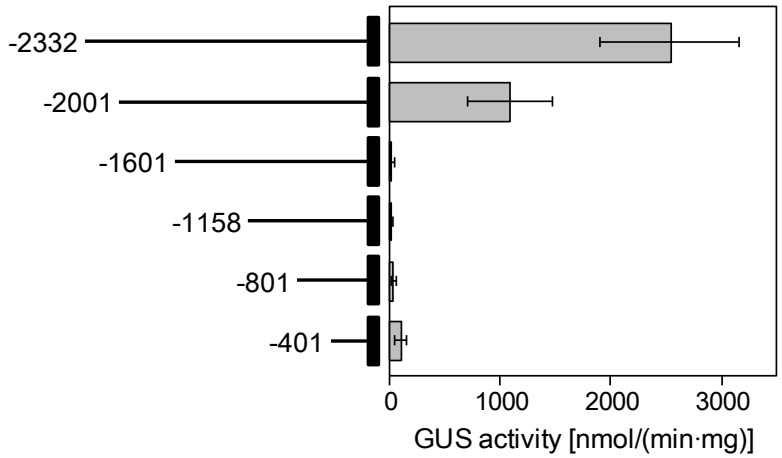


Fig. 2-4. 5′-deletion analysis of the *GAL10* promoter.
Left column) Constructs with different 5′ upstream deletions of the promoter region are shown. For each construct, the length of the fragment upstream from the transcription start site is shown on the left end. Right column) GUS activity levels with the deleted constructs in *M. alpina* transformants are shown. All transformants were cultivated for 3 days on SC agar medium containing 2% galactose substituted for glucose. The average GUS activity of the undeleted construct (2332 bp) was set at 100% and has been used to define the relative GUS activity of individual deletion constructs. Bars represent the mean values with standard deviations of GUS activity in 10 individual transformant lines for each construct.

DISCUSSION

In this chapter, the author investigated the promoter regions of the *GAL1* and *GAL10* genes as inducible promoter candidates for an oleaginous fungus *M. alpina*. The enzymes coded by these genes are involved in the galactose-metabolic pathway; GAL1 catalyzes phosphorylation of galactose, and GAL10 catalyzes epimerization from uridine diphosphate galactose to uridine diphosphate glucose. The promoter regions of genes homologous to of *GAL1* and *GAL10* have been reported as galactose-inducible promoters in various microorganisms including

Saccharomyces cerevisiae [52]. Promoters of other genes involved in the galactose-metabolic pathway, such as *GAL4* and *GAL7*, also have been used as inducible promoters in such microorganisms [53-57]. Although *GAL4* and *GAL7* homologous were not found in the *M. alpina* genome database, discovery of these genes could result in isolation of other galactose-inducible promoters.

In general, useful inducible promoters exhibit the following features: (i) easily controlled by the presence or absence of components in the medium and (ii) fully repressed in the absence of inducer in the medium. For the convenience of induction in submerged cultivation, another useful feature of an inducible promoter was searched: (iii) induced by addition of the inducer into the medium, rather than by replacement of medium. The GUS reporter assay revealed that *GAL1* and *GAL10* promoters were both regulated by the presence/absence of galactose in the medium (Table 2-2). In particular, GUS activity regulated by the *GAL10* promoter was extremely low in the medium without galactose. On the other hand, GUS activity was detectable even when other sugars were present in the medium containing galactose (Table 2-2). This result suggests that the *GAL10* promoter activity can be fully repressed during cultivation in the medium containing sugars other than galactose, and then easily induced by the addition of galactose into the medium. Therefore, the author focused on *GAL10* promoter and carried out further investigation.

To investigate the function of the *GAL10* promoter in submerged cultivation, raffinose was used as a sole carbon source in pre-culture medium, because raffinose did not affect the induction of the *GAL10* promoter in medium with/without galactose (Table 2-2). When galactose was added to the medium, in which transformants with the *GAL10* promoter fused with the *GUS* gene were pre-cultivated, GUS activity was elevated 10 h after the addition of galactose (Fig. 2-1), was maintained for 2–3 days, and then declined (Fig. 2-2). The same tendency was observed in all cultivation phases of transformants (Fig. 2-2). This phenomenon might be caused by galactose assimilation resulting in a concentration decrease in the medium. Continued addition of galactose to the medium might achieve extended periods of induced expression.

In terms of industrial application, the inducibility of the *GAL10* promoter in GY medium was also investigated, a conventional nutrient-rich medium for *M. alpina* cultivation. The *GAL10* promoter was able to induce GUS activity when galactose was added into GY medium

regardless of cultivation phase, as well as in the synthetic medium (Fig. 2-3). However, the induced GUS activity was lower and the induction response was slower than in synthetic medium (compare Fig. 2-2 and Fig. 2-3). This effect is likely caused by glucose in GY medium, because slightly repression of GUS activity with the *GAL10* promoter by glucose was observed even in the presence of galactose (Table 2-2). In agreement with this findings, it has been reported for other microorganisms that glucose represses expression regulated by promoters that can be induced by carbon sources such as galactose and xylose [58-61]. In addition, the difference in nitrogen sources and trace elements between synthetic and complex media might also affect regulation and induction kinetics of this promoter. Further investigation of cultivation conditions could result in high levels of activity and/or prolonged induction with the *GAL10* promoter for potential use. Recently, functional lipids such as PUFAs have been recognized for their beneficial effects on human health [62], and *M. alpina* has been utilized for the production of various PUFAs through molecular bleeding [14, 18-20]. The ability of the *GAL10* promoter that can be induced even in complex medium as well as in synthetic medium will be a great advantage for industrial lipid production by *M. alpina*.

The 5′ deletion analysis of the *GAL10* promoter region revealed that a relatively long length (over 2,000 bp) was required to regulate high GUS activity (Fig. 2-4). This result suggests that transcription factor binding sites, enhancer elements and induction factor binding sites of the *GAL10* promoter are located in the far upstream region. A more detailed deletion analysis will lead to identification of functionally essential regulatory elements and elucidation of the inducible regulatory mechanisms of this promoter. Such information of inducible promoters is also useful for practical applications. For example, in *Saccharomyces cerevisiae*, the introduction of multiple copies of the consensus sequence, which is essential for the galactose-inducible promoters has been reported to improve inducibility of promoters [63].

SUMMARY

The putative promoter regions of two genes encoding galactose metabolic enzymes, GAL1 and GAL10, were cloned from the genome of *M. alpina* 1S-4. The *GUS* reporter gene assay in *M. alpina* 1S-4 revealed that regulation of these promoters was dependent on the presence of galactose in the medium both with and without other sugars. With the *GAL10* promoter, an

approximately 50-fold increase of GUS activity was demonstrated by addition of galactose into the culture media at any cultivation phase. The 5′ deletion analysis of the *GAL10* promoter revealed that a promoter region of over 2,000 bp length was required for an inducible response and high-level activity. The *GAL10* promoter will be a the valuable tool for gene manipulation in *M. alpina* 1S-4.

CHAPTER III
Omega-3 eicosatetraenoic acid (ETA) production by molecular breeding of the mutant strain S14 derived from *Mortierella alpina* 1S-4

As mentioned in the general introduction to this thesis, ω3-PUFAs are found in natural sources. Especially, α-linolenic acid (ALA, C18:3ω3), eicosapentaenoic acid (EPA, C20:5ω3) and docosahexaenoic acid (DHA, C22:6ω3) have been well studied because of their sufficient natural supply. Recently, stearidonic acid (SDA, C18:4ω3) and ω3-docosapentaenoic acid (DPA, C22:5ω3) have been reported to be accumulated in several natural oils [64, 65] and their sources are being developed, therefore research on their physiological function will be advancing in the near future. On the other hand, ω3-eicosatetraenoic acid (ETA, C20:4ω3) is hard to find in nature. In addition, there are only few reports of ETA production at a low level by overexpression of Δ6 desaturase gene in *E. plantagineum* [66], by mutation in *Mortierella alpina* [67], and molecular breeding in *Arabidopsis thaliana* [68]. Although ETA has been expected to show beneficial effects on human health just like eicosanoids, the detailed bioactivity of ETA has remained almost unknown because its sources were scarce.

Mortierella alpina 1S-4, an oleaginous fungus, is known as an industrial strain that produces arachidonic acid (ARA, C20:4ω6) commercially [14]. To date, considerable accumulation of EPA have been reported by overexpressing endogenous ω3-desaturase gene in *M. alpina* 1S-4 as a novel alternative source of ω3-PUFAs [42]. The industrial production of various kinds of PUFAs have been also succeeded by using mutants derived from *M. alpina* 1S-4 through chemical mutagenesis [17, 69]. *M. alpina* S14 is a Δ5-desaturation activity-defective mutant derived from *M. alpina* 1S-4, after treating the parental spores with a chemical mutagen [70] (Fig. 3-1). The strain S14 produces only a trace (about 1%) amount of ARA, and the ratio of dihomo-γ-linolenic acid (DGLA, C20:3ω6) to total fatty acids is markedly high, accounting for as much as 43% [71] and has been applied to the industrial production of DGLA [72]. ETA can be biosynthesized by ω3- (e.g. similar to Δ17-) desaturation from DGLA; therefore, the author hypothesized that this strain would be a good host strain for ETA production by expressing ω3- or Δ17-desaturase gene.

In this chapter, the author describe evaluation of ETA production using expression of the endogenous ω3-desaturase gene and the heterologous Δ17-desaturase gene in *M. alpina* S14.

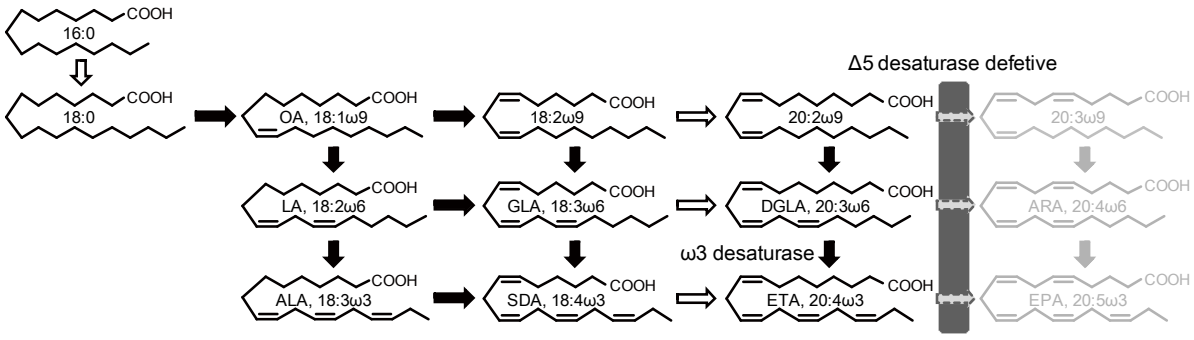


Fig. 3-1. A biosynthetic pathway of PUFAs in the mutant strain S14 derived from *Mortierella alpina* 1S-4.
OA, oleic acid; LA, linoleic acid; GLA, γ-linolenic acid; DGLA, dihomo-γ-linolenic acid; ARA, arachidonic acid; ALA, α-linolenic acid; SDA, stearidonic acid; ETA, ω3 eicosatetraenoic acid; EPA, eicosapentaenoic acid.

MATERIALS AND METHODS

Strains, media, and growth conditions

M. alpina S14, a Δ5-desaturation activity-defective mutant, has been previously isolated from *M. alpina* 1S-4 deposited in the Graduate School of Agriculture of Kyoto University *[40] an*d was used as a control strain in this chapter. The media described in chapter I were used. All cultivation was performed as described in chapter I unless otherwise mentioned.

Isolation of uracil auxotrophs of *M. alpina* S14

Isolation of uracil auxotrophs was performed as described previously [21]. Mutant S14 was incubated on Czapek-Dox agar medium at 28ºC for 1 month, and allowed to sporulate at 12ºC for 1 month. Spores of ST1358 were harvested from the surface of Czapek-Dox (2.6×10^8 spores/225 cm^2); 2.6×10^7 spores were spread on a GY agar medium containing 5-FOA (1.0 mg/mL) and uracil (0.05 mg/mL). By means of 5-FOA positive selection, uracil auxotrophs acquired that acquired 5-FOA resistance could be isolated.

Fatty acid analysis

All strains were inoculated in GY medium and then the culture was carried out at 12ºC or 28ºC with reciprocal shaking (120 strokes/min) for a desired period. The mycelia were harvested by suction filtration and dried at 120ºC. The dried cells were weighed and transmethylated with 10% methanolic HCl and dichloromethane at 55ºC for 2 h, containing 0.2 mg of *n*-tricosanoic acid as an internal standard. The resultant fatty acid methylesters were extracted with *n*-hexane, concentrated and then analyzed using gas chromatography.

Isolation of the *ura5* genomic gene of uracil auxotrophs of S14

The *ura5* genomic gene was amplified using forward primer ura5upF (5′-TTTCTGATGTG TCTCCCACC-3′) and reverse primer ura5downR (5′-TTCCAACAGAACCTTCCCTCG-3′) with uracil auxotrophic S14 genomic DNA as the template. A 700-bp PCR product was cloned into the pUC118 vector using Reagent Set for Mighty Cloning Kit (Takara, Shiga, Japan), and then sequenced with a Beckman-Coulter CEQ8000 system (Beckman- Coulter, Fullerton, CA, USA) using M13 primers.

Construction of a transformation vector for *M. alpina* S14 *ura5*⁻ strain

Transformation vectors pSDura5ω3 and pSDura5ω3×2 were constructed by the modification of pSDura5 [9, 10]. The ω3-desaturase gene was amplified using a forward primer, w3F2PciI (5′-GGGAATATTAAGCTTACATGTCCCC-3′) and a reverse primer, w3R2BamHI (5′-GCCGGATCCAAATTGTTAATGCTTG-3′) at 56ºC with *M. alpina* 1S-4 cDNA as a template. The 2 primers contained a *Pci*I and a *Bam*HI site, respectively (underlined). About 1.3-kb of PCR product was ligated to the pT7 Blue T-Vector (Novagen, Darmstadt, Germany), resulting in construction of a plasmid named pT7ω3. Its sequence was checked. The ω3-desaturase gene was digested with *Pci*I and *Bam*HI, followed by ligation into pBlueshtp treated with *Nco*I and *Bam*HI to construct pBluesω3 [73]. The ω3-desaturase expression unit including a promoter and a terminator was cut out by *Eco*RI from pBluesω3 and ligated into pSDura5 digested with the same enzyme to generate pSDura5ω3 and pSDura5ω3×2 (Fig. 3-2). The latter plasmid possessed two ω3-desaturase expression units.

Saprolegnia diclina Δ17 desaturase gene (*Sdd17m*) was synthesized with optimized codon usage to reflect the codon bias of *M. alpina* 1S-4 (obtained from the Kazusa database; http://www.kazusa.or. jp/codon/), with additional *Spe*I and *Bam*HI restriction enzyme sites at the 5′ and 3′ ends, respectively. The *Sdd17m* expression cassette, with the *SSA2* promoter and *SdhB* terminator, was generated by fusion PCR with *Xba*I and *Nhe*I restriction sites at the 5′ and 3′ ends of the cassette, respectively. This cassette was then digested with *Xba*I and *Nhe*I and ligated into pBIG35ZhSSA2pSdd17m, which had been digested with same restriction enzymes (Fig. 3-2).

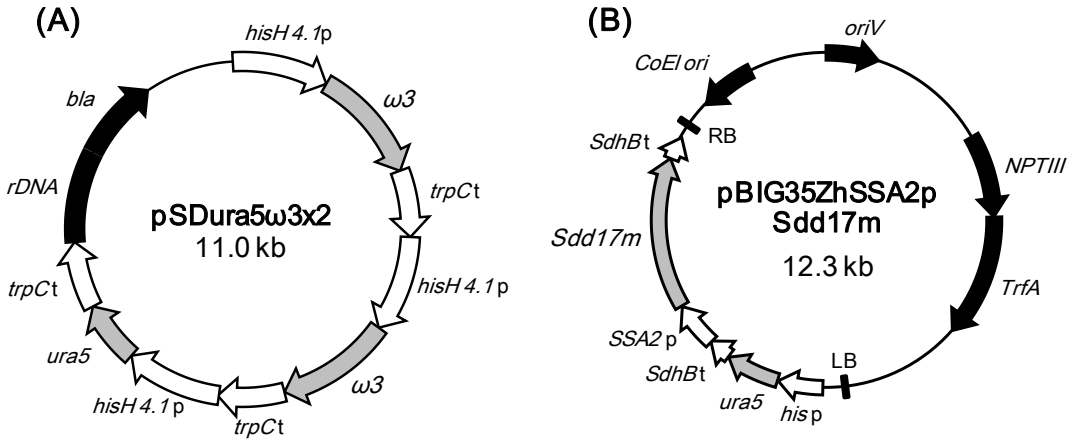


Fig. 3-2. Vector constructs used for expression of (A) ω3-desaturase and (B) *sdd17m* in *M. alpina* S14. *ω3*, *Mortierella alpina* ω3-desaturase; *hisH 4.1*p, *M. alpina* histone H4.1 promoter; *trpC* t, *Aspergillus nidulans trpC* transcription terminator; *rDNA, M. alpina* 1S-4 18S rDNA fragment*;* bla, ampicillin resistance gene; *ura5*, orotate phosphoribosyl transferase gene of *M. alpina*; *SSA2* p, *M. alpina SSA2* promoter; *SdhB* t, *M. alpina SdhB* transcription terminator; *Sdd17m*, codon-optimized Δ17 fatty acid desaturase gene from S*aprolegnia diclina*; *NPT*III, neomycin phosphotransferase III gene; *TrfA*, TrfA locus, which produces 2 proteins that promote replication of the plasmid; *ColEI ori*, ColEI origin of replication; *oriV,* pRK2 origin of replication; RB, right border; LB, left border.

Transformation of *M. alpina* S14

Transformation by microprojectile bombardment was performed as follows; a spore suspension from the *M. alpina* S14 *ura5*⁻ strain was freshly prepared from cultures growing on Czapek-Dox agar medium supplemented with 0.05 mg/mL uracil; the suspension was filtered through Miracloth (Calbiochem) [40] and spread on a uracil-free SC medium. A PDS-1000/He Particle Delivery System (Bio-Rad Laboratories Inc., CA, USA) was used for the

transformation. Tungsten particles (0.4 μm in diameter) coated with pSDura5, pSDura5ω3 and pSDura5ω3×2 were prepared according to the manufacturer's manual. Bombardment was performed in the plate placed on the device under a helium pressure of 1,100 psi (7,580 kPa). After the bombardment, the plate was incubated at 28ºC (3–6 days).

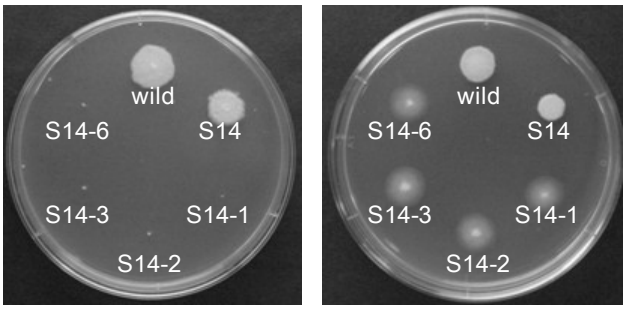
Transformation by the ATMT method was performed as described in chapter I.

RESULTS

Isolation and characterization of uracil auxotrophs of *M. alpina* S14

M. alpina S14, a Δ5-desaturase defective mutant derived from *M. alpina* 1S-4, was used as the host strain for heterologous gene expression. The strain S14 accumulates a higher amount of DGLA, a precursor for ETA, than does the wild-type strain 1S-4. To develop a transformation system using *M. alpina* S14, four uracil auxotrophic S14 mutants (S14-1, 2, 3, and 6) was obtained. The mutants grew on the SC medium with uracil, but not on uracil-free SC medium (Fig. 3-3A).

(A)



(–) uracil (+) uracil

(B)

Strain	Gene mutation	Derived result
S14-1	N.D.*	-
S14-2	G 211 A G 212 T	G 71 I
S14-3	A 1 G	Deficiency of start codon
S14-6	N.D.*	-

*: not determined

Fig. 3-3. Characterization of uracil auxotrophic strains of *M. alpina* S14.
(A) Growth of *M. alpina* 1S-4, S14 and uracil auxotrophic S14 on SC medium with or without uracil. (B) A mutation site of the *ura5* gene in *M. alpina* uracil auxotrophic S14.

These S14 uracil auxotrophs were evaluated as a host strain for molecular breeding based on growth and fatty acid production and composition. As a result, all 4 uracil auxotrophs showed vigorous growth and as much fatty acid production and composition as the wild-type strain S14 (data not shown).

To assess whether the homologous *ura5* gene was suitable as a selective marker for uracil auxotrophic mutants, the *ura5* gene in the genome of these mutants was sequenced [5]. As shown in Fig. 3-3B, in case of a mutant S14-2, the mutation of base substitution was observed in the *ura5* gene: the substitution of G for A and G for T were observed at the +211 and +212 nucleotide positions, leading to amino acid replacement, G 71 I. A base-pair change was detected in that of S14-3: the substitution of A to G at +1 caused the deficiency of start codon. S14-1 and -6 were found to have no mutations point in their *ura5* gene.

Consequently, the strain S14-2 was used as a host strain for transformation in subsequent studies because of the evident multiple mutational points in its *ura5* gene.

Transformation of the *M. alpina* S14 uracil auxotroph with pSDura5ω3×2 and fatty acid analysis

The vector pSDura5-ω3×2 was introduced into the *M. alpina* S14 uracil auxotroph using the microprojectile bombardment method and selected one stable transformant (ω3#1). Subsequently, the ETA production of the 1S-4 wild-type strain, S14 host strain, and the transformant ω3#1 was evaluated during cultivation at 12°C (Fig. 3-4). The amount of ETA in ω3#1 remarkably increased with the elapse of cultivation time compared to wild-type 1S-4 and host S14. The ETA contents of ω3#1 reached 42.1% in the total fatty acids, while those of wild-type 1S-4 and S14 were just 0.5% and 13.1%: ETA content of ω3#1 was up to 84.2-fold and 3.2-fold higher compared to wild-type 1S-4 and S14, respectively. ALA and SDA contents of ω3#1 were up to 3.7% and 14.2% of total fatty acid on Day 5. In contrast, no accumulation of ω3-PUFAs including ETA was observed in ω3#1 when cultivated at 28°C (data not shown).

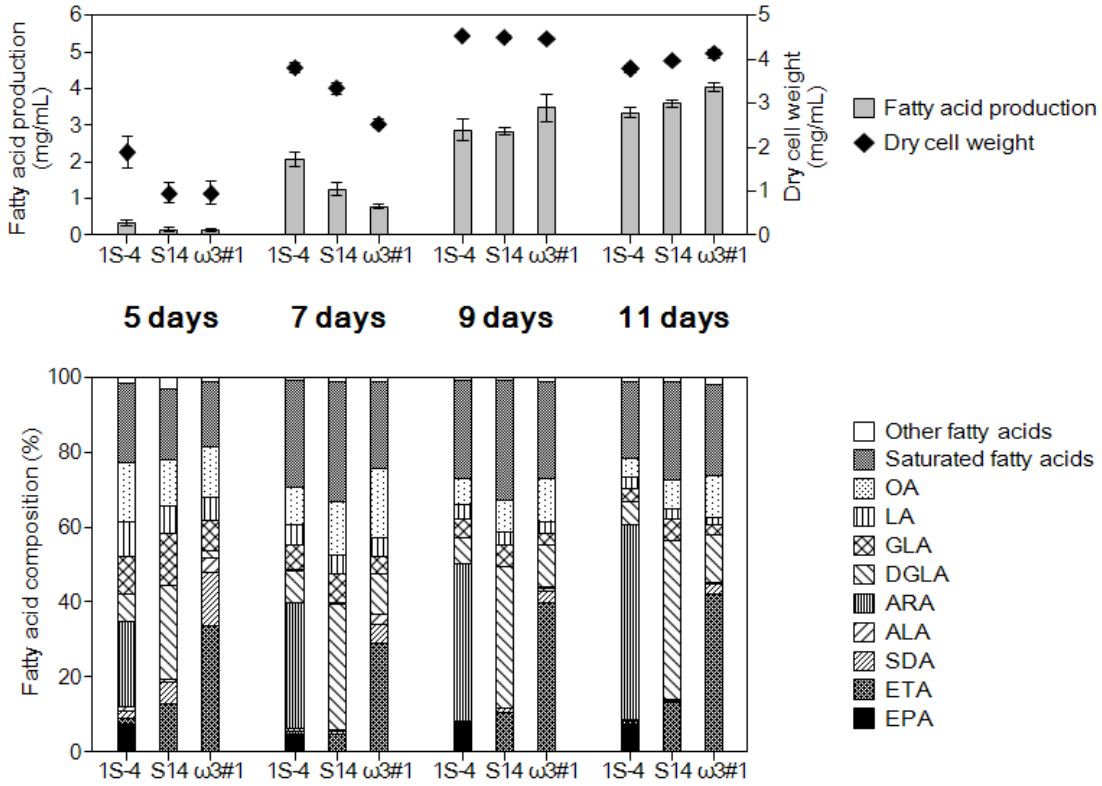


Fig. 3-4. Time course of growth, fatty acid production, and composition of the *M. alpina* 1S-4 wild-type strain, S14 host strain, and its transformant ω3#1 overexpressing ω3-desaturase gene. All strains were cultivated in 10 mL of the GY liquid medium at 12°C for 5, 7, 9, and 11 days. The data are shown as mean ± SD from 3 individual experiments. For all other abbreviations, see the legend of Fig. 3-1.

Transformation of the *M. alpina* S14 uracil auxotroph with pBIG35ZhSSA2pSdd17m and fatty acid analysis

In order to produce ETA in *M. alpina* S14 at a normal temperature, the *Saprolegnia diclina* fatty acid Δ17-desaturase gene (*Sdd17m*) was used. *S. diclina* is an oleaginous microorganism producing omega-3 PUFAs at a normal temperature, and Sdd17 is able to catalyze a desaturation at ω3 position of DGLA resulting in ETA biosynthesis [74] as well as endogenous ω3 desaturase in *M. alpina*. The expression vector pBIG35ZhSSA2pSdd17m (Fig. 3-2), carrying the codon-optimized *Sdd17* gene (*Sdd17m*), was constructed and introduced into the *M. alpina* S14 uracil auxotroph using the ATMT method. As a result, five stable transformants were randomly selected and used for further studies.

The selected transformants and the host strain S14 were cultivated in GY medium at 28°C, and the time course of their fatty acid production and composition were analyzed (Fig. 3-5). All transformants showed accumulation of ETA, which was not detected in the host strain S14 at normal temperature. Transformant #7 exhibited the highest ETA content (24.9% of total fatty acids on day 10) among all transformants. The trace amount of ALA and SDA was detected in transformants.

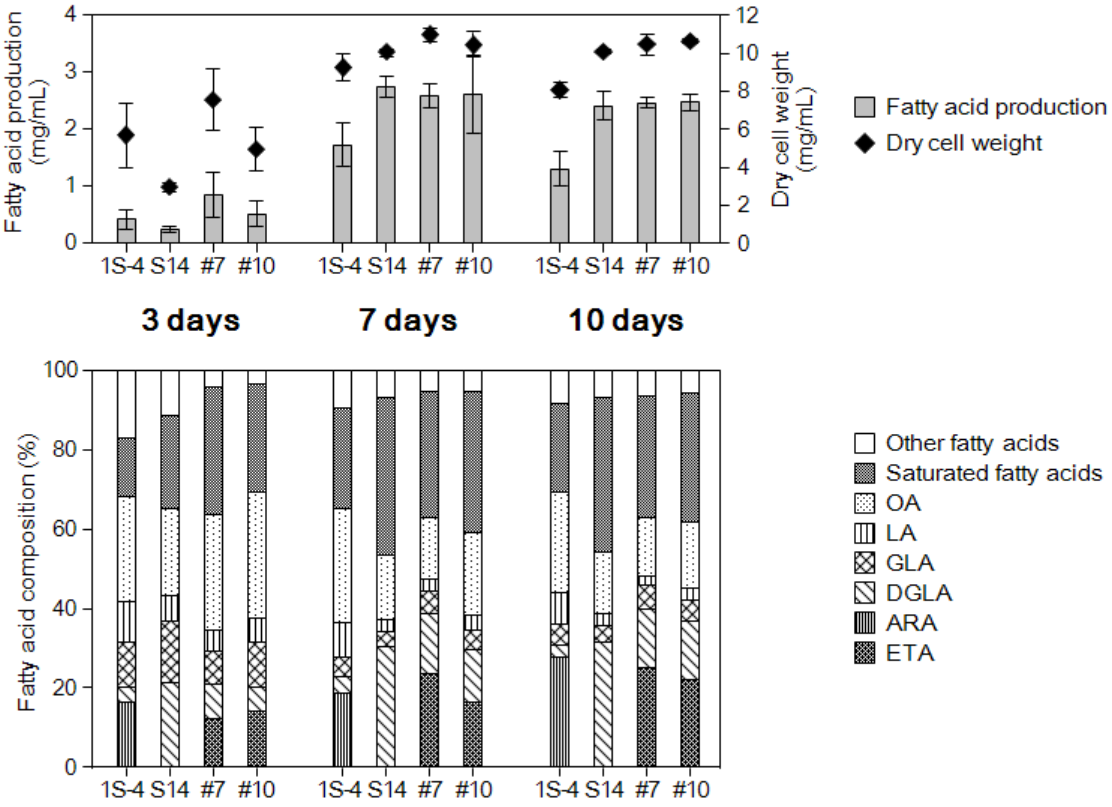


Fig. 3-5. Time course of growth, fatty acid production, and composition of the *M. alpina* 1S-4 wild-type strain, S14 host strain and its transformants expressing the *Sdd17m* gene.
All strains were cultivated in 4 mL of the GY liquid medium at 28°C for 3, 7, and 10 days. The data are shown as mean ± SD from 3 individual experiments. For other all abbreviations, see the legend of Fig. 3-1.

DISCUSSION

The oleaginous filamentous fungus *Mortierella alpina* 1S-4 is known as an industrial strain that produces arachidonic acid (ARA) commercially [14]. Until now, the industrial production of PUFAs using *M. alpina* 1S-4 and its mutant strains has been succeeded [17, 69]. This strain is also known to accumulate considerable amounts of EPA, when cultivated at low temperatures, which cause the expression of the endogenous ω3-desaturase gene and increase the activity of the gene product [75]. Based on these observations, the author hypothesized that *M. alpina* has a potential of ω3-PUFAs accumulation and employed this fungus as a host strain to produce a rare ω3-PUFA: ETA.

M. alpina S14 is a Δ5-desaturation activity-defective mutant derived from *M. alpina* 1S-4 [70]; *M. alpina* S14 also exhibits a higher accumulation of DGLA, a precursor of ETA, than strain 1S-4. Therefore, the author surmised that this strain would be suitable for evaluation of endogenous ω3- and heterologous Δ17-desaturase gene expression, which both can be converted to DGLA to ETA.

First, some *ura5*$^{-}$ mutants which were derived from the strain S14 were obtained, and then the strain S14-2 was selected as the host strain for transformation. The endogenous ω3-desaturase gene was introduced into the uracil auxotroph, strain S14-2, and the transformants showed considerable production of ETA (Fig. 3-4). Other rare ω3-PUFAs such as SDA were also accumulated in the transformants. This result indicates that it is possible to obtain some transformants producing other unique PUFAs by constructing a transformation system with other useful mutants derived from *M. alpina* 1S-4.

Thus, the potency of *M. alpina* as an ETA source was demonstrated via overexpression of the endogenous ω3-desaturase gene. For industrial production, however, low-temperature cultivation is not suitable because of its high running cost compared to normal-temperature cultivation. Therefore, the transformation with *Sdd17m* gene which enables the strain to produce ω3-PUFAs at a normal room temperature was subsequently tested. For the introduction of the *Sdd17m* gene, the ATMT method [42] was used, instead of the microprojectile bombardment method which often causes undesired mutations in host cells and results in unstable transformants because of the physical damage by high pressure employed to transfer plasmid DNA, and random multiple integrations of plasmids into the chromosomal DNA. The ATMT

method results in a single-copy integration of T-DNA at a random location in chromosomal DNA and the increased transformation frequency under mild conditions for *M. alpina* cells. Moreover, application of stronger promoters, not the conventional histone promoter, is expected to lead to the further improvements in ETA production.

In order to produce ETA at normal temperature, the *Sdd17m* gene were introduced into the uracil auxotrophic S14 using the ATMT method under the control by a strong promoter *SSA2* p. As a result, some transformants exhibited ETA production at 28°C (Fig. 3-5), and it was confirmed that the Sdd17 protein was functional in *M. alpina* S14. On the other hand, it was found that the activity of Sdd17 to convert DGLA to ETA might be lower than that of ARA to EPA because residual accumulation of DGLA was observed in the transformants while efficient conversion of ARA to EPA was reported [74]. It was also suggested that the Δ15-desaturation activity of Sdd17 for C18 substrates is extremely low because ALA and SDA accumulation were hardly detected in transformants (Fig. 3-5). Additional expression of heterologous Δ15-desaturase and endogenous ω3-desaturase might lead to enhanced ETA biosynthesis and productivity. In addition, this study is the first report to show that the *SSA2* promoter can be used for modification of the PUFA production in *M. alpina*. Recently, several useful promoters of *M. alpina* have been newly identified. By applying them, further efficient production of ω3-PUFAs might be possible using *M. alpina*.

SUMMARY

The endogenous ω3-desaturase gene or the heterologous *Saprolegnia diclina* Δ17 desaturase (*Sdd17m*) gene were overexpressed in *M. alpina* S14, a Δ5-desaturation activity-defective mutant derived from *M. alpina* 1S-4. Transformants introduced with the endogenous ω3-desaturase gene showed ETA at 42.1% content in the total lipids that was 84.2-fold and 3.2-fold higher than that of the wild-type strain 1S-4 and host strain S14, respectively, when cultivated at 12°C. No accumulation of ETA was observed at 28°C. In contrast, transformants with the heterologous *Sdd17m* gene showed 24.9% of the content of total lipids at 28°C. These results indicated that these *M. alpina* S14 transformants are promising strains for the production of ETA, which is hard to obtain from natural sources.

CHAPTER IV
Eicosapentaenoic acid (EPA) production by an oleaginous fungus *Mortierella alpina* expressing heterologous Δ17-desaturase gene under normal temperature

Mortierella alpina 1S-4 is known to accumulate considerable amounts of eicosapentaenoic acid (EPA, C20:5ω3) when cultivated at low temperatures, which cause the expression of the endogenous omega3-desaturase gene and the activity of its gene product to increase [75]. Although *M. alpina* is a promising alternative source of EPA, there are some problems with commercial EPA production using this strain when cultivated under low temperatures, such as low growth and fatty acid productivity. Nonetheless, some groups have reported that it is possible to produce omega-3 fatty acid-containing lipids under normal temperatures through the expression of hetelologous genes involved in omega-3 fatty acid biosynthesis in oleaginous organisms [76, 77].

In this chapter, the author describe the investigation of heterologous gene expression in *M. alpina* for the production of EPA at a normal temperature.

MATERIALS AND METHODS

Strains, media, and growth conditions

M. alpina ST1358, an ω3-desaturation activity-defective mutant, has been previously isolated from *M. alpina* 1S-4 deposited in the Graduate School of Agriculture of Kyoto University [40] and was used as a control strain in this chapter. The media described in chapter I were used. For jar-fermentation, the transformant was precultured in a 500-ml Erlenmeyer flask containing 100 ml GY medium, with shaking for 5 days at 28°C, and the subsequent main culture was carried out in a 5-l jar-fermentor (Able, Tokyo, Japan) with a working volume of 2.5 l medium at 28°C, an inoculation rate of 4%, an agitation speed of 700 rpm, and an aeration rate of 2.5 l/min. Glucose (2.4%), yeast extract (1.0%) and soy bean oil (0.1%) were used as the initial ingredients. For other conditions for the jar-fermentation, see the legend to Fig. 4-5.

Cloning and sequencing of the *M. alpina* ST1358 ω3-desaturase gene

The ω3-desaturase genomic gene was amplified with the forward primer w3overupF (5′-TTTTCCTTCCTCCCGCCAGAGTCATA-3′) and the reverse primer w3overdownR (5′-CTGCAGTTCTGTATTGACGCTTTTCG-3′), using ST1358 genomic DNA as the template. The PCR product, which was approximately 1.3 kb in size, was cloned into pUC118 using the Reagent Set for Mighty Cloning Kit (Takara, Shiga, Japan), the insert was then sequenced with a Beckman-Coulter CEQ8000 system (Beckman-Coulter, Fullerton, CA, USA) using M13 primers.

Isolation of uracil auxotrophs of *M. alpina* ST1358

Isolation of uracil auxotrophs was performed as described previously [21]. *M. alpina* ST1358 was incubated on Czapek-Dox agar medium at 28ºC for 1 month, and allowed to sporulate at 12ºC for 1 month. Spores of strain ST1358 were harvested from the surface of Czapek-Dox (2.6×10^8 spores/225 cm^2); 2.6×10^7 spores were spread on a GY agar medium containing 5-fluoroorotic acid (1.0 mg/ml) and uracil (0.05 mg/ml).

Isolation of *ura5* genomic gene from uracil auxotrophs of *M. alpina* ST1358

The *ura5* genomic gene was amplified with the forward primer ura5upF (5′-CCGCAACCC ATCAGCACACA-3′) and the reverse primer ura5downR (5′-GGACCTTATCCCATTTAGATT TGCC-3′), using uracil auxotrophic ST1358 genomic DNA as the template. A 700 bp PCR product was cloned into the pUC118 vector using the Reagent Set for Mighty Cloning Kit; and the insert was then sequenced with a Beckman-Coulter CEQ8000 system using M13 primers.

Fatty acid analysis

Analysis of fatty acids and lipids were performed as described in chapter III.

Construction of the *Sdd17m* expression vector

Saprolegnia diclina Δ17 desaturase gene (*Sdd17m*) was synthesized with optimized codon usage to reflect the codon bias of *M. alpina* 1S-4 (obtained from the Kazusa database; http://www.kazusa.or. jp/codon/), with additional *Spe*I and *Bam*HI restriction enzyme sites at the 5′ and 3′ ends, respectively. The *Sdd17m* expression cassette, with a histone promoter and *SdhB*

terminator, was generated by fusion PCR with *Xba*I and *Nhe*I restriction sites at the 5′- and 3′-ends of the cassette, respectively. This cassette was then digested with *Xba*I and *Nhe*I and ligated into pBIG35ZhSdd17m, which had been digested with same restriction enzymes (Fig. 4-1).

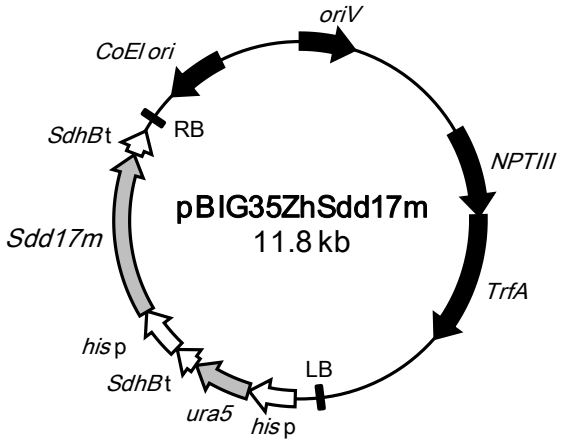


Fig. 4-1. The vector construct used for the expression of *sdd17m* in *Mortierella alpina* ST1358.
Sdd17m, codon-optimized Δ17 fatty acid desaturase gene from *Saprolegnia diclina*; *his* p, *M. alpina* histone H4.1 promoter short fragment; *SdhB* t, *M. alpina SdhB* transcription terminator; *ura5*, orotate phosphoribosyl transferase gene of *M. alpina*; *NPTIII*, neomycin phosphotransferase III gene; *TrfA*, TrfA locus, which produces 2 proteins that promote replication of the plasmid; *ColEI* ori, ColEI origin of replication; *oriV*, pRK2 origin of replication; RB, right border; LB, left border.

Transformation of *M. alpina* ST1358 *ura5*⁻ strain

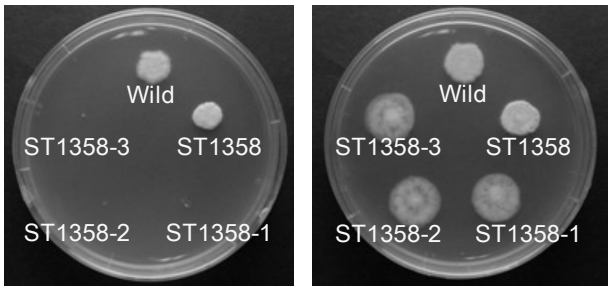
Transformation of *M. alpina* ST1358 *ura5*⁻ strain was performed by the ATMT method as described in chapter I.

RESULTS

Isolation and characterization of uracil auxotrophs of *M. alpina* ST1358

In this chapter, *M. alpina* ST1358, an ω3-desaturase defective mutant derived from *M. alpina* 1S-4, was used as the host strain for heterologous gene expression. Strain ST1358 accumulates a higher amount of ARA than the wild strain 1S-4 and is deficient in the productivity of ω3 PUFAs [78]. To develop a transformation system using *M. alpina* ST1358, we obtained three uracil auxotrophic ST1358 mutants (ST1358-1, 2 and 3) that grow on SC medium with uracil, but not on uracil-free SC medium (Fig. 4-2A). Growth and fatty acid production and composition in the ST1358 uracil auxotrophs were examined to select one as a host strain for transformation. All the three uracil auxotrophs grew in a rich medium ato the same extent as wild 1S-4 and ST1358. There was no significant difference in fatty acid production or composition between ST1358 and these uracil auxotrophs (data not shown). These results indicate that all uracil auxotrophs could be used as a host strain for transformation.

(A)



(–) uracil (+) uracil

(B)

Strain	Gene mutation	Derived result
ST1358-1	A163 deletion	Framesift
ST1358-2	T2C	Deficiency of start codon
ST1358-3	A1T	Deficiency of start codon

Fig. 4-2. Characterization of uracil auxotrophic strains of *Mortierella alpina* ST1358.
(A) Growth of *M. alpina* 1S-4, ST1358 and uracil auxotrophic ST1358 on SC medium with (+) or without (-) uracil.
(B) Mutation site of the *ura5* gene in the *M. alpina* uracil auxotrophic ST1358.

To detrmine which strain had a *ura5* mutation that would be able to maintain stable uracil auxotrophy, the sequence of the *ura5* gene in each mutant was analyzed. As shown in Fig. 4-2B, in ST1358-1, the adenine at +163 nucleotide position in *ura5* had been deleted, leading to a frameshift mutation. In ST1358-2 and ST1358-3, the start codon had been eliminated by the substitution of T to C at +2 and A to T at +1, respectively.

Consequently, strain ST1358-1 was used as the host strain for transformation in subsequent studies.

Transformation of the *M. alpina* ST1358 uracil auxotroph with *Sdd17m*

In order to convert ARA accumulated in *M. alpina* ST1358 to EPA, the *Saprolegnia diclina* fatty acid Δ17-desaturase gene (*Sdd17*) was used. *S. diclina* is an oleaginous microorganism producing EPA at a normal temperature, and Sdd17 is able to catalyze a desaturation at ω3 position of ARA [74] as well as endogenous ω3 desaturase in *M. alpina*. The expression vector pBIG35ZhSdd17m (Fig. 4-1), carrying the codon-optimized *Sdd17* gene (*Sdd17m*), was constructed and introduced into the *M. alpina* ST1358 uracil auxotroph using the ATMT method. As result, eight stable transformants were randomly selected and used for further studies.

Cultivation and fatty acid analysis of the transformants carrying *Sdd17m*

The stable transformants and the host strain were cultivated in GY medium for 7 days at 28°C and 12°C, and then their fatty acid productivity and composition were evaluated (Fig. 4-3). When cultivated at 28°C, all the transformants produced EPA, which was hardly detectable in the host strain (Fig. 4-3A). At 12°C, the content of EPA in total fatty acids produced by transformants were also higher than that of the host strain (Fig. 4-3B). EPA content of transformant #8 and #9 was over 20% of total fatty acid at 12°C. Based on the fatty acid productivity and the EPA content, transformants #4, #6, #8 and #9 were selected for subsequent studies.

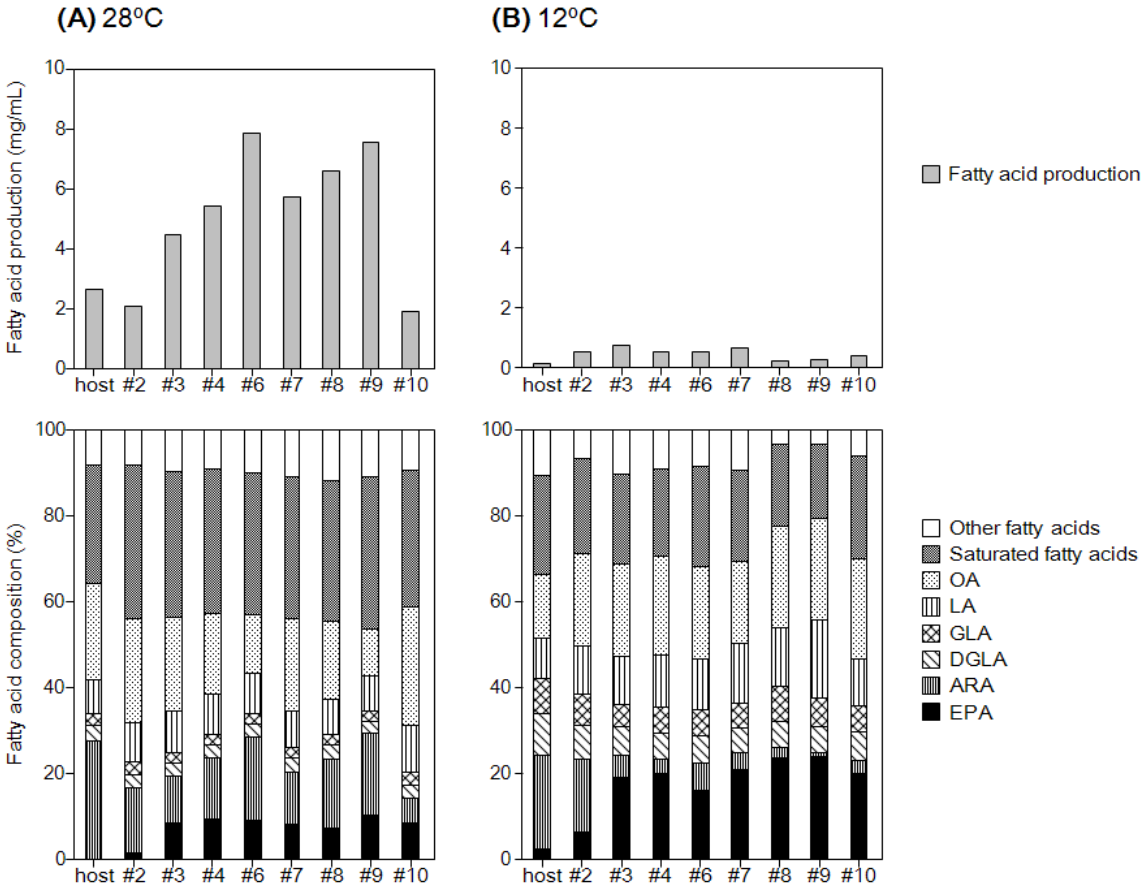


Fig. 4-3. Fatty acid production and composition of the *Mortierella alpina* ST1358 host strain and its transformants expressing *Sdd17m*.
All strains were cultivated in 10 ml of GY liquid medium for 7 days at (A) 28˚C and (B) 12˚C. OA, oleic acid; LA, linoleic acid; GLA, γ-linolenic acid; DGLA, dihomo-γ-linolenic acid; ARA, arachidonic acid; EPA, eicosapentaenoic acid.

The selected transformants, the host strain ST1358, and the wild-type strain 1S-4 were cultivated in GY medium at 28°C, and the time course of their fatty acid production and composition were analyzed (Fig. 4-4). As reported in the previous paper, ST1358 showed a higher accumulation of ARA than the wild-type strain 1S-4 but did not accumulate EPA. On the

other hand, except transformant #9, all transformants showed increasing accumulation and content of EPA during cultivation. Transformant #6 exhibited high PUFA productivity and resulted in the highest EPA content (26% of total fatty acids on day 10) among all transformants.

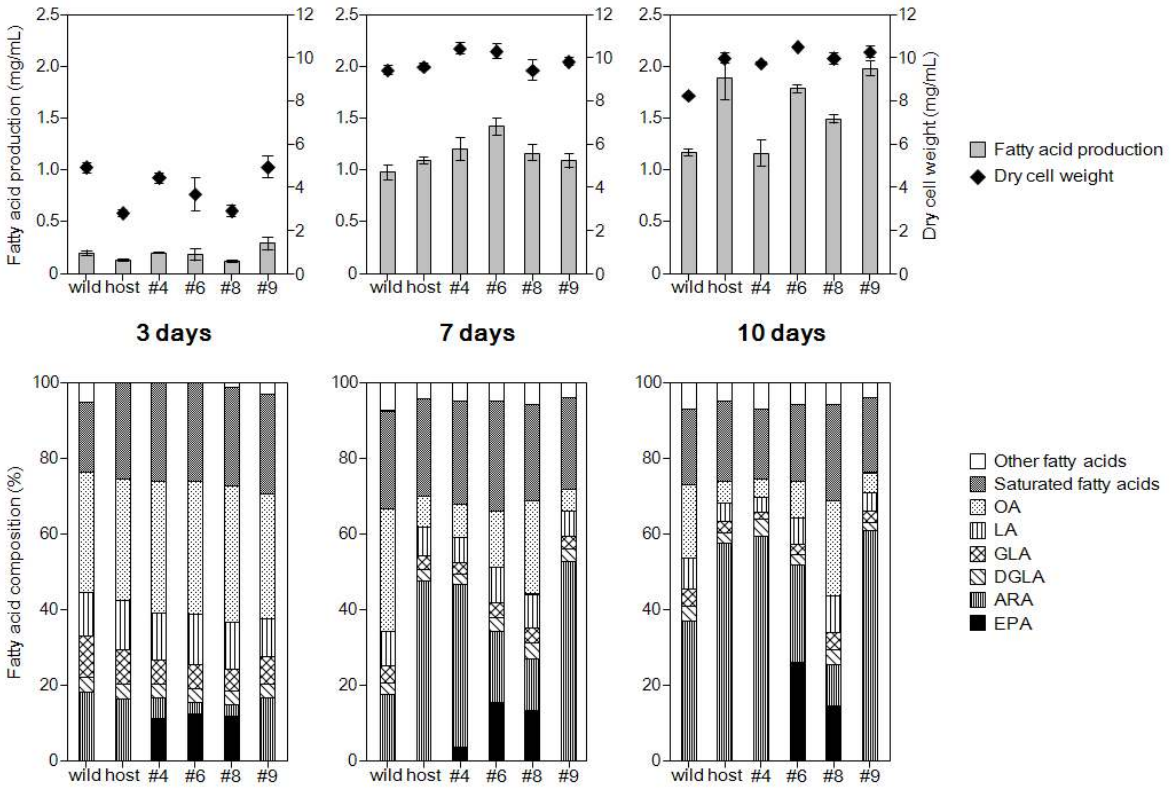


Fig. 4-4. Time course of growth, fatty acid production and composition of the *Mortierella alpina* 1S-4 wild-type strain, the ST1358 host strain and its transformants expressing *Sdd17m*.
All strains were cultivated in 4 ml of GY liquid medium at 28˚C for 3, 7, and 10 days. Values represent mean values with standard deviations from three individual experiments. For all abbreviations, see the legend for Fig. 4-3.

EPA production by expressing of *Sdd17m* in transformant #6 in a 5-l jar fermentor

Bench-scale EPA fermentation using *M. alpina* ST1358 transformant #6 in a 5-l jar fermentor was carried out. The transformant expressing the *Sdd17m* gene was cultivated with feeding of glucose in 2.5 l GY medium with aeration and agitation for 12 days at 28°C. The time course analysis of growth, fatty acid production and composition, and glucose concentration in the medium are shown in Fig. 4-5. The production of total fatty acids was low at first, then increased markedly to reach approximately 7.0 g/l culture broth on day 3, and is maintained at the level until day 12. EPA content increases with growth until it reached approximately 20% of total fatty acids. EPA content was almost unchanged after day 7.

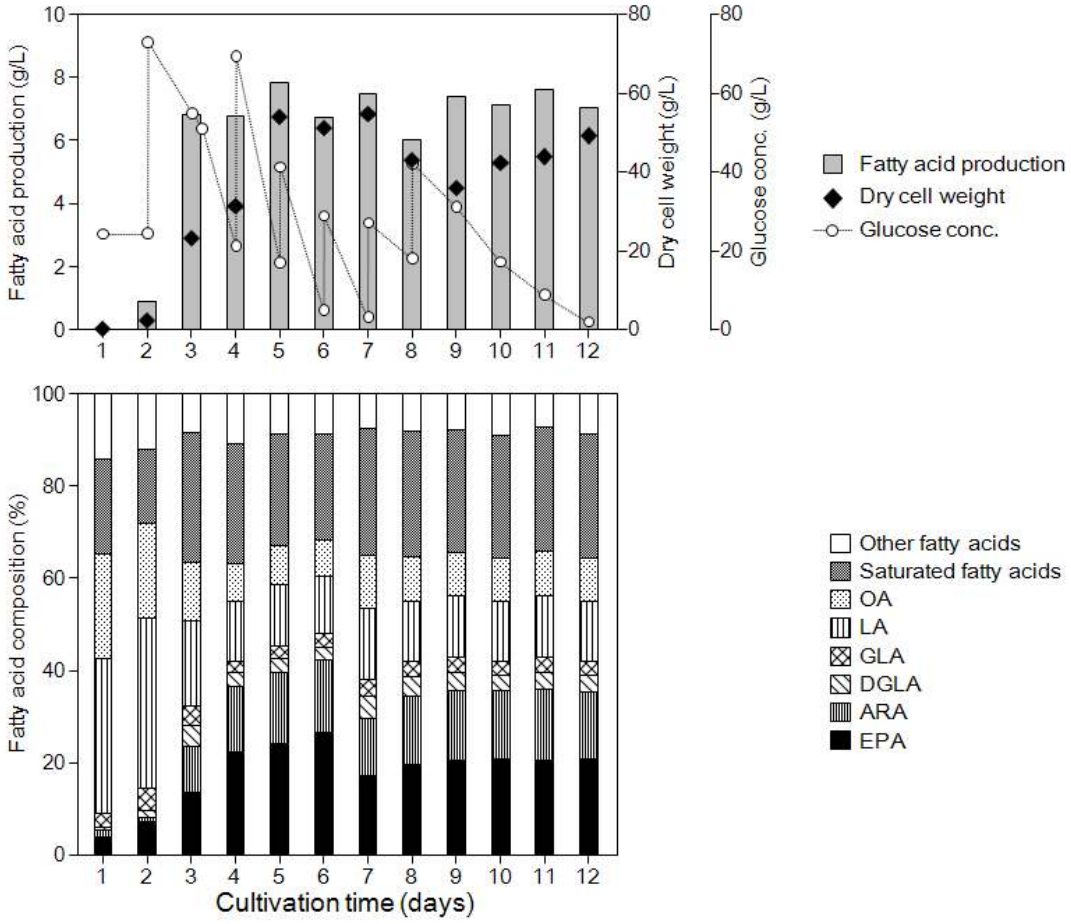


Fig. 4-5. Time course of growth, glucose consumption, fatty acid production and composition of the *Mortierella alpina* ST1358 transformant expressing *Sdd17* cultivated in a 5-l jar fermentor. *M. alpina* ST1358 *sdd17m* #6 was cultivated in 2.5 l medium (2.4% glucose, 1.0% yeast extract and 0.1% soybean oil) at 28˚C for 12 days with aeration at 2.5 l/min and agitation at 700 rpm. Glucose was added periodically to the medium as shown. For all abbreviations, see the legend for Fig. 4-3.

DISCUSSION

M. alpina ST1358 is a mutant derived from *M. alpina* 1S-4 after treating the parental spores with chemical mutagenesis twice [78]. Strain ST1358 exhibited a deficiency in the production of the omega-3 PUFAs and a higher accumulation of ARA, a precursor of EPA, than the wild-type strain 1S-4. Therefore, the author considered that this strain would be suitable to evaluate heterologous Δ17-desaturase gene expression, which has a function similar to that of the endogenous ω3 desaturase gene which produces EPA from ARA. The low ω3-desaturation activity and high ARA productivity observed in this strain might be caused by a mutation in the regulatory region of ω3-desaturase gene or in other genes related to PUFA biosynthesis, as no mutations were found in the ω3-desaturase gene in this strain.

Using methods described in the previous report [21], the author succeeded in deriving *ura5*$^-$ mutants from strain ST1358 and selected one of the *ura5*$^-$ mutants, ST1358-1, as a host strain for transformation. Both the growth and PUFA productivity of strain ST1358-1, which has a frameshift mutation in *ura5*, are slightly better than those of the other *ura5*$^-$ mutants and are almost equal to those of the parent strain, ST1358.

Recently, the importance of the ratio of omega-3 and omega-6 PUFAs in the diet has been noticed and oils with a high omega-3/omega-6 lipid ratio have been in demand [79]. Previously it has been reported that it was possible to produce an EPA content up to 35% of total lipids by overexpressing the endogenous ω3-desaturase gene in *M. alpina*; but in that report, residual accumulation of ARA, an omega-6 PUFA, have been also observed due to the preference of endogenous ω3-desaturase for C18 substrates [42]. In this chapter, the *Sdd17m* gene (codon-optimized *Sdd17* gene) was used to produce EPA in *M. alpina* ST1358. Sdd17 is a Δ17-desaturase of *Saprolegnia diclina*, which has been reported to preferentially convert C20 substrates including ARA than C18 substrates [74], and is expected to accumulate less ARA during EPA production than endogenous ω3-desaturase. In addition, low contents of PUFA expect for EPA could respond to the demand for food and medical purposes as well as contribute to reduction of costs in purification step in industrial EPA production.

Strain ST1358-1 was transformed with *Sdd17m* using the ATMT method. The transformants exhibited EPA production at a normal temperature (Fig. 4-3A). Moreover, the transformants produce a higher EPA content at a low temperature than the parent strain (Fig.

4-3B). These results indicate that the Sdd17 protein was functionally worked in *M. alpina* ST1358. In transformants, ARA was almost fully converted into EPA under the low temperature, whereas residual accumulation of ARA was observed at the normal temperature. The use of stronger promoters for *Sdd17m* expression might improve EPA production and content during cultivation at a normal temperature.

All the transformants exhibited levels of growth and fatty acid productivity that were comparable to those seen in the wild-type strain (Fig. 4-4) except for transformant #9, in which the introduced *Sdd17m* gene might be omitted. In terms of industrial application, a bench-scale fermentation with a 5-l jar fermentor was performed (Fig. 4-5). Fatty acid analysis showed the high level of EPA content up to 20% of the total fatty acids.

Up to date, the production of various kinds of PUFA has been achieved in *M. alpina* by molecular breeding. However, all of them were performed using its endogenous genes, that is, by over expression or repression. This study is the first report of the use of heterologous gene expression for the modification of the PUFA biosynthesis pathway in *M. alpina*. Recently, various genes involved in biosynthesis of PUFAs and their derivatives in oleaginous organisms have been identified. By applying these gene resources, production of further diverse kinds of PUFAs might be possible using *M. alpina*.

Many investigations for EPA production has been already reported in various organisms by their molecular breeding. For example, recombinant production of EPA at 15% of total lipids in *E. coli* has been reported [80]. In plants, the EPA production at 20–30% of total lipids has been reported in soybean, tobacco and camellia [11, 81]. In microorganisms, *Yarrowia lipolytica* has achieved higher level of EPA production that are up to 50% of total lipids [77]. In fungi, although *Pythium irregulare* has been reported to accumulate EPA at 10% of total lipids [82], this report is the first example of EPA production at a normal temperature using an industrial strain of oleaginous fungus. In this chapter, the content of EPA of the total fatty acids was about 20%, which was still lower than in previous reports of other high EPA-producing organisms. Higher production of EPA will be achieved through further studies, including studies on the expression of other heterologous genes related to PUFA biosynthesis, applying high-expression promoters, and blocking undesired fatty acid synthesis by metabolic engineering.

SUMMARY

The heterologous *Sdd17m* gene was expressed in *M. alpina* ST1358, an ω5-desaturation activity-defective mutant derived from *M. alpina* 1S-4. EPA accumulation was observed in transformants at both 28°C and 12°C. The EPA content in total lipids produced by transformants was over 20% at 28°C. Bench-scale fermentation with a 5-l jar fermentor showed that EPA content reached 20% of total fatty acids, and final EPA production reached 1.6 g/l. These results provide a platform technology for the industrial production of EPA at a normal temperature using *M. alpina* as a promising source for EPA.

CHAPTER V
Isolation and characterization of a docosahexaenoic acid (DHA)-phospholipids producing microorganism *Crypthecodinium* sp. D31

Docosahexaenoic acid (DHA, C22:6ω3) is one of the most important dietary compounds because of its various specific functions, such as reducing the risk of coronary heart disease [83, 84], lowering blood cholesterol levels [85], and reducing the risk of certain cancers [86]. DHA is also an important component of cell membranes, especially in the brain and retina of mammals [87]. Especially, DHA in the phospholipid form is of particular interest compared with the triacylglycerol form, because of its advantages such as few side effects and a higher capacity for transport into and accumulation in the human brain [88-90]. Clinical studies have shown that DHA-phospholipids is effective for the activation and the maintenance of brain functions, *e.g.* enhancement of learning ability [91], maintenance of memories [92] and increasing sleeping hour [93]. In addition, DHA in the phospholipid form is more suitable for industrial storage because it is not easily oxidized compared with the triacylglycerol form. Due to these advantages, the demand for DHA-phospholipids has increased.

Currently, the major sources of DHA are fish oils. However, these sources have some disadvantages, including limited supply, lower DHA content and a peculiar taste arising from fish smells [94]. In addition, DHA typically exists in triacylglycerol form in fish oils. Recently, single cell oils from marine microorganisms such as thraustochytrids, labyrinthulids and dinoflagellates have been noticed as alternative sources of DHA [95-97]. These microorganisms can be cultivated easily on a large scale and produce considerable amounts of high-quality DHA. Although many marine organisms that produce significant amounts of DHA have been reported [96, 98, 99], they mainly produce DHA as a component of triacylglycerols, and there are many potential strains of marine microorganisms yet to be explored. To respond to the increasing demand for DHA, and for DHA-phospholipids in particular, microorganisms that can produce DHA in a more suitable form at higher yields are required.

In this chapter, the author describe the isolation of a new candidate strain for high DHA-phospholipids production, *Crypthecodinium* sp. D31, and the evaluation of its DHA productivity and lipid profile.

MATERIALS AND METHODS

Strains, media and growth conditions

Marine microorganisms were isolated from fallen mangrove leaves collected from several brackish areas in Okinawa and Ishigaki islands in Japan by using pine pollen as a bait [95]. The collected zoospores were cultivated on dGPY agar medium (2 g/L glucose, 1 g/L polypeptone, 0.5 g/L yeast extract and 15 g/L agar) containing 200 mg/L chloramphenicol, at a salinity equivalent to 50% of that of seawater (18 g/L Daigo's artificial seasalts; Wako Chemical. Co. Ltd., Tokyo, Japan) until colonies appeared. GPY medium containing 20 g/L glucose, 10 g/L polypeptone and 5 g/L yeast extract, at a salinity equivalent to 50% of that of seawater, was used for liquid cultivation followed by analysis of fatty acid composition, unless otherwise noted.

Fatty acid analysis

For total fatty acid analysis, all the strains were inoculated into a test tube containing 4 mL of GPY medium and were cultivated at 28°C with shaking (300 rpm) for 2–14 days. The cultured cells were harvested by centrifugation at 3000 × *g* for 10 min and were washed twice with distilled water. The cells were dried, methyl-esterified and analyzed as described in Chapter III.

Lipid class analysis

For analysis of lipid classes, all the strains were inoculated into a 500 ml-flask containing 100 mL GPY medium and were cultivated at 28°C with shaking (120 rpm) for 14 days. The cultured cells were harvested by centrifugation at 3000 x *g* for 10 min, washed twice with distilled water and disrupted with metal corn by using a Multi-beads shocker (Yasui Kikai, Osaka, Japan) at 1700 rpm for 30 sec two times. Lipids were extracted from the cells with chloroform-methanol (1:1) as described previously [100].

Total lipids were fractionated by thin-layer chromatography (TLC) into neutral lipids and polar lipids on a silica gel coated-glass plate (Merck Ltd., Germany) with hexane-diethyl ether-acetic acid (40:60:1) and chloroform-diethyl ether-methanol-acetic acid (4:3:2:1), respectively, as solvents. After spraying the plates with 0.01% primuline in 80% acetone, the

appeared spots were detected under UV (260 nm), scratched, and collected in test tubes. Each fractionated lipid on the collected silica gel pieces was directly methyl-esterified, and analyzed as described in the preceding section.

Isolation of genomic DNA and identification of 18S rDNA sequences from strain D31

The cells were cultivated, harvested and ground into a powder by treatment with liquid nitrogen. Then, the genomic DNA was extracted using a standard phenol/chloroform method [101]. The 18S rDNA region was amplified by polymerase chain reaction (PCR) with the forward primer 18S1 F (5′-TACCTGGTTGATCCTGCCAG-3′) and the reverse primer 18S12 R (5′-CCTTCCGCAGGTTCACCTAC -3′) [100] with D31 genomic DNA as the template. The resultant PCR fragment was cloned into the pUC118 plasmid (Takara bio Inc., Tokyo, Japan) according to the manufacturer's instructions, and the insert was sequenced with a Beckman-Coulter CEQ8000 system (Beckman-Coulter, Fullerton, CA, USA) by using M13 primers. The determined sequence was compared with 18S rDNA sequences of various microorganisms stored in the DNA Data Bank of Japan (DDBJ) by using the Basic Local Alignment Search Tool (BLAST; http://blast.ncbi.nlm. nih.gov/ Blast.cgi).

RESULTS

PUFA profiles of DHA-producing isolates

Approximately 300 strains of microorganisms were isolated, and 34 of these strains produced DHA. These strains exhibited provided various fatty acid compositions (containing arachidonic acid, eicosapentaenoic acid, docosatetraenoic acid and DHA as polyunsaturated fatty acids) and productivities (7.87 to 502 mg/L of total fatty acid production and 8.7% to 66.7% of DHA content in total fatty acids) (Fig. 5-1). In particular, the isolated strain D31 had a unique fatty acid composition; DHA was the only polyunsaturated fatty acid (shown in Fig. 5-2) and the ratio of DHA was over 60% of its total fatty acids, while about 40% in *Shizochytrium limacinum* SR21 previously isolated as a high DHA-producing strain [102] that was used as a reference strain in Fig. 5-1.

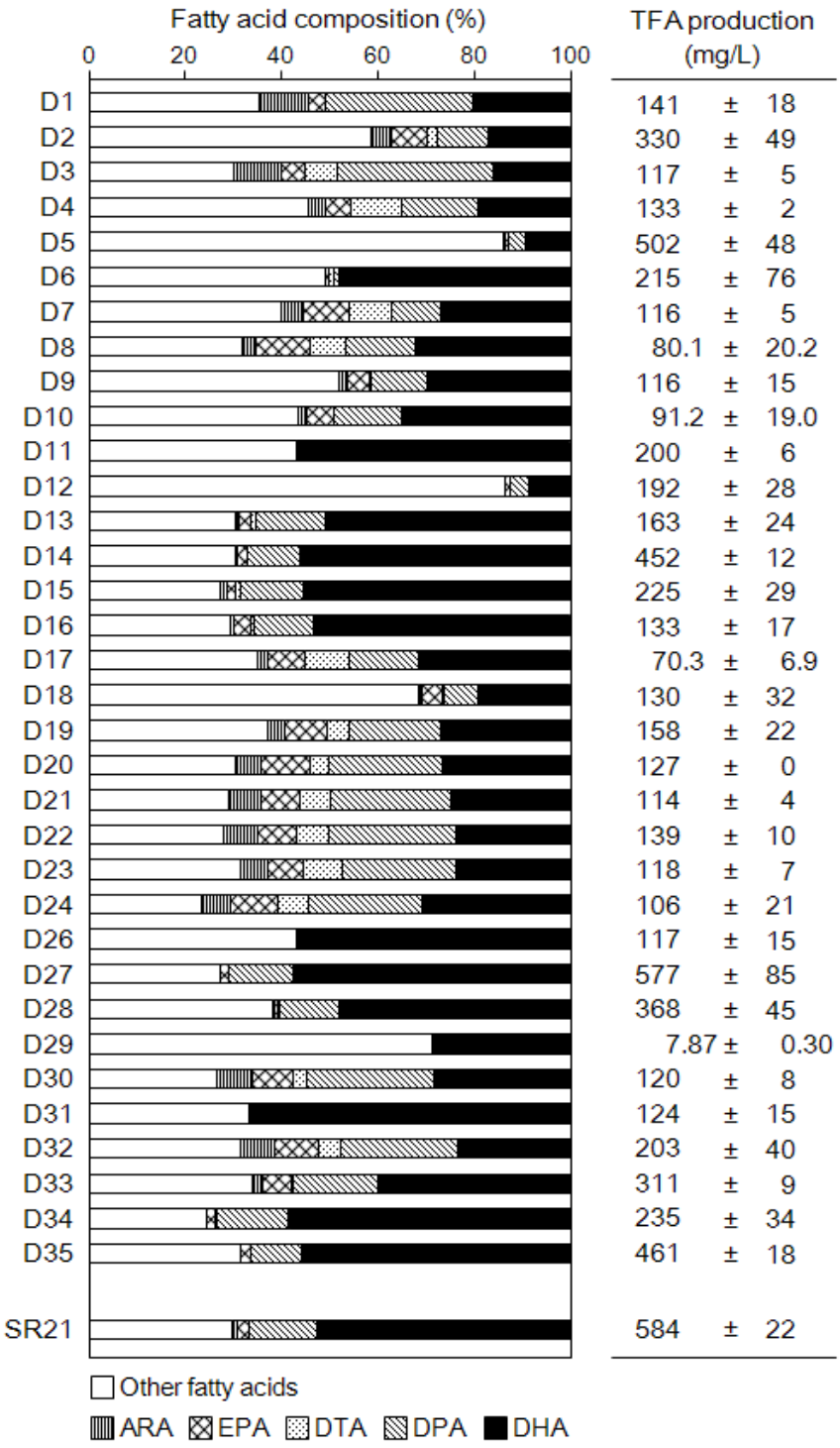


Fig. 5-1. Comparison of fatty acid profiles produced by the isolates. All the strains were cultivated in GPY liquid medium for 7 days.
"Other fatty acids" includes all fatty acids with a chain length <20 carbon atoms. TFA, total fatty acid; ARA, arachidonic acid; EPA, ω3 eicosapentaenoic acid; DTA, ω6 docosatetraenoic acid; DPA, ω6 docosapentaenoic acid; DHA, docosahexaenoic acid. SR21 refers to *Scizochytrium limacinum* SR21 that was used as a reference strain. TFA values are means of triplicate analyses ± standard deviation.

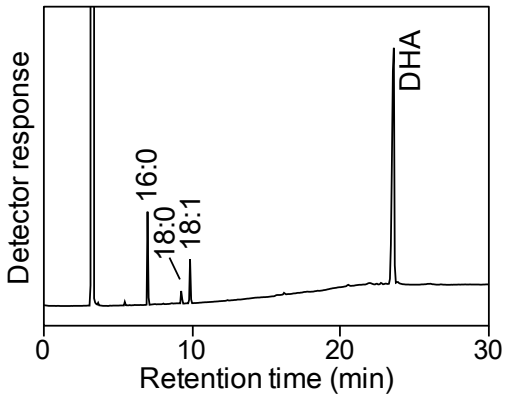


Fig. 5-2. Gas chromatogram of total fatty acids from the isolated strain D31 after methyl esterification. DHA, docosahexaenoic acid.

Identification and phylogenetic classification of strain D31

The almost complete length of 18S rDNA sequence (approximately 1700 bp) from strain D31 was amplified, cloned and sequenced. BLAST analysis against nucleotide sequences from various microorganisms in DDBJ indicated that the 18S rDNA sequence of strain D31 exhibited 99% homology with that of *Crypthecodinium cohnii* (data not shown). Moreover, in BLAST analysis using the 26S rDNA sequence, strain D31 also showed high homology (96.4%) with *C. cohnii* (data not shown). These 18S and 26S rDNA sequences of strain D31 were deposited in DDBJ with accession numbers AB811790 and AB811791, respectively. Finally, this strain was identified as the related species of *Crypthecodinium cohnii* based on its morphological (yellowish-white creamy circular colonies with raised elevation and wavy edges) and microscopy (swimming vegetative cells with flagella) characteristics and the results of the molecular phylogenetic analysis of the 26S rDNA sequence (Fig. 5-3). The strain D31 was deposited in the National Institute of Technology and Evaluation, Biological Resource Center, Japan (NBRC) with accession number NBRC109771.

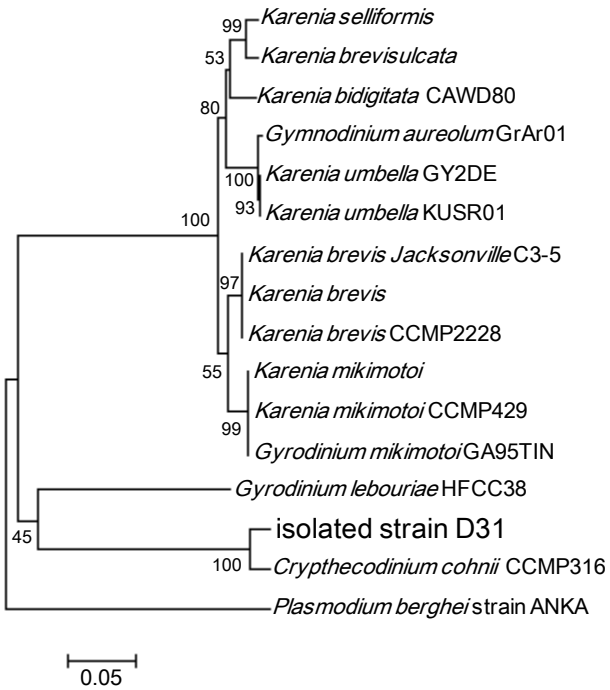


Fig. 5-3. Phylogenetic tree based on the 26S rDNA gene sequences from strain D31 and other dinoflagellates, with *Plasmodium berghei* as an outgroup (based on the data from TechnoSuruga Laboratory Co., Ltd.).
The numbers at each internal branch show the bootstrap values.

Effects of cultivation conditions on DHA productivity of *Crypthecodinium* sp. D31

To characterize and evaluate the DHA productivity of *Crypthecodinium* sp. D31, the effects of various cultivation conditions (carbon sources, nitrogen sources, salinity and initial pH of cultivation medium) were investigated. The cells of *Crypthecodinium* sp. D31 were cultivated for 10 days unless otherwise noted, because this strain showed maximum DHA production on day 10 when cultivated on the standard GPY medium (data not shown).

The effect of the carbon source on growth and DHA production is shown in Table 5-1. D-Glucose, D-fructose, acetic acid, ethanol and glycerol promoted cell growth, whereas other saccharides, organic acids and sugar alcohols did not promote growth. Ethanol and glycerol elicited DHA productivity comparable to that seen for glucose, and DHA production with glycerol as the carbon source was the highest.

Table 5-1. Effect of carbon sources on growth and DHA production in *Crypthecodinium* sp. D31[a].

Carbon source (2%)	DCW (g/L)	TFA (mg/L)	DHA (mg/L)
No carbon source	0.44 ± 0.08	3.99 ± 0.06	2.48 ± 0.08
Glucose	3.08 ± 0.42	131 ± 6	75.8 ± 4.4
Fructose	1.64 ± 0.14	24.4 ± 4.1	15.1 ± 1.7
Galactose	0.86 ± 0.31	11.7 ± 0.2	8.22 ± 0.50
Mannose	0.73 ± 0.22	10.9 ± 0.1	6.23 ± 0.11
Xylose	0.54 ± 0.01	3.13 ± 0.02	1.91 ± 0.10
Sucrose	0.64 ± 0.09	4.98 ± 0.99	2.62 ± 0.07
Maltose	0.80 ± 0.21	3.81 ± 0.23	2.32 ± 0.10
Lactose	0.67 ± 0.04	4.03 ± 0.13	2.57 ± 0.05
Raffinose	0.95 ± 0.57	3.43 ± 0.22	2.28 ± 0.23
Dextrin	0.78 ± 0.11	2.88 ± 0.32	1.77 ± 0.11
Starch	0.67 ± 0.01	4.62 ± 0.38	2.81 ± 0.22
Cellulose	not measurable [b]	3.44 ± 0.35	1.89 ± 0.25
Sorbitol	0.71 ± 0.04	6.11 ± 1.19	3.57 ± 0.70
Mannitol	0.71 ± 0.22	9.47 ± 1.56	5.28 ± 0.13
Citric acid	0.29 ± 0.01	4.75 ± 4.22	2.34 ± 2.19
Acetic acid	0.92 ± 0.12	20.6 ± 1.1	13.9 ± 0.9
Lactic acid	0.83 ± 0.11	4.42 ± 0.70	2.81 ± 0.33
Pyruvic acid	0.50 ± 0.07	3.37 ± 1.81	2.26 ± 1.16
Succinic acid	0.82 ± 0.14	8.98 ± 0.29	6.50 ± 0.10
Fumaric acid	0.53 ± 0.02	2.69 ± 0.49	1.73 ± 0.41
Maleic acid	0.54 ± 0.03	2.48 ± 0.47	1.43 ± 0.15
Malic acid	0.64 ± 0.10	2.00 ± 0.77	1.42 ± 0.59
Methanol	0.59 ± 0.16	3.69 ± 0.00	2.24 ± 0.00
Ethanol	2.38 ± 0.07	61.7 ± 3.2	38.4 ± 0.7
Glycerol	6.90 ± 0.20	188 ± 16	103 ± 12

[a]Cultivated in the medium containing a mixture of 1% polypeptone and 0.5% yeast extract as the nitrogen source, at a salinity equivalent to 50% of that of seawater.
[b]Because insoluble cellulose in the medium was not able to separate with cultivated cells when DCW measuring.
DCW, dry cell weight; TFA, total fatty acids; DHA, docosahexaenoic acid.
Values are means of triplicate analyses ± standard deviation.

The effect of the nitrogen source on growth and DHA production is shown in Table 5-2. The mixture of polypeptone and yeast extract, used as the basic nitrogen source in GPY medium, was preferable for growth and DHA production. Inorganic nitrogen sources, including $(NH_4)_2SO_4$, $NaNO_3$, urea and so on, did not support sufficient growth of *Crypthecodinium* sp. D31 (data not shown).

Table 5-2. Effect of nitrogen sources on growth and DHA production in *Crypthecodinium* sp. D31[a].

Nitrogen source (1.5%)	DCW (g/L)	TFA (mg/L)	DHA (mg/L)
No nitrogen source	-[b]	-	-
Yeast extract	1.33 ± 0.31	85.7 ± 16.9	51.3 ± 5.9
Polypeptone	-	-	-
Peptone	1.00 ± 0.50	41.2 ± 0.9	25.1 ± 3.3
Tryptone	0.50 ± 0.00	41.5 ± 4.9	27.1 ± 2.9
Tryptose	2.00 ± 0.89	44.0 ± 7.3	30.7 ± 4.4
Malt extract	1.25 ± 0.25	43.1 ± 5.0	27.5 ± 1.9
Marine broth	2.59 ± 0.18	106 ± 6	52.5 ± 2.1
Meat extract	1.25 ± 0.00	70.8 ± 2.6	38.1 ± 0.0
Neopeptone	0.50 ± 0.10	35.4 ± 1.4	21.5 ± 2.4
1% Polypeptone + 0.5% yeast extract	3.08 ± 0.42	131 ± 7	75.8 ± 4.4

[a]Cultivated in the medium containing 2% glucose as a carbon source, at a salinity equivalent to 50% of that of seawater.
[b]-, Trace amounts.
DCW, dry cell weight; TFA, total fatty acids; DHA, docosahexaenoic acid.
Values are means of triplicate analyses ± standard deviation.

The effect of the concentration of artificial seawater (with a salinity of 0–200% that of seawater) is shown in Table 5-3. Seawater salinities of 25%, 50% and 100% were effective for the growth and DHA production, especially 50% seawater salinity was optimal. On the other hand, growth and DHA production were suppressed in the case of 0% and 200% seawater salinity.

Table 5-3. Effect of salinity of the medium on cell growth and DHA production in *Crypthecodinium* sp. D31[a].

Salinity (%) [b]	DCW (g/L)	TFA (mg/L)	DHA (mg/L)
0	0.28 ± 0.08	13.8 ± 0.2	8.43 ± 0.42
25	1.59 ± 0.16	52.6 ± 5.7	28.7 ± 2.3
50	2.28 ± 0.16	98.6 ± 11.5	58.0 ± 5.8
100	1.63 ± 0.04	67.2 ± 6.4	40.5 ± 2.2
200	0.26 ± 0.04	8.32 ± 0.42	4.85 ± 0.18

[a]Cultivated in the medium containing 2% glucose as a carbon source and a mixture of 1% polypeptone and 0.5% yeast extract as the nitrogen source.
[b]Salinity of seawater is treated as 100%.
DCW, dry cell weight; TFA, total fatty acids; DHA, docosahexaenoic acid.
Values are means of triplicate analyses ± standard deviation.

The effect of the initial pH of the cultivation medium is shown in Table 5-4. The growth could not be measured in the pH range from 7.0 to 9.0 because of the formation of precipitants in the medium. DHA production was approximately constant in the acidic pH range (pH 3.0–6.0) and decreased in medium that had a pH 6.0.

Table 5-4. Effect of initial medium pH on growth and DHA production in *Crypthecodinium* sp. D31[a].

Initial pH[b]	DCW (g/L)	TFA (mg/L)	DHA (mg/L)
3.0	4.19 ± 0.44	230 ± 69	123 ± 33
4.0	3.13 ± 0.12	156 ± 20	84.9 ± 7.5
5.0	4.06 ± 0.06	223 ± 17	120 ± 11
5.5	4.13 ± 0.38	226 ± 10	124 ± 14
6.0	3.38 ± 0.50	191 ± 16	94.3 ± 12.8
6.5	2.61 ± 0.25	128 ± 10	65.4 ± 5.7
7.0	1.63 ± 1.13	97.4 ± 2.7	49.0 ± 4.0
7.5	not measurable	83.8 ± 7.9	44.9 ± 1.7
8.0	not measurable	49.6 ± 4.1	26.4 ± 3.2
9.0	not measurable	-	-

[a]Cultivated in GPY liquid medium (containing 2% glucose as a carbon source and a mixture of 1% polypeptone and 0.5% yeast extract as the nitrogen source, at a salinity equivalent to 50% of that of seawater).
[b]pH was adjusted with HCl or NaOH. Without adjustment, the pH of GPY medium was about 6.5.
DCW, dry cell weight; TFA, total fatty acids; DHA, docosahexaenoic acid.
Values are means of triplicate analyses ± standard deviation.

Crypthecodinium sp. D31 produced 375 mg/L of DHA when cultivated under optimized conditions, i.e., glycerol as the carbon source, a mixture of yeast extract and polypeptone as the nitrogen sources, salinity at 50% that of seawater, and pH 5.5.

Lipid class composition

Total lipids from *Crypthecodinium* sp. D31 and *Schizochytrium limacinum* SR21, a previously characterized industrial strain [102], used as the reference strain, were fractionated by TLC and the lipid class compositions were evaluated (Table 5-5). In *Crypthecodinium* sp. D31, polar lipids accounted for 69.4% of the total lipids, whereas they accounted for only 10.2% of the total lipids in *S. limacinum* SR21. *Crypthecodinium* sp. D31 accumulated DHA mainly in polar lipids (79.4% of total DHA), particularly as phosphatidylcholine (71.4% of the total polar DHA), in contrast to *S. limacinum* SR21 which preferentially accumulated DHA in the neutral lipid triacylglycerol form.

Table 5-5. Lipid class composition (%) of *Crypthecodinium* sp. D31 and *Schizochytrium limacinum* SR21[a].

	Crypthecodinium sp. D31		*S. limacinum* SR21	
Lipid class	in TFA	in DHA	in TFA	in DHA
Total lipid composition (%)				
Neutral lipids	30.6 ± 1.1	20.6 ± 0.5	89.8 ± 1.2	83.4 ± 3.7
Polar lipids	69.4 ± 1.1	79.4 ± 0.5	10.2 ± 1.2	16.6 ± 3.7
Neutral lipid composition (% in neutral lipids)				
Triacylglycerols	66.3 ± 3.4	86.0 ± 2.7	90.5 ± 0.3	89.3 ± 2.1
1,3-diacylglycerols	6.4 ± 1.3	-	3.9 ± 0.8	4.1 ± 1.1
1,2-diacylglycerols	8.7 ± 2.1	4.7 ± 1.6	0.5 ± 0.1	0.2 ± 0.0
Monoglycerols	9.2 ± 1.6	-	0.7 ± 0.1	0.9 ± 0.2
Free fatty acids	10.4 ± 1.2	10.2 ± 1.4	4.7 ± 0.5	6.0 ± 0.9
Polar lipid composition (% in polar lipids)				
Phosphatidic acid	4.6 ± 0.7	3.0 ± 0.4	24.3 ± 1.9	3.0 ± 0.8
Phosphatidylethanolamine	10.5 ± 2.0	2.2 ± 0.7	16.8 ± 3.2	1.6 ± 0.2
Phosphatidylserine	-	-	0.2 ± 0.0	-
Phosphatidylinositol	3.0 ± 0.6	2.0 ± 0.4	0.9 ± 0.1	1.5 ± 0.1
Phosphatidylcholine	32.4 ± 2.7	71.4 ± 2.0	23.6 ± 2.9	73.0 ± 1.6
Other polar lipids	49.4 ± 0.6	21.4 ± 2.7	34.1 ± 2.2	20.8 ± 2.2

[a]Both strains were cultivated in GPY liquid medium. *S. limacinum* SR21 was used as a reference strain.
TFA, total fatty acids; DHA, docosahexaenoic acid.
Values are means of triplicate analyses ± standard deviation.

DISCUSSION

Thirty-four strains of DHA-producing microorganisms were isolated from brackish areas in Japan; these strains had various fatty acid compositions. the author focused on strain D31, which showed the highest DHA content among the strains isolated in this study. The DHA content of strain D31 accounted for over 60% of the total fatty acids (Fig. 5-1), and was higher than that of previously reported DHA-producing marine microorganisms (40–50% DHA), including the industrial strain *Scizochytrium limacinum* SR21 [95, 96, 102]. By the phylogenetic analysis, D31 was identified as a related species to the heterotrophic dinoflagellate *Crypthecodinium cohnii*. *C. cohnii* is known to be a DHA-producing microorganism, but the DHA content of this dinoflagellate is limited to approximately 50% of the total fatty acids [97].

Our data showed that DHA exists mainly as a component of polar lipids in *Crypthecodinium* sp. D31. The phospholipids produced by *Crypthecodinium* sp. D31 accounted for approximately 70% of the total lipids, while *S. limacinum* SR21 and other reported *Crypthecodinium* strains have relatively low phospholipid content account for 10.2% (Table 5-5) or 28.5% [103] of the total lipids, respectively. Recently, DHA-containing phospholipids have been noticed because of their various specific functions [91-93] and advantages over triacylglycerides, such as fewer side effects and better transportability and accumulation in the human brains [88-90]. *Crypthecodinium* sp. D31 is an attractive candidate for the industrial production of high-purity DHA-containing phospholipids.

In general, DHA-producing microorganisms have been known to synthesize DHA via the conventional fatty acid synthase (FAS) pathway and/or the polyketide synthase-like (PKS) pathway [104]. In microorganisms with the FAS pathway, DHA is synthesized via alternating steps of desaturation and elongation; therefore, various kinds of PUFAs including precursors of DHA are produced [105]. On the other hand, in microorganisms with only the PKS pathway, their fatty acid profiles are most likely to be simpler because DHA is synthesized without intermediate products [94]. *Crypthecodinium* sp. D31 was found to have a simple fatty acid composition with DHA as the sole polyunsaturated fatty acid. This result, in agreement with that of a previous report [97], suggests that this strain adopts only the PKS pathway for DHA synthesis.

By investigation of the effects of carbon sources in the cultivation medium on growth and DHA production of *Crypthecodinium* sp. D31, this strain was found to produce DHA well in the medium containing ethanol or glycerol. This result suggests that these carbon sources are easily metabolized and utilized by the DHA biosynthetic pathways in this strain. In particular, glycerol is likely to be used as the backbone for triacylglycerol (TAG) because DHA-TAG production increased when this strain was cultivated in the glycerol-containing medium (data not shown). Glycerol is a byproduct of biodiesel production [106] and there has been increasing interest regarding the processes for its utilization. *Crypthecodinium* sp. D31 is able to efficiently produce DHA from glycerol, therefore it may be suitable for the utilization of excess glycerol. Ethanol was also utilized well by *Crypthecodinium* sp. D31, but acetate and other organic acids were relatively not so, although ethanol is known to be metabolized via the acetic acid in general. This finding suggests that *Crypthecodinium* sp. D31 does not metabolize these free

organic acids efficiently or that it may possess an alternative ethanol-metabolic pathway instead of the conversion of ethanol into acetate.

Although the DHA production by *Crypthecodinium* sp. D31 was still lower (approximately 0.38 g/L) than that by other DHA producers such as *Schizochytrium* sp. SR21 (approximately 4.2 g/L [107]), two useful features, that *Crypthecodinium* sp. D31 can produce DHA as the only long-chain PUFA in its total fatty acids and high content of phospholipid DHA, will be of great advantage for industrial fields.

SUMMARY

Thirty-four strains of docosahexaenoic acid (DHA)-producing microorganisms were newly isolated from brackish areas in Japan. These strains showing various compositions of fatty acids. Especially, the fatty acids produced by one of the strains, named D31, had a high DHA content (over 60% of the total fatty acids) and the simple fatty acid composition (16:0, 18:0, 18:1 and DHA without any other polyunsaturated acids). Although most oleaginous microorganisms accumulate DHA as triacylglycerol, the strain D31 accumulated DHA mainly as a polar lipid (79.4% of total DHA), especially as phosphatidylcholine (71.4% of polar DHA). This strain D31 was identified as the related species of *Crypthecodinium cohnii* on the basis of phylogenetic analysis. *Crypthecodinium* sp. D31 showed high DHA productivity when cultivated in a medium containing glycerol as the carbon source and a mixture of yeast extract and polypeptone as the nitrogen sources, with a salinity that was equivalent to 50% of that of seawater and a pH in the acidic range (<pH 6.0). *Crypthecodinium* sp. D31 is considered as a promising producer of high-purity DHA-containing phospholipids.

CONCLUSIONS

In this thesis, the author described screening, selection, biochemical analysis, development of gene manipulation tools and molecular breeding of oleaginous microorganisms for ω3-PUFA production. The results described in each chapter are summarized as follows:

CHAPTER I

This chapter described the selection and characterization of promoters based on genomic approach for the molecular breeding of oleaginous fungus *Mortierella alpina* 1S-4.

The promoter regions of 28 genes in *Mortierella alpina* 1S-4 were selected and cloned on the basis of expression sequence tag (EST) abundance data. The activity of each promoter was evaluated by using the *β*-glucuronidase (*GUS*) reporter gene. Eight of these promoters were shown to enhance GUS expression more efficiently than a histone promoter, which is conventionally used for the gene manipulation in *M. alpina*. Especially, the predicted protein 3 (*PP3*) and the predicted protein 6 (*PP6*) promoters demonstrated approximately 5-fold higher activity than the histone promoter. The activity of some promoters changed along with the cultivation phase of *M. alpina* 1S-4. Seven promoters with constitutive or time-dependent, high-level expression activity were selected, and deletion analysis was carried out to determine the promoter regions required to retain activity. The promoters described in this chapter will be useful tools for gene manipulation in this strain.

CHAPTER II

This chapter described galactose-dependent promoters for potential use in an oleaginous fungus *Mortierella alpina* 1S-4. The putative promoter regions of two genes encoding galactose metabolic enzymes, GAL1 and GAL10, were cloned from the genome of *M. alpina* 1S-4. The *GUS* reporter gene assay in *M. alpina* 1S-4 revealed that regulation of these promoters was dependent on the presence of galactose in the medium both with and without other sugars. With the *GAL10* promoter, an approximately 50-fold increase of GUS activity was demonstrated by addition of galactose into the culture media at any cultivation phase. The 5′ deletion analysis of the *GAL10* promoter revealed that a promoter region of over 2,000 bp length was required for an inducible response and high-level activity. The *GAL10* promoter will be a the valuable tool

for gene manipulation in *M. alpina* 1S-4.

CHAPTER III

This chapter described ω3-eicosatetraenoic acid (ETA) production by molecular breeding of the mutant strain S14, a Δ5-desaturation activity-defective mutant derived from *M. alpina* 1S-4.

The endogenous ω3-desaturase gene or the heterologous *Saprolegnia diclina* Δ17 desaturase (*Sdd17m*) gene were overexpressed in *M. alpina* S14. Transformants introduced with the endogenous ω3-desaturase gene showed ETA at 42.1% content in the total lipids that was 84.2-fold and 3.2-fold higher than that of the wild-type strain 1S-4 and host strain S14, respectively, when cultivated at 12°C. No accumulation of ETA was observed at 28°C. In contrast, transformants with the heterologous *Sdd17m* gene showed 24.9% of the content of total lipids at 28°C. These results indicated that these *M. alpina* S14 transformants are promising strains for the production of ETA, which is hard to obtain from natural sources.

CHAPTER IV

This chapter described eicosapentaenoic acid (EPA) production at a normal room temperature by molecular breeding of the mutant strain ST1358, an ω5-desaturation activity-defective mutant derived from *M. alpina* 1S-4.

The heterologous *Sdd17m* gene was expressed in *M. alpina* ST1358. EPA accumulation was observed in transformants at both 28°C and 12°C. The EPA content in total lipids produced by transformants was over 20% at 28°C. Bench-scale fermentation with a 5-l jar fermentor showed that EPA content reached 20% of total fatty acids, and final EPA production reached 1.6 g/l. These results provide a platform technology for the industrial production of EPA at a normal temperature using *M. alpina* as a promising source for EPA.

CHAPTER V

This chapter described the screening, isolation and characterization of docosahexaenoic acid (DHA)-producing microorganisms.

Thirty-four strains of docosahexaenoic acid (DHA)-producing microorganisms were newly isolated from brackish areas in Japan. These strains showing various compositions of fatty acids.

Especially, the fatty acids produced by one of the strains, named D31, had a high DHA content (over 60% of the total fatty acids) and the simple fatty acid composition (16:0, 18:0, 18:1 and DHA without any other polyunsaturated acids). Although most oleaginous microorganisms accumulate DHA as triacylglycerol, the strain D31 accumulated DHA mainly as a polar lipid (79.4% of total DHA), especially as phosphatidylcholine (71.4% of polar DHA). This strain D31 was identified as the related species of *Crypthecodinium cohnii* on the basis of phylogenetic analysis. *Crypthecodinium* sp. D31 showed high DHA productivity when cultivated in a medium containing glycerol as the carbon source and a mixture of yeast extract and polypeptone as the nitrogen sources, with a salinity that was equivalent to 50% of that of seawater and a pH in the acidic range (<pH 6.0). *Crypthecodinium* sp. D31 is considered as a promising producer of high-purity DHA-containing phospholipids.

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PUBLICATIONS

CHAPTER I

Okuda, T., Ando, A., Sakuradani, E., Kikukawa, H., Kamada, N., Ochiai, M., Shima, J. and Ogawa, J. Selection and characterization of promoters based on genomic approach for the molecular breeding of oleaginous fungus *Mortierella alpina* 1S-4. *Submitted for publication.*

CHAPTER II

Okuda, T., Ando, A., Sakuradani, E., Kikukawa, H., Kamada, N., Ochiai, M., Shima, J. and Ogawa, J. Characterization of galactose-dependent promoters from an oleaginous fungus *Mortierella alpina* 1S-4. *Submitted for publication.*

CHAPTER III

Okuda, T., Ando, A., Negoro, H., Kikukawa H., Sakuradani, E., Shima, J., Shimizu, S. and Ogawa, J. Omega-3 eicosatetraenoic acid (ETA) production by molecular breeding of the mutant strain S14 derived from *Mortierella alpina* 1S-4. *Submitted for publication.*

CHAPTER IV

Okuda, T., Ando, A., Negoro, H., Muratsubaki, T., Kikukawa, H., Sakuradani, E., Shima, J. and Ogawa, J. Eicosapentaenoic acid (EPA) production by an oleaginous fungus *Mortierella alpina* expressing heterologous Δ17-desaturase gene under normal temperature. *Submitted for publication.*

CHAPTER V

Okuda, T., Ando, A., Sakuradani, E. and Ogawa, J. Isolation and characterization of a docosahexaenoic acid-phospholipids producing microorganism *Crypthecodinium* sp. D31. *J Am Oil Chem Soc* **90 (12)**, 1837-1844 (2013).