Cloning and Sequencing of the *ura3* and *ura5* Genes, and Isolation and Characterization of Uracil Auxotrophs of the Fungus *Mortierella alpina* 1S-4

Seiki Takeno,1 Eiji Sakuradani,1 Shoichi Murata,1 Misa Inohara-Ochiai,2 Hiroshi Kawashima,3 Toshihiko Ashikari,2 and Sakayu Shimizu1,†

1Laboratory of Fermentation Physiology and Applied Microbiology, Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan
2Institute for Advanced Technology, Suntory Ltd., Osaka 618-0001, Japan
3Institute for Food & Beverage Health Care Science Laboratory, Suntory Ltd., Osaka 618-0001, Japan

Received June 9, 2003; Accepted October 28, 2003

The oil-producing fungus *Mortierella alpina* 1S-4 is an industrial strain. In order to prepare host strains for a transformation system for this fungus, six uracil auxotrophs were obtained by means of random mutation with N-methyl-N′-nitro-N-nitrosoguanidine (MNNG). When the activities of orotate phosphoribosyl transferase (OPRTase, EC 2.4.2.10) and orotidine-5′-phosphate decarboxylase (OMPdecase, EC 4.1.1.23) were examined in the mutants and wild strain, OPRTase activity was found to be completely absent in all mutants, on the other hand, OMPdecase activity was intact. The genomic DNA and cDNA of the *ura5* gene encoding OPRTase and the *ura3* gene encoding OMPdecase were cloned and sequenced. The Ura5p deduced amino acid sequence of this fungus showed highest similarity to that of *Vibrio cholerae* classed among prokaryote. Furthermore, the mutational points in the *ura3* genes of two selected mutants were identified; a base-replacement and a base-insertion.

Key words: *Mortierella alpina*; uracil auxotrophy; cloning; *ura5* gene; *ura3* gene

Polysaturated fatty acids (PUFAs) play important roles as structural components of membrane phospholipids, and as precursors of the eicosanoids of signaling molecules including prostaglandins, thromboxanes, and leukotrienes.1,2) The principal PUFAs are dihomo-γ-linolenic acid (n-6 PUFA; Δ5, Δ11, Δ14–20:3), arachidonic acid (AA, n-6 PUFA; Δ5, Δ8, Δ11, Δ14–20:4), and eicosapentaenoic acid (n-3 PUFA; Δ5, Δ8, Δ11, Δ14, Δ17–20:5). All mammals synthesize eicosanoids that are involved in inflammatory responses, reproductive function, immune responses, and regulation of blood pressure.3) Therefore, studies on PUFAs are important in both the medical and pharmaceutical fields.

We screened for microorganisms capable of accumulating lipids containing PUFAs,4) and found a filamentous fungus, *Mortierella alpina* 1S-4, belonging to the Zygomycetes. This strain is unique in that it produces some C-20 PUFAs (e.g., dihomo-γ-linolenic acid, AA, and eicosapentaenoic acid). We have studied the fatty acid metabolism in this strain and succeeded in its application to the industrial production of a triacylglycerol with a high content of AA.5) Therefore, this fungus is a good model for analyzing fatty acid desaturation and elongation systems from both fundamental and applied standpoints.

Up to now, many host-vector systems in filamentous fungi have been developed with a variety of selectable markers. One of the useful selectable markers is a gene encoding an enzyme relating to pyrimidine metabolism. The ability to synthesize pyrimidines is common to prokaryotes and eukaryotes, however, the pathway has undergone a number of changes and modifications during evolution.6) The last two steps in the biosynthesis of pyrimidines are catalyzed by orotate phosphoribosyl transferase (OPRTase, EC 2.4.2.10) and orotidine-5′-phosphate decarboxylase (OMPdecase, EC 4.1.1.23), respectively. In mammals and plants, both enzymatic activities are combined on a single polypeptide chain and thus appear as bifunctional enzymes.7) In a bacterium, *Salmonella typhimurium*,8) a filamentous fungus, *Neurospora crassa*,9) and a yeast, *Saccharomyces cerevisiae*,10) the two enzymatic activities are exhibited by independent enzymes, that is, the *ura5* and *ura3* genes encode OPRTase and OMPdecase, respectively. The nucleotide sequences have been identified in a number of organisms.

We have aimed to establish a transformation system for this fungus and to clarify the mechanism underlying PUFAs biosynthesis by means of genetic manipulation. In this study, uracil auxotrophs required as host strains

---

1 To whom correspondence should be addressed. Tel: +81-75-753-6115; Fax: +81-75-753-6128; E-mail: sim@kais.kyoto-u.ac.jp

Abbreviations: PUFA, polysaturated fatty acid; AA, arachidonic acid; MNNG, N-methyl-N′-nitro-N-nitrosoguanidine; 5-FOA, 5-fluoorotic acid; OPRTase, orotate phosphoribosyl transferase; OMPdecase, orotidine-5′-phosphate decarboxylase
were obtained and their characteristics were investigated by means of enzymatic analyses. 5-Fluoroorotic acid (5-FOA) is frequently used for the positive selection of OMPdecase- or OPRTase-deficient strains.\(^{11–14}\) Since in this fungus, the genes encoding these proteins were not sequenced, we isolated uracil auxotrophs of \textit{M. alpina} 1S-4, cloned the \textit{ura3} gene encoding OMPdecase and the \textit{ura5} gene encoding OPRTase of \textit{M. alpina} 1S-4, and identified the mutational points in the uracil auxotrophs.

**Materials and Methods**

Enzymes and chemicals. Restriction enzymes and other DNA-modifying enzymes were obtained from Takara Bio Inc. (Shiga, Japan) and New England Biolabs (Beverly, MA, USA). Orotidine-5'-monophosphate decarboxylase (OMPdecase, EC 4.1.1.23) was purchased from Sigma (St. Louis, MO, USA). All other chemicals were of the highest purity commercially available.

Strains, media, and growth conditions. The wild strain \textit{M. alpina} 1S-4 was maintained on Potato Dextrose Agar (PDA) medium (Difco, Detroit, MI, USA), and the uracil auxotrophs derived from \textit{M. alpina} 1S-4 on the PDA medium containing 5-fluoroorotic acid (5-FOA, 0.5 mg/ml) and uracil (0.05 mg/ml) (5-FOA/uracil medium). \(5\text{~mm}\) Tris-HCl containing 5 \(\mu\text{M}\) \(\beta\)-mercaptoethanol (pH 7.5). The mycelia were suspended in 2 volumes of the same buffer and then disrupted by passage, twice, through a French pressure cell (Amino, New York, USA) at 35 MPa. The extract was centrifuged at 2,500 \(\times\) \(g\) for 15 min to remove cell debris and intact cells. The supernatant (cell-free extract) was further ultracentrifuged at 100,000 \(\times\) \(g\) for 60 min to remove membranous components and organelles. The resultant supernatant corresponded to the soluble fraction. All steps were done at 4°C unless otherwise stated.

Measurement of enzyme activities and the protein content. OMPdecase activity was measured as described by Yoshimoto \textit{et al.},\(^{18}\) and orotate phosphoribosyltransferase (OPRTase, EC 2.4.2.10) as described by Umezu \textit{et al.}\(^{19}\) Protein contents were measured with a Bio-Rad Protein Assay kit (Bio-Rad Laboratories Inc., Hercules, CA, USA).

Preparation of genomic DNA and construction of a cDNA library. Preparation of genomic DNA of \textit{M. alpina} strains was done by the method described previously.\(^{20}\) Purification of mRNA and cDNA synthesis were also done by the methods described previously.\(^{21,22}\)

Isolation of the \textit{ura5} cDNA and \textit{ura5} genomic gene of \textit{M. alpina} 1S-4. Two highly degenerate primers were synthesized for the screening of the \textit{ura5} cDNA: a sense primer, 5'-TTYGHCICCIGCITAYAARGGHATYCC-3', and an antisense primer, 5'-CCCTCDCCRTGRTCYT-TIGGYCTYT-3', where I indicates inosine. Both the primers were designed based on the \textit{ura5} gene sequences of \textit{Glomerella graminicola}, \textit{Saccharomyces cerevisiae}, and \textit{Sordaria macrospora}. The sequences of the sense and antisense primers correspond to the regions of the \textit{ura5} gene that encode FGPAYKGIP and KEAKDHGEG, respectively. These primers were used in a Biometra T Gradient thermal cycler (Biometra GmbH, Göttingen, Germany) with a program of 1 min at 94°C, 1 min at 52°C, and 2 min at 72°C, for 35 cycles, followed by extension for 10 min at 72°C. The about 130-bp PCR-amplified products were ligated into pCR™2.1 (Invitrogen Co., Carlsbad, CA, USA) and then used to transform \textit{E. coli} DH5\(a\) and NM514 were used for DNA manipulation and cultivated at 37°C.

Preparation of cell-free extracts and soluble fractions. Cell-free extracts of \textit{M. alpina} strains were prepared by a slight modification of the method described by Wyll and Ratledge.\(^{17}\) Four-day-grown mycelia were harvested by suction filtration and washed extensively with 100 mM Tris-HCl containing 5 mM \(\beta\)-mercaptoethanol (pH 7.5). The mycelia were suspended in 2 volumes of the same buffer and then disrupted by passage, twice, through a French pressure cell (Amino, New York, USA) at 35 MPa. The extract was centrifuged at 2,500 \(\times\) \(g\) for 15 min to remove cell debris and intact cells. The supernatant (cell-free extract) was further ultracentrifuged at 100,000 \(\times\) \(g\) for 60 min to remove membranous components and organelles. The resultant supernatant corresponded to the soluble fraction. All steps were done at 4°C unless otherwise stated.
Based on the resultant sequence of the ura5 cDNA, the ura5 genomic gene was amplified by PCR, with the following primers: a sense primer, 5'-CCGCAACCCATCAGCACACA-3', and an antisense primer, 5'-GGACCTTATCCCATTTAGATTTGCC-3', and sequenced. The same strategy was used to identify the mutation point in the ura5 gene of the M. alpina 1S-4 ura5' strain. The 5'-untranslated regions (UTRs) were sequenced with a TaKaRa LA PCR TM in vitro Cloning Kit (Takara Bio Inc.). The first PCR was done with the primer 5'-GAGTCCACGAAAACTGCTGATGAGCA-GCAC-3', and a second one with primer 5'-ATCGTCATGACCAGACCTTGCCCTCCAA-3'.

Isolation of ura3 cDNA and ura3 genomic DNA of M. alpina 1S-4. Two highly degenerate primers were synthesized based on the amino acid sequences of the common OMPdecase internal peptides of Rhizopus niveus and Aspergillus oryzae. The amino acid sequence KFADIGNTV was used to design oligonucleotide pools 5'-AARTTYGCIGAYATYGGHAAYACIGT (sense strand) and RGVIIVDAG to obtain 5'-CKICCIACRA-TRATVACRTCIGCDCC-3' (antisense strand). The letter I indicates inosine in the oligonucleotide sequences. These primers were used in a Biometra T Gradient thermal cycler with a program of 1 min at 94°C, 1 min at 55°C for 1 min, and 72°C for 2 min, for 30 cycles, followed by extension for 10 min at 72°C. The reaction mixture comprised 0.5 μg of genomic DNA, 100 pmol of each oligonucleotide pool, and 2.5 U of Ex-Taq polymerase (Takara Bio Inc.) in a total volume of 50 μl. The about 450-bp PCR-amplified products were ligated into pCR™2.1, and then used to transform E. coli DH5α. The resulting clone was proved to have a part of the ura3 gene by means of DNA sequencing. This fragment was used as a probe to screen a ura3 cDNA library. Positive clones were excised in vivo, and their inserts were sequenced. Based on the resultant sequence of the cDNA, the ura3 genomic gene was amplified by PCR, with the following primers: a sense primer, 5'-ACATCTCACTCTGCTTCAA-3' and an antisense primer, 5'-CAAAACATAGACTTGAGAACTCTC-3', and sequenced.

Southern blot analysis and DNA sequencing. Southern blot analysis was done with a Gene Images™ Alkphos Direct™ labeling and detection system (Amersham Bioscience, Buckinghamshire, UK) following the standard procedure. Nucleotide sequences were identified by the dideoxy chain termination method with a CEQ Dye Terminator Cycle Sequencing kit (Beckman Coulter Inc., Fullerton, CA, USA) and an automated sequencer DNA Analysis System CEQ 2000XL (Beckman Coulter Inc.). Sequence data were analyzed with Genetyx-Mac ver. 11.2 (Software Development, Japan).

Nucleotide sequence accession numbers. The nucleotide sequences of the ura3 and ura5 genes cloned from M. alpina 1S-4 have been assigned DDBJ accession nos. AB109469 and AB109468, respectively.

Results

Isolation and characterization of uracil auxotrophs of M. alpina 1S-4
We used MNNG for random mutagenesis in order to obtain uracil auxotrophic mutants of M. alpina 1S-4. As a result, we succeeded in obtaining six isolates that can grow on SC medium with uracil but not on uracil-free SC medium (Fig. 1). On the contrary, the wild strain of M. alpina 1S-4 could grow on uracil-free SC medium, but not on 5-FOA/uracil medium (data not shown). All

Fig. 1. Growth of M. alpina 1S-4 and Its Uracil Auxotrophs on Synthetic Medium.
Wild strain M. alpina 1S-4 and its uracil auxotrophs were inoculated onto SC medium with or without uracil. Cultivation was done for 2 days at 28°C.
isolates showed vigorous growth as well as the wild strain in the presence of uracil (data not shown). The six resultant isolates must be uracil auxotrophs due to OMPdecase- and/or OPRTase-deficiency. The activities of both enzymes in the uracil auxotrophs were measured in order to find whether or not the uracil auxotrophy of the mutants is due to OMPdecase- or OPRTase-deficiency (Table 1). In comparison of the OMPdecase activity of *M. alpina* 1S-4 with that of uracil auxotrophs, uracil auxotrophy was not thought to be due to OMPdecase-deficiency. A noticeable finding was that no OPRTase activity was detected in any isolates. All isolates, therefore, should be defined as OPRTase-deficient mutants (*ura5/C0* strains).

### Isolation and sequencing of the *ura5* gene

All resultant mutants were *ura5/C0* strains. When these strains are used as host cells, *ura5* gene of *M. alpina* 1S-4 should be isolated to be used as a selective marker. Two highly degenerate primers were designed from highly conserved regions of other microbes. A 130-bp fragment was amplified by means of PCR with genomic DNA of *M. alpina* 1S-4 as a template. The amplified fragment was subcloned into pCR™2.1 and nucleotide sequence was analyzed. The amino acid sequence of the predicted translation product showed high similarity to those of other OPRTase proteins. The *ura5* cDNA was obtained by means of plaque hybridization with this fragment as a probe. Based on the *ura5* cDNA sequence, the *ura5* genomic gene was amplified and sequenced (Fig. 2). The *ura5* genomic gene contains an ORF with a length of 654 bp without introns, starting with an ATG codon and ending with a TAA codon. The *ura5* genomic gene was suggested to encode a protein consisting of 217 amino acids with a molecular weight of 24,000.

A computer-aided homology search for amino acid sequences in other enzymes in a database revealed that the deduced amino acid sequence of the *ura5* genomic gene exhibits 65, 50, and 43% identity with those of the OPRTases of *Vibrio cholerae*,23) *S. cerevisiae*,24) *G. graminicola*,25) and 29 and 30% identity with those of the UMP synthases of *Arabidopsis thaliana*26) and man (Fig. 3).27) It is proposed that Lys-106 serves as the active center, and that the region from Val-123 to Ala-135 is a motif sequence. A novel information was that the deduced amino acid sequence of the OPRTase of this fungus showed the highest identity with that of a bacterium, *Vibrio cholerae* than those of other eukaryotes.

### Isolation and sequencing of the *ura3* gene

Considering the novel similarity of the deduced OPRTase amino acid sequence of this fungus, we tried to compare the OMPdecase sequence of this fungus with those of other organisms. Therefore, the *ura3* gene was isolated and sequenced. Two conserved amino acid sequences in *Rhizopus niveus*28) and *Aspergillus oryzae*29) OMPdecases were used to design oligonucleotide sequences as primers. Since *R. niveus* is a zygomycete like *M. alpina*, the amino acid sequence of *R. niveus* OMPdecase was chosen. An about 450-bp fragment was amplified by PCR with these primers and 1S-4 genomic DNA as a template. The amplified fragment was subcloned into pCR™2.1 and the nucleotide sequence was analyzed. The deduced amino acid sequence of this fragment was confirmed to be similar to that of heterologous *ura3* genes. Therefore, the amplified fragment in pCR™2.1 was considered to have been derived from the *M. alpina* ura3 gene. Positive plaques were obtained by plaque hybridization with this fragment as a probe. The sequence revealed one complete cDNA with a length of 789 bp encoding a protein consisting of 262 amino acids with a molecular weight of 29,000 (Fig. 4). Based on the *ura3* cDNA sequence, the *ura3* genomic gene comprises 1073 bp from a start codon to a stop codon. In comparison of the nucleotide sequence of the *ura3* cDNA with that of the genomic DNA, two introns were found (nucleotide positions 181–291 and
**Fig. 4.** Complete Nucleotide Sequence of the *M. alpina* 1S-4 ura3 Genomic Gene and Its Deduced Amino Acid Sequence. The two introns (181–291 and 404–576) are underlined. The start codon, ATG, and stop codon, TAA, are highlighted as reverse characters.

**Fig. 3.** Comparison of the Deduced Amino Acid Sequences of the OPRTases from *M. alpina* 1S-4, *Vibrio cholerae*, *Saccharomyces cerevisiae*, and *Glomerella graminicola*, and the UMP Synthases from *Arabidopsis thaliana* and Man.

The alignment was done by introducing gaps (hyphens) to achieve maximum homology using the GENETYX-MAC ver. 11.2 program. Conserved residues are highlighted as reverse characters. An asterisk indicates a base-pair change mutational point in Ura5p of *ura5*—No. 6 strain.

**Fig. 4.** Complete Nucleotide Sequence of the *M. alpina* 1S-4 ura3 Genomic Gene and Its Deduced Amino Acid Sequence. The two introns (181–291 and 404–576) are underlined. The start codon, ATG, and stop codon, TAA, are highlighted as reverse characters.
404–576), which conform to the GT-AG rule as to RNA splicing, in the genomic DNA.

A computer-aided homology search for amino acid sequences in other enzymes in a database revealed that the deduced amino acid sequence of the ura3 cDNA exhibits 67, 65, 53, and 50% identity with those of the OMPdecases of Phycomyces blakesleeanus,(30) R. niveus,(28) Candida dubliniensis,(31) and Saccharomyces cerevisiae,(7) and 53% identity with those of the uridine monophosphate (UMP) synthases of mouse(32) and man,(27) respectively (Fig. 5). The three conserved amino acid residues, Asp-91, Lys-93, and Asp-96, involved in the active site of OMPdecase, were present in that of M. alpina 1S-4.

Identification of mutational points of the ura5 gene in uracil auxotrophs

All mutants are uracil auxotrophs due to a deficiency of OPRTase activity, so we tried to identify the mutational points of the ura5 genes encoding OPRTase in the mutants. We selected the No. 2 and No. 6 strains as hosts for Mortierella transformation, because of their clear phenotype of uracil auxotrophy. In case of mutant No. 2, a frameshift mutation was detected in the ura5 gene: an additional base was inserted at the +93 nucleotide position, leading to drastic changes in the overall amino acid sequence and resulting in an inactive enzyme. On the other hand, a base-pair change mutation was observed in that of mutant No. 6: the exchange of guanine to adenine was observed at the +398 nucleotide position, leading to G133D, which is indicated by an asterisk in Fig. 3. The ura3 genomic genes of the two mutants were also sequenced but no mutations were found.

Discussion

M. alpina 1S-4 is not only used in the oil industry and but also noticeably for studies of PUFA biosynthesis in microorganisms. Therefore, establishment of a transformation system for this fungus has been desired. First, we tried to find useful antibiotics that inhibit the growth
of this fungus, but never found any. For example, hygromycin B, reported to be a strong inhibitor for fungi, was also not sufficient in the case of this fungus. Taking note of not only antibiotics but also auxotrophy as a selectable marker, we succeeded in constructing some uracil auxotrophs capable of growing on 5-FOA/uracil-containing medium. All the mutants showed a high degree of uracil requirement phenotypically (Fig. 1). It is thought that this property is stably maintained and not easily reversed because we have empirically observed such stabilities among MNNG-derived mutants. In addition, spores of all mutants also showed auxotrophy. All mutants showed the same properties, such as the morphological aspects or the fatty acid composition, in comparison with those of wild strain (data not shown). These observations were needed prior to establishment of a transformation system for this fungus for industrial production of the useful lipids. To investigate the activities of OPRTase and OMPdecase, a soluble fraction was prepared by means of measurement of hexokinase (EC 2.7.1.1), a typical cytosolic enzyme, as an index. The enzyme activity was detected in the soluble fractions of M. alpina strains, which suggested that a soluble fraction was successfully prepared. As a result, all mutants were proved to have no OPRTase activity. Although this screening method involving 5-FOA was reported to be successful for obtaining both ura3 and ura5 mutants of other fungi,11–14,33) all mutants obtained in this study were ura5 ones (Table 1). Although a similar tendency has been observed for the basidiomycete Phanerochaete chrysosporium34) or hyperthermophilic archaea,35,36) the reason for this phenomenon remains unclear.

The ura3 genomic gene of M. alpina 1S-4 comprises 1,073 bp and is in accordance with the GT-AG rule, in which is expected to be present in M. alpina 1S-4. In addition, it has been reported that the amino acid sequence of Ura5p has 75.3% identity with that of Ura10p in S. cerevisiae.39)

Genetic analyses were done to identify mutational points on M. alpina 1S-4 ura5− strain genome DNA. Two types of mutations were found: a base insertion and a base replacement. A base replacement was observed in the ura5 genomic gene of the ura5− No. 6 strain. The base replacement is thought to lead to replacement of an amino acid within its motif sequence (DBGET PROSITE accession number, PS00103), which is responsible for inactivation of this enzyme. On the other hand, the base insertion seen in the ura5 genomic gene is expected to lead to drastic changes in the overall amino acid sequence, suggesting the enzyme’s inactivation. In fact, a quite different amino acid sequence was expected and two stop codons were present within its ORF. Since MNNG is known to be a strong alkylating agent that causes base substitutions, a base replacement often occurs. Indeed, many mutants have been obtained by MNNG treatment and their mutational patterns identified in this laboratory, and all the mutations obey the base replacement rule (data not shown). Therefore, a base addition on MNNG mutation may occur only rarely. It has been reported that 5-bromouracil, 5-chlorouracil, or 5-iodouracil serves as a mutagen, a pyrimidine base being incorporated into DNA. Considering this report, a high concentration of 5-FOA on screening of uracil auxotrophs may cause a base insertion in the ura5 gene. There has been a report that most uracil auxotrophs of Phanerochaete chrysosporium with UV-radiation mutagenesis are ura5-deficient ones.34) The ura5 gene in M. alpina 1S-4 may easily undergo mutation with any mutagen.

A transformation system and genetic manipulation of M. alpina 1S-4 must be established on the basis of these data. A transformation system with ura5− strains as hosts and the homologous ura5 gene as a selectable marker may allow elucidation of the biosynthetic pathways for PUFAs or the mechanism underlying lipid accumulation in mycelia.

Acknowledgments

This work was supported in part by the New Energy and Industrial Technology Development Organization (NEDO) and Grants-in-Aid for Scientific Research (No. 15658024 for S. S.) from the Ministry of Education, Science, Sports, and Culture, Japan.

References

2) Smith, W. L., and Borgeat, P., The eicosanoids:


16) Wynn, J. P., and Ratledge, C., Evidence that the rate-limiting step for the biosynthesis of arachidonic acid in Mortierella alpina is at the level of the 18:3 to 20:3 elongase. Microbiology, 146, 2325–2331 (2000).


28) Mattern, I. E., Unkles, S., Kinghorn, J. R., Pouwels,


