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# Genetic Analysis of Cytochrome $b_5$ from Arachidonic Acid-Producing Fungus, *Mortierella alpina* 1S-4: Cloning, RNA Editing and Expression of the Gene in *Escherichia coli*, and Purification and Characterization of the Gene Product[1]

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**Information on the amino acid sequences of the internal peptide fragments of cytochrome $b_5$ from *Mortierella hygrophila* was used to prepare synthetic oligonucleotides as primers for the polymerase chain reaction. A 100-base DNA fragment was thus amplified, by using a genomic gene from *Mortierella alpina* 1S-4 as a template, which produced polyunsaturated fatty acids such as arachidonic acid. The amplified DNA fragment was used as the probe to clone both a 523-base cDNA fragment and a 2.1-kilobase *Sal*I-*Nru*I genomic fragment coding for the whole *M. alpina* 1S-4 cytochrome $b_5$. On the basis of nucleotide sequences of both cytochrome $b_5$ genomic gene and cDNA, the genomic cytochrome $b_5$ gene was found to consist of four exons and three introns. A novel type of RNA editing, in which the cDNA included either guanine insertion or adenine→guanine substitution at one base upstream of poly(A), was interestingly observed. The deduced amino acid sequence of *M. alpina* 1S-4 cytochrome $b_5$ showed significant similarities with those of cytochrome $b_5$s from other organisms such as rat, chicken, and yeast. The soluble form of the cytochrome $b_5$ gene was expressed to 16% of the total soluble protein in *Escherichia coli*. The holo-cytochrome $b_5$ accounted for 8% of the total cytochrome $b_5$ in the transformants. The purified cytochrome $b_5$ showed the oxidized and reduced absorbance spectra characteristic of fungal microsomal cytochrome $b_5$.**

**Key words: cloning, cytochrome $b_5$, gene expression, *Mortierella*, RNA editing.**

Cytochrome $b_5$ is a small hemoprotein which is an integral component of the microsomal membranes of higher plants, animals, and yeasts (*1*). This protein is composed of three domains: a hydrophilic heme-containing catalytic domain of about 100 amino acids at the N-terminus (*2, 3*); a membrane-binding hydrophobic domain containing about 30 amino acids at the C-terminus of the molecule (*4*); and a membrane-targeting region represented by the 10-amino-acid sequence located at the C-terminus of the membrane-binding domain (*5*). A hydropathy plot of the rabbit liver cytochrome $b_5$ sequences demonstrated a single hydrophobic stretch of 23 amino acids at the C-terminus which has been proposed as the membrane anchor of the protein (*6*). It functions as an electron carrier in a number of microsomal oxidation/reduction reactions, including fatty acid desaturation (*7, 8*), cholesterol biosynthesis (*9*), and reduction of cytochrome P-450 (*10*). Among these reactions, the fatty acid biosynthesis reaction around the membrane of the endoplasmic reticulum has been reported. In animals, cytochrome $b_5$ is reduced by NADH-cytochrome $b_5$ reductase and used as the electron donor in the desaturation of acyl-CoA substrates (*11*), while in plants, cytochrome b5 is involved in the desaturation of acyl-complex lipids (*12*).

The amino acid sequences for cytochrome $b_5$ from a number of organisms have been determined (*13*), and the crystal structure of the heme-containing segment of the tryptic digested cytochrome $b_5$ from calf liver has been reported (*14*). Recently, heterologous expression systems have been employed for the synthesis of recombinant membrane-bound cytochrome $b_5$ (*15-18*). Computer modeling studies (*19, 20*) have demonstrated the electron-transfer properties of cytochrome $b_5$ required for "docking" with proteins such as cytochrome *c*.

Although cytochrome $b_5$ from plants and animals has been extensively studied, fungal studies have not been carried out at both protein and gene levels; our previous report concerned the purification of cytochrome $b_5$ from *Mortierella hygrophila* (*21*). We also reported the production of polyunsaturated fatty acids (PUFA), such as arachidonic acid, dihomo-$\gamma$-linolenic acid, Mead acid, and eicosapentaenoic acid, by *M. alpina* 1S-4 and its derivative

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Abbreviations: PUFA, polyunsaturated fatty acid; aa, amino acid.

mutants; in particular, arachidonic acid has been industrially produced using this fungus (*22-25*). Considering the high production of PUFA by this fungus, we anticipate that this strain has unique and potential desaturation systems containing cytochrome $b_5$. It is therefore of interest to analyze this system genetically.

We succeeded in cloning cytochrome $b_5$ cDNA and its genomic gene from *M. alpina* 1S-4 fungi. We also report here a unique type of RNA editing and the expression of the active fungal cytochrome $b_5$ in *Escherichia coli*.

## MATERIALS AND METHODS

*Strains and Plasmids*—*M. alpina* 1S-4 was previously isolated from soil samples. *E. coli* JM109 [*recA*1, *endA*1, *gyrA*96, *thi*, *hsdR*17, *supE*44, *relA*1, Δ(*lac-proAB*)/F′(*traD*36, *proAB*$^+$, *lacI*$^q$, *lacZ*ΔM15)] was the host for pBluescript II transformation and phage M13 propagation. λ phage infection in constructing the cosmid library was performed using *E. coli* XL-1 Blue MR [Δ(*mcrA*)182, Δ(*mcrCB-hsdSMR-mrr*)172, *endA*1, *gyrA*96, *lac*, λ$^-$, *lec*, *recA*, *relA*1, *supE*44, *thi*-1]. λ phage infection in constructing the cDNA library was performed using *E. coli* NM514 [*hsdR*514 (*rk*$^-$*mk*$^-$), *argH*, *galE*, *galX*, *strA*, *lycB*7, (*Hfl*$^+$)]. Expression of the cytochrome $b_5$ gene was performed using *E. coli* BL21(DE3) [F$^-$, *ompT*, *hsdS*$_B$-($r_B^-m_B^-$), *gal*, *dcm*(DE3)] bearing the T7 RNA polymerase gene (λ DE3 lysogen) for expression of target proteins (*26*).

*Media*—*M. alpina* 1S-4 was cultured in GY medium, which consisted of 20 g of glucose and 10 g of yeast extract (pH 6.0)/liter. M13 phage was propagated in 2×YT medium consisting of 16 g of tryptone, 10 g of yeast extract, and 5 g of sodium chloride. *E. coli* transformants containing the *M. alpina* 1S-4 cytochrome $b_5$ gene were grown in Luria-Bertani medium (LB medium) consisting of 10 g of tryptone, 10 g of sodium chloride, and 5 g of yeast extract (pH 7.2)/liter.

*Enzymes and Chemicals*—Restriction endonucleases and other DNA-modifying enzymes were obtained from Takara Shuzo and Toyobo. [α-$^{32}$P]dCTP (110 TBq/mmol) and [γ-$^{32}$P]ATP (180 TBq/mmol) were from Amersham Japan. A Kilo-sequence Deletion kit and a Supercos 1 cosmid vector kit were purchased from Takara Shuzo and Stratagene, respectively. A cDNA synthesis module kit and a Complete rapid cloning system λgt10 kit were obtained from Amersham (England). Isopropyl-β-D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) were obtained from Wako Pure Chemicals (Tokyo). All other chemicals were of the highest purity commercially available.

*Genomic DNA Preparation*—Total DNA of *M. alpina* 1S-4, which was grown in 400 ml of GY medium for 4 days, was prepared by a modification of the method of Malardier *et al.* (*27*). The following procedure was carried out. Approximately 10-20 g (wet mass) of mycelia was added to a stainless-steel blender cup precooled by filling with liquid nitrogen and then blended. The blended mycelium was suspended in 50-100 ml of lysis buffer consisting of 2% cetyl trimethyl ammonium bromide (CTAB), 0.1 M Tris-HCl (pH 8.0), 1.4 M NaCl, and 1% polyvinylpyrrolidone. The mixture was incubated for 3-4 h at 65°C. Total genomic DNA was purified by extracting the lysate with phenol/chloroform (1:1; v/v), isopropanol-precipitated, RNase-treated, and re-precipitated with ethanol. The resultant precipitated genomic DNA was dissolved in TE buffer consisting of 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA (pH 8.0).

*Construction of Cosmid Library*—The procedure for constructing the cosmid library basically followed the protocol of the Supercos 1 cosmid vector kit (*28*). The chromosomal DNA from *M. alpina* 1S-4 was partially digested with *Sau*3AI. The digested chromosomal DNA was ligated into the *Bam*HI sites of the Supercos 1 cosmid vector. The ligated DNA was packaged *in vitro*, and the packaged phage were propagated in *E. coli* XL-1 Blue MR.

*Cloning of Cytochrome $b_5$ Gene from M. alpina 1S-4*—Oligonucleotide primers were synthesized based on the amino acid sequences of the cytochrome $b_5$ internal peptides from *M. hygrophila* (*21*). The amino acid sequence Gly-Phe-Ile-Asp-Glu-His-Pro-Gly-Gly-Glu-Glu-Val was used to design oligonucleotide pool 5′-GGATT(C/T)ATCG-ATGA(A/G)CA(C/T)CCIGG(A/C/G/T)GG(A/C/G/T)G-A(A/G)GA(A/G)G-3′ (sense strand), and Phe-Glu-Asp-Val-Gly-His-Ser-Asp-Glu-Ala-Arg-Asp-Ile-Met to design 5′-CATGATGTCCCGGGC(C/T)TC(A/G)TC(A/G/T)(C/G)(A/T)(A/G)TGICCIAC(A/G)TC(C/T)TC(A/G)AA-3′ (antisense strand). These oligonucleotides were synthesized by the phosphoramidite method using an Applied Biosystems Model 381A automatic synthesizer (California, USA). DNA was amplified by the polymerase chain reaction (PCR) using a thermal cycler (Perkin-Elmer Cetus Instruments, USA). Reaction mixture contained 5 μg of chromosomal DNA, 200 pmol of each oligonucleotide pool, and *Thermus thermophilus* DNA polymerase (TOYOBO, Osaka) in a volume of 100 μl. Thirty-five thermal cycles were performed of 95°C for 1 min, 55°C for 2 min, and 72°C for 1 min. The gel-purified PCR product [100 base pairs (bp)] was cloned into the *Acc*I-*Xma*I sites of M13mp18 replicative form DNA. To clone the full-length cytochrome $b_5$ gene, the PCR-synthesized products incorporating [α-$^{32}$P]dCTP were used as a radiolabeled probe (*29*). Nucleotides were sequenced by chain termination using Sequenase version 2 (United States Biochemical). Deoxy-ITP or 2′-deoxy-7-deaza-GTP was used as a substitute for dGTP during M13 sequencing to minimize compression.

*Screening for the Cosmid Library*—Colony hybridization was performed essentially as described by Ausubel *et al.* (*30*). The nitrocellulose filter (Schleicher and Schuell, Germany) was placed on the LB agar plate on which colonies from the cosmid genomic library were grown. The replica filter was cultured on a new LB agar plate containing 100 μg/ml ampicillin at 37°C overnight. The nitrocellulose filter was placed on a 3MM Chr paper (Whatman, USA) saturated with 0.5 M NaOH and 1.5 M NaCl for 5 min, then neutralized by placing on a 3MM Chr paper saturated with 1.5 M NaCl, 0.5 M Tris-HCl (pH 7.2), and 1 mM EDTA. The filter was briefly washed in 2×SSC (1×SSC=0.15 M NaCl, 15 mM sodium citrate), then the air-dried filter was baked at 80°C for 2 h in a vacuum oven. Colony hybridization was performed by using the $^{32}$P-labeled 100-bp PCR-synthesized products containing part of the *M. alpina* 1S-4 genomic gene as a probe. Hybridization was performed at 42°C overnight in a solution consisting of 40% (v/v) formamide, 5×SSC, and 0.1% (w/v) SDS. The filters were washed at room temperature in the hybridization buffer described above and then in 2×SSC and 0.1% SDS.

*Poly(A)$^+$ RNA Preparation*—Total RNA was extracted by AGPC (acid guanidinium-phenol-chloroform) method (*31*) as follows: liquid nitrogen was added to 5 g of fresh mycelium cultured in GY medium at 28°C, and then the mycelia were ground using stainless-steel blender. The ground-mycelia were mixed with 50 ml of solution D (4 M guanidine thiocyanate, 2 M sodium citrate, 0.1 M 2-mercaptoethanol, and 0.5% sodium *N*-lauroyl sarcosinate), 5 ml of 2 M sodium acetate, 50 ml of water-saturated phenol and 20 ml of chloroform/isoamyl alcohol (49:1; v/v) vigorously for 10 s and kept on ice for 15 min. After centrifugation at 10,000×*g* for 20 min, the supernatant was mixed with the same volume of isopropyl alcohol and centrifuged at 10,000×*g* for 20 min. The pellet was suspended in 3 ml of solution D, then the same volume of isopropyl alcohol was added. After centrifugation at 10,000×*g* for 10 min, the pellet was washed with 75% ethanol and dissolved in diethyl pyrocarbonate (DEPC)-treated water.

Poly(A)$^+$ RNA was prepared from the total RNA by chromatography on an oligo(dT)-cellulose column in an mRNA purification kit (Pharmacia, Sweden).

*Construction of cDNA Library and Screening*—Commercial kits [*i.e.*, cDNA synthesis module and Complete rapid cloning system λgt10 (Amersham)] were used for cDNA synthesis and construction of a cDNA library. Complete cDNA was synthesized from the prepared poly-(A)$^+$ RNA. The cDNA was ligated with the *Eco*RI-digested λgt10 arms, packaged *in vitro*, and infected into *E. coli* NM514. Three positive plaques were obtained from 50,000 recombinant phages. One recombinant, which contained the longest cDNA of the cytochrome $b_5$, was selected. The *Eco*RI insert from purified positive lDNA was then subcloned into pBluescript II for the large scale preparation of DNA.

*Northern Analysis*—Northern analysis was performed according to standard procedures (*32*). Nitrocellulose filters were hybridized at 42°C in a solution containing 40% (v/v) formamide, 5×SSC and 0.1% SDS overnight. The filters were washed at room temperature in the same hybridization solution and then in 2×SSC containing 0.1% SDS.

*DNA Sequence Analysis*—All DNA fragments containing a cytochrome $b_5$ gene were cloned into pBluescript II vectors. Nucleotide sequence analysis was performed on dideoxy chain-terminating method (*33*). A Taq dye primer sequencing kit was used with an autosequencer (DNA Sequencer 373A, Applied Biosystems).

*Constructions of Vectors for the Expression of Cytochrome $b_5$ in E. coli*—A 523-bp cDNA encoding cytochrome $b_5$ was isolated from the *M. alpina* 1S-4 cDNA library. To express the full-length form of *M. alpina* 1S-4 cytochrome $b_5$, we improved the sequence upstream from ATG codon by PCR with the cytochrome $b_5$ cDNA as a template and two oligonucleotides as primers. The sense primer (5′-TCGCCATATGGCCGAACTTAAG-3′) contained an *Nde*I recognition site (which is underlined) and 15 nucleotides of the cytochrome $b_5$ cDNA starting with the ATG start codon. The antisense primer (5′-TAGGATCCCTAAAGCAGCACCTTC-3′) contained a *Bam*HI recognition site and 13 nucleotides of the cytochrome $b_5$ cDNA (complementary to the nucleotide sequence) terminating with the TAG stop codon. The plasmid designated as pMCB30CF was constructed by ligation of the PCR product with pET-16b/*Nde*I-*Bam*HI and was transformed into *E. coli* BL21-(DE3). To express the soluble form of *M. alpina* 1S-4 cytochrome $b_5$, we constructed another expression vector containing the cytochrome $b_5$ that excluded its C-terminal region (36 amino acid residues). The resultant soluble form was composed of 94 amino acid residues. The sense primer was the same as that used for the construction of pMCB30-CF. The antisense primer (5′-CTGGATCCTCATGAGGAGGGAGACTTG-3′) contained a *Bam*HI recognition site, a modified TGA stop codon, and 16 nucleotides of the 3′-terminus of the cytochrome $b_5$ cDNA (complementary to the nucleotide sequence). The plasmid designated as pMCB40CS was constructed by ligation of the PCR product with pET-16b/*Nde*I-*Bam*HI and was transformed into *E. coli* BL21(DE3). Both plasmids were sequenced to verify that they had correctly encoded the full-length form and the C-terminal-excluded form of cytochrome $b_5$.

*Spectrophotometric Assay and Densitometric Scanning of Cytochrome $b_5$*—Spectrophotometric measurements were performed with a recording spectrophotometer UV-240 (Shimadzu, Kyoto) at room temperature. Absorbance spectra were monitored by scanning the sample between 400 and 600 nm. In bacterial lysates and during protein purification, cytochrome $b_5$ content was determined by measuring the reduced minus oxidized difference spectrum and taking the extinction coefficient of 125 $mM^{-1} \cdot cm^{-1}$ between 424 and 409 nm (*21*). The absorbance coefficients were defined from *M. hygrophila* microsomal cytochrome $b_5$. The cytochrome $b_5$ was reduced by adding a few milligrams of solid sodium hydrosulfite.

Quantification of cytochrome $b_5$ on the slab gels after separation by SDS-PAGE was carried out by densitometric scanning with an LKB 2222 Ultroscan XL (Pharmacia, Sweden).

*Protein Determination*—Protein was determined by the Coomassie Brilliant Blue G-250 dye-binding method of Bradford (*34*) using a dye reagent supplied by Bio-Rad.

*Gel Electrophoresis*—SDS-PAGE was carried out by the method of Laemmli (*35*) with 15% (w/v) acrylamide gels. After electrophoresis, the gel was stained for protein with Coomassie Brilliant Blue R-250, followed by decolorization in 10% (v/v) acetic acid and 30% (v/v) methanol.

*Protein Purification*—For the purification of the soluble form of cytochrome $b_5$, a single colony of *E. coli* was inoculated into a 500-ml flask containing 100 ml of LB medium and 100 μg/ml ampicillin, and cultured at 37°C overnight. Cells were harvested by centrifugation at 5,000×*g* for 10 min at 4°C and the resulting pellet was resuspended in 4 ml of ice-cold buffer (5 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 7.9), followed by sonication for 10 min (19 kHz; Insonator Model 201M, Kubota, Tokyo). The lysate was centrifuged at 12,000×*g* for 20 min to remove cells debris. The supernatant was centrifuged at 39,000×*g* for 20 min to remove insoluble proteins, and the resulting supernatant was filtered through a 0.45 micron membrane to prevent clogging of the resin. The supernatant was applied to a His-Bind Resin column (Novagen, USA) equilibrated with 50 mM $NiSO_4$ (typically 2.5 ml bed volume). The column was then washed with 15 ml of a solution consisting of 60 mM imidazole, 500 mM NaCl, and 20 mM Tris-HCl (pH 7.9), in order to throw out unbound proteins. Cytochrome $b_5$ was eluted with a solution

consisting of 1 M imidazole, 500 mM NaCl, and 20 mM Tris-HCl (pH 7.9). Each fraction eluted was assayed by SDS-PAGE. Fractions containing cytochrome $b_5$ were pooled, loaded onto a column of Superdex 75 (Pharmacia), and eluted with a buffer consisting of 50 mM $NaH_2PO_4$ (pH 8.0), 300 mM NaCl, 10% (w/v) glycerol. The cytochrome $b_5$ was concentrated by Centricon-10 (Amicon, Beverly, MA). All column chromatographies were carried out at 4°C, except for Superdex 75, which was performed at room temperature.

*N-Terminal Amino Acid Sequence Analysis*—The target bands of cell-free extracts separated by SDS-PAGE were directly electroblotted onto a PVDF membrane (Bio-Rad, USA) according to a standard procedure (*36*). After equilibration of a membrane and an acrylamide gel in transfer buffer containing *N*-cyclohexyl-3-aminopropanesulfonic acid (CAPS), the target protein was transferred onto a PVDF membrane by an electroblotting system Typ-Nr. B 33 (Biometra, Germany). The electroblotted membrane was used directly for the N-terminal sequence analysis by automatic Edman degradation with an Applied Biosystems Model 476A amino acid analyzer (*37*).

## RESULTS

*Cloning Cytochrome $b_5$ Genomic DNA by PCR Amplification*—The amino acid sequences of the partial peptide fragments from *M. hygrophila* IFO 5941 were previously determined in our laboratory (*21*). The amino acid sequences of two of these peptide fragments were similar to those of other organisms such as rat (*5, 38*), chicken (*39*), and yeast (*13*). Based on this sequence information, we designed primers for PCR (described in "MATERIALS AND METHODS"). PCR was performed in high stringency with these primers and the template of *M. alpina* 1S-4 chromosomal DNA. A 100-bp fragment was amplified, and its nucleotide sequence was determined. The amino acid sequence deduced from the nucleotide sequence of this fragment was consistent with those of both the K-6 and the K-10 internal peptide fragments of *M. hygrophila* cytochrome $b_5$ in 83% of 12 amino acid residues (*21*).

*Analysis of Cytochrome $b_5$ Expression Level in M. alpina 1S-4 by Northern Hybridization*—Total RNA was prepared from mycelia grown on GY medium at 28°C for either 1 or 3 days. The 100-bp DNA fragment (described above) was used as the probe for Northern blot against the total RNA. The RNA (*i.e.*, lanes 1-4 in Fig. 1) prepared from cells grown for each period gave a hybridization band of about 0.6 kb with the probe. It was transcribed much more in the mycelium cultured for 1 day.

*Cloning of Cytochrome $b_5$ cDNA from M. alpina 1S-4*—The PCR-amplified 100-bp fragment was used to screen a cytochrome $b_5$ cDNA from the cDNA library prepared from *M. alpina* 1S-4 grown at 28°C for 1 day. Three positive plaques were obtained from 50,000 recombinants in the cDNA library. After selection of one of the positive plaques, plaque-hybridization screening isolated one positive plaque. This positive clone contained a 630-bp insert DNA. Figure 2 shows the complete nucleotide sequence of the cytochrome $b_5$ cDNA and its deduced amino acid sequence. The cytochrome $b_5$ cDNA was composed of 523 bp nucleotides and contained polyadenyl residues 70 bp downstream from its stop codon TAG.

*Cloning of Cytochrome $b_5$ Genomic Gene from M. alpina 1S-4*—The PCR-amplified 100-bp fragment was used to screen a cytochrome $b_5$ genomic gene from the genomic cosmid library of *M. alpina* 1S-4. From 3,000 recombinants in the genomic library, three positive clones were obtained. We selected one of the positive clones, which contained a 28-kb insert DNA. This positive clone was designated pMCB10.

A DNA fragment containing the cytochrome $b_5$ genomic gene, which was 2.1-kb in length and had *SalI*-*NruI* sites at the both ends, was cloned from the cosmid clone pMCB10. The whole nucleotide sequence encoding cytochrome $b_5$ was determined. Both the genomic gene and the cDNA nucleotide sequences of cytochrome $b_5$ in *M. alpina* 1S-4 suggested that the cytochrome $b_5$ gene was composed of four exons and three introns (Fig. 2). The three introns contained 326-, 234-, and 358-bp nucleotides, respectively. GT and AG nucleotides were present at the 5′- and 3′-terminus, respectively, of every intron. A putative polyadenylation signal AATATA is present 18 bp upstream of the poly(A). Moreover, surprisingly, we discovered that one guanine residue was inserted or substituted for an adenine residue one base upstream of poly(A) in the *Mortierella* cytochrome $b_5$ cDNA.

*Structural Analysis and Proposed Topology of the M. alpina 1S-4 Cytochrome $b_5$*—We compared putative amino acid sequences of cytochrome $b_5$ from *M. alpina* 1S-4 with those of other organisms such as rat (*5, 38*), chicken (*39*), and yeast (*13*). The amino acid sequence of *M. alpina* 1S-4 cytochrome $b_5$ showed 48, 40, and 39% identities with rat, chicken, and yeast, respectively, over 100 amino acids of the N-terminus (Fig. 3). By contrast, there was no significant identity among those sequences over 30 amino acids of the C-terminus. Highly by homologous amino acids existed only between *M. alpina* 1S-4 and yeast (*13*) cytochrome $b_5$ on aligned sequences. Two His residues highlighted in

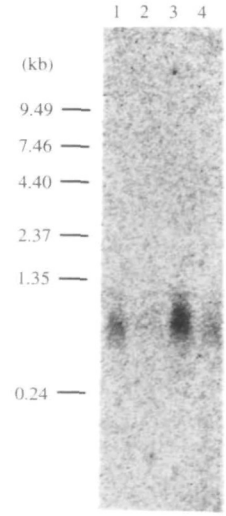


Fig. 1. **Analysis of *M. alpina* 1S-4 total RNA by Northern hybridization.** Total RNAs were obtained from mycelia cultured at 28 C for 1 or 3 days in GY medium. Lanes 1 and 2 were loaded with 30 and 10 $\mu$g of total RNA prepared from mycelia grown for 3 days. Lanes 3 and 4 were loaded with 30 and 10 $\mu$g of total RNA prepared from mycelia grown for 1 day. PCR-amplified 100-bp DNA fragment was used as a probe. An RNA ladder marker consisting of 9.49, 7.46, 4.40, 2.37, 1.35, and 0.24 kb fragments was used.

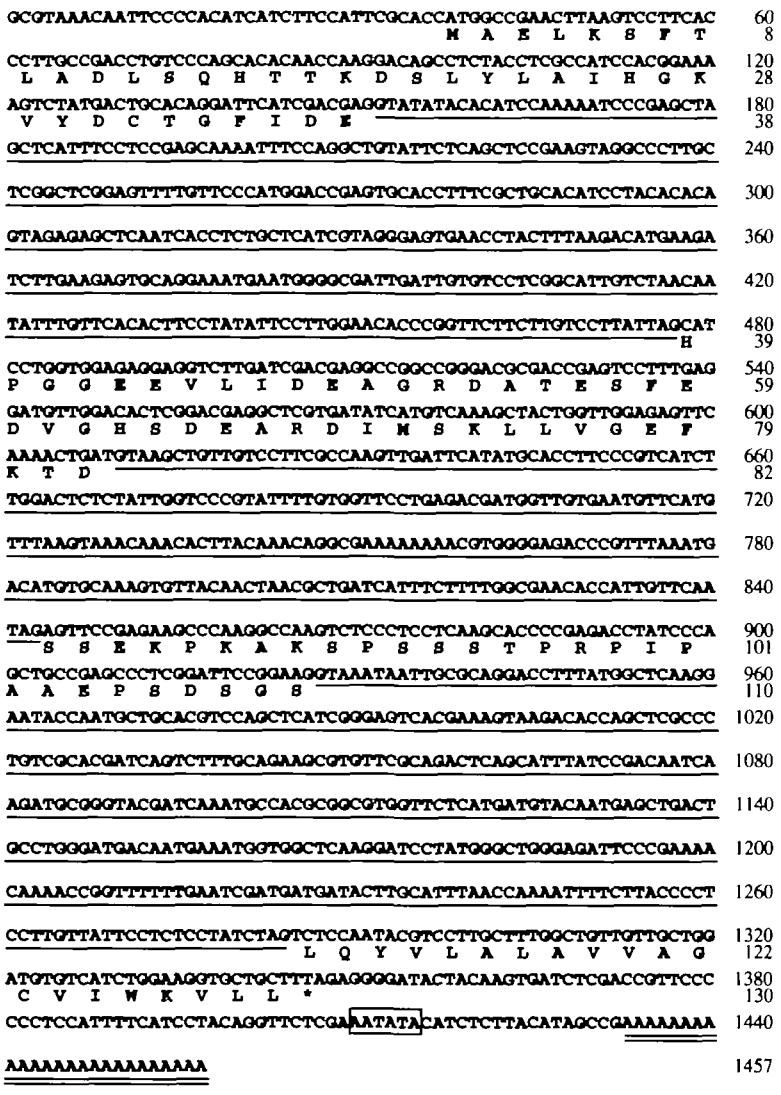

Fig. 2. **Complete nucleotide sequence of the *M. alpina* 1S-4 cytochrome $b_5$ DNA and its deduced amino acids.** Three introns having GT- and AG-ends in the cytochrome $b_5$ gene are underlined. A relevant stop codon is indicated by an asterisk. The putative polyadenylation signal is boxed, and the additional poly(A) tail is indicated by double underlines. In the cytochrome $b_5$ cDNA, one guanine and the succeeding poly(A) are present just after cytidine corresponding to this residue (nucleotide position: 1431) of the genomic DNA.

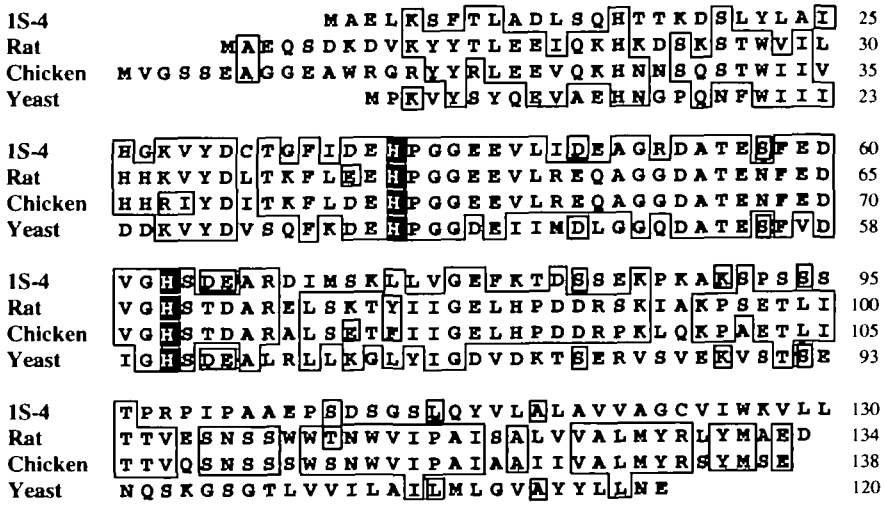

Fig. 3. **Homology of amino acid sequences of cytochrome $b_5$ among 4 different organisms.** Identical amino acids are enclosed in boxes. Two histidine residues for heme binding are highlighted in reversed characters. 1S-4, *M. alpina* 1S-4, and Yeast, *Saccharomyces cerevisiae*.

reverse letters in Fig. 3 were conserved in all four organisms and are considered to become heme iron ligands.

Hydropathy profiles of the deduced amino acid sequences of the *M. alpina* 1S-4 and rat cytochrome $b_5$s are shown in Fig. 4. The hydropathic index was calculated by the method of Kyte and Doolittle (*40*) with a window size of five amino

acid residues. The shapes of the two graphs resemble each other.

*Characterization of Recombinant Cytochrome $b_5$ Expressed in E. coli*—The vector pET-16b is designed to allow the expression of a cloned gene in the *E. coli* strain BL21(DE3) by use of an IPTG-inducible bacteriophage T7 RNA polymerase/promoter system (*26*). In the experiments using this vector, however, the recombinant cytochrome $b_5$ accumulated in the *E. coli* cells irrespective of induction by IPTG, and no increase in cytochrome content was observed when IPTG was present. Therefore, IPTG was not added to the culture medium in the following experiments.

The expression of the full-length cytochrome $b_5$ was first analyzed in the recombinant *E. coli*. SDS-PAGE analysis of the precipitates of the sonicated cell-free extracts from the transformants containing the full-length cytochrome $b_5$ gene revealed the prominently stained band corresponding to a molecular mass of 22 kDa. The full-length cytochrome $b_5$ protein was precipitated during the centrifugation of cell-free extracts, suggesting that the protein forms inclusion bodies. Therefore, we next tried to express the soluble form of cytochrome $b_5$ that lacks its C-terminal region in *E. coli*. The vector for expression of the soluble form of cytochrome $b_5$ was constructed by use of a pET system with a T7 promotor. The cells containing the soluble form of cytochrome $b_5$ appeared red. SDS-PAGE analysis of the supernatants of cells from the transformants containing the soluble form of cytochrome $b_5$ revealed a prominently stained band corresponding to molecular masses of 18 kDa. Protein bands obtained from both the full-length and the soluble form of cytochrome $b_5$ on SDS-PAGE were directly electroblotted onto PVDF membranes, and their N-terminal amino acid sequences were determined. Both forms of cytochrome $b_5$ had an N-terminal sequence of Gly-(His)$^{10}$-Ser-Ser-Gly-His-Ile-Glu-Gly-Arg-His-Met-Ala-Glu-Leu-Lys-Ser and contained many proteins lacking the N-terminal methionine derived from the vector pET-16b. These sequences exactly matched those deduced from the cDNA sequence. Densitometric scanning for SDS-PAGE of the cell-free extracts derived from the soluble form of cytochrome $b_5$ demonstrated that the amount of cytochrome $b_5$ was 16% of the total cellular proteins. In contrast, the full-length form of cytochrome $b_5$ corresponded to 0.3% of the total cellular protein and 5.7% of the total proteins of cellular precipitates. Transformant cells containing the pET-16b expression vector without the cytochrome $b_5$-coding sequence did not contain either band.

Spectrophotometric determination of cytochrome con-

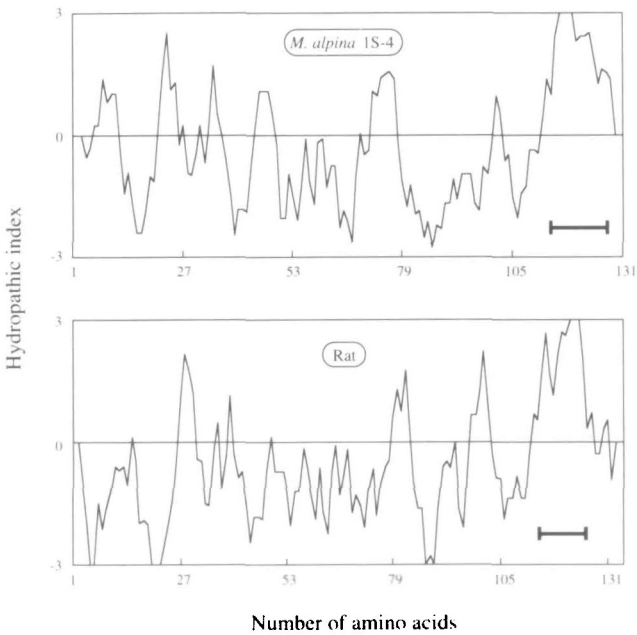


Fig. 4. **Comparison of hydropathy profiles from products derived from the *M. alpina* 1S-4 and rat cytochrome $b_5$ cDNAs.** Hydropathy profiles of the deduced amino acid sequences of *M. alpina* 1S-4 cytochrome $b_5$ cDNA (top panel) and rat cytochrome $b_5$ cDNA (bottom panel) are shown. Putative membrane binding domains conserved between them are indicated by solid bars.

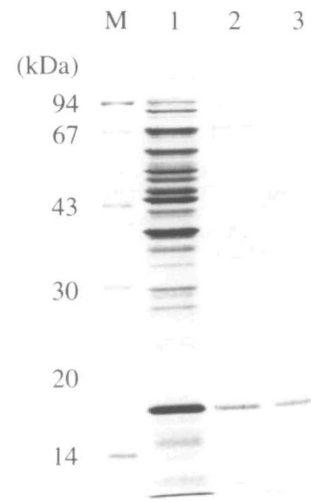


Fig. 5. **SDS-PAGE of the purified recombinant cytochrome $b_5$.** Lane 1, supernatants in extracts of the cells transformed with pET-16b containing cDNA encoding the soluble form of cytochrome $b_5$; lane 2, fractions eluted in His-Tag column chromatography; lane 3, fractions eluted in FPLC Superdex 75 column chromatography. Lane M was loaded with the following molecular mass standards: phosphorylase (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa), and $\alpha$-lactalbumin (14 kDa).

TABLE I. **Purification of the soluble form of cytochrome $b_5$ expressed in *E. coli*.** Cytochrome $b_5$ content was assessed from the Soret absorbance maximum of the oxidized cytochrome $b_5$ using the absorbance coefficient of 125 mM $^{-1}$·cm $^{-1}$ for *M. hygrophila* cytochrome $b_5$ determined by Kouzaki *et al.* (*21*).

| Step | Total protein (mg) | Total cytochrome $b_5$ (nmol) | Specific content (nmol of $b_5$/mg of protein) | Fold | Yield (%) |
|---|---|---|---|---|---|
| Supernatant of extracts | 14.7 | 15.6 | 1.06 | 1.0 | 100 |
| His-Bind Resin | 1.35 | 4.53 | 3.36 | 3.2 | 29 |
| Superdex 75 | 8.00 × 10$^{-1}$ | 0.302 | 37.8 | 36 | 1.9 |

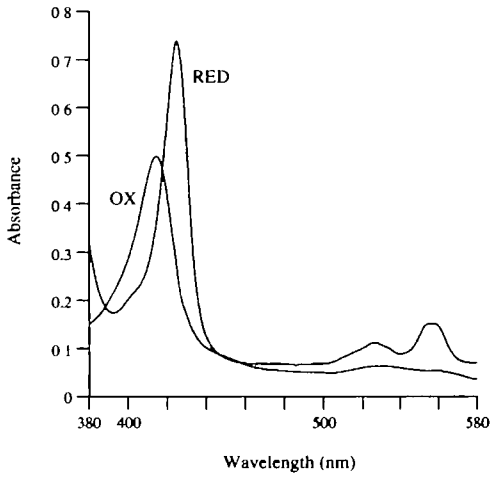


Fig. 6. **Absorbance spectra of the soluble form of cytochrome $b_5$.** Purified cytochrome $b_5$ was suspended in 100 $\mu$l of 0.1 M potassium phosphate buffer (pH 7.2). The absorbance spectrum of the oxidized cytochrome $b_5$ was recorded (OX). A few crystals of sodium dithionite were added, and the spectrum of the resultant reduced cytochrome $b_5$ was recorded (RED).

tent revealed that cells expressing full-length cytochrome $b_5$ did not exhibit the absorption spectra peculiar to cytochrome $b_5$, whereas cells expressing the soluble form did. The content of cytochrome $b_5$ which exhibited that spectra was 13.5 $\mu$g/mg of the total cell protein. This spectrophotometric method gave values for cytochrome content considerably lower than those by densitometric scanning of SDS-PAGE separation of total cell proteins. This method, however, detects only the cytochrome $b_5$ holoprotein (*i.e.*, the cytochrome apoprotein with the heme group), whereas the densitometric method (although not strictly quantitative) measures total cytochrome apoprotein. The expression of the full-length cytochrome $b_5$ gene resulted in the formation of almost all apoproteins, whereas that of the C-terminal-lacking cytochrome $b_5$ gene produced the holoproteins, which accounted for 8% of the total cytochrome $b_5$ accumulated in the cells. The soluble form of cytochrome $b_5$ was accumulated in the cytosol of the *E. coli* transformants and could be released simply by sonication and subsequent purification procedures.

*Purification of the Soluble Form of Cytochrome* $b_5$—By the spectrophotometric assay, the soluble form of cytochrome $b_5$ was purified 36-fold with a yield of 1.9% from the supernatant of cell-free extracts of the transformant cells containing the C-terminal-lacking cytochrome $b_5$ gene (Table I). The cytochrome $b_5$ eluted from the Superdex 75 column was purified to homogeneity, as shown by SDS-PAGE (Fig. 5). Reduced and oxidized difference spectra of the purified protein showed absorbance maxima characteristic of cytochrome $b_5$ (Fig. 6).

## DISCUSSION

In previous studies (*22*), *M. alpina* 1S-4 was considered to be the best producer of arachidonic acid. A number of fatty acid-biosynthetic mutants derived from *M. alpina* 1S-4 are also able to accumulate several available PUFAs in its body through various biosynthetic pathways. PUFAs are potentially of great value, because they are converted to eicosanoids (prostaglandins, leukotrienes, thromboxane, and so on), which function as essential bioactive compounds in mammals. Cytochrome $b_5$, which is a component of the microsomal electron transport system, is closely related to fatty acid desaturation. Here, we report for the first time the cloning of the cytochrome $b_5$ gene from filamentous fungi.

To investigate whether cytochrome $b_5$ constantly is expressed in *M. alpina* 1S-4, Northern hybridization was performed with mycelia cultivated at 28°C on GY medium for either 1 or 3 days. The 1-day culture corresponds to the logarithmic growth phase of *M. alpina* 1S-4, and the 3-day culture to the stationary growth phase. As shown in Fig. 1, the cytochrome $b_5$ appears to be highly expressed at the beginning of cultivation. This is consistent with the beginning of the high PUFA accumulation in the early cultivation period in *M. alpina* 1S-4, and it suggests that the cytochrome $b_5$ plays an important role in PUFA synthesis in this fungus.

The predicted cytochrome $b_5$ from *M. alpina* 1S-4 is composed of 130 amino acids. This finding is similar to the data sequence of reported rat liver cytochrome $b_5$. The cDNA sequence demonstrated that a transcriptional initiation point exists 36 bp upstream of its initiation codon ATG. In general, the ACCATGG sequence containing an initiation codon is identified as an optimal sequence for initiation by eucaryotic ribosomes (*41*). As the cytochrome $b_5$ cDNA from *M. alpina* 1S-4 also has this ACCATGG sequence, mRNA for *M. alpina* 1S-4 cytochrome $b_5$ could be translated swiftly. We could not find the TA-rich 6-nucleotides sequence that is generally observed in a promoter of eucaryotes and therefore expect other kinds of promoter to be present. We found that GT nucleotides at 5′-terminus of each intron and AG nucleotides at 3′-terminus in the *Mortierella* cytochrome $b_5$ gene were common to those in eucaryotes. Moreover, the three introns of the *M. alpina* 1S-4 cytochrome $b_5$ were longer than those of other fungi such as the subgenus *Aspergillus*. This suggests that *M. alpina* 1S-4, which belongs to the Zygomycetes, is a higher species among fungi, since higher eucaryotes generally have longer introns. There were no genes involved in fatty acid desaturation around the cytochrome $b_5$ gene.

A corollary of the central dogma of molecular biology is that genetic information passes from DNA to RNA on a DNA template and is finally translated to a protein. Eukaryotic messenger RNA (mRNA) undergoes several co- and posttranscriptional modifications. The 5′ end of the molecule is generally capped, the 3′ end polyadenylated, and up to 98% of the internal sequences can be eliminated by splicing. In conflict with the central dogma, RNA editing (*e.g.*, specific insertion, deletion, or substitution of residues in RNA) to create an RNA with a sequence different from its own template, has been reported (*42-44*). Here, we reported either insertion of guanine or substitution of guanine for adenine in the *M. alpina* 1S-4 cDNA. This is the first mode of RNA editing. In addition, the same phenomenon was also found in another gene (a desaturase gene) of *M. alpina* 1S-4 (unpublished data). There may be further examples of such RNA editing in this fungus. These findings of the RNA editing are significant, and this information also could contribute to elucidation of the genetics of the fungi kingdom, which lags far behind that of the animal and plant kingdoms.

The putative amino acid sequence of cytochrome $b_5$ from *M. alpina* 1S-4 showed similarity to all five internal peptide sequences of cytochrome $b_5$ from *M. hygrophila* (*21*). The amino acid sequence of the *M. alpina* 1S-4 cytochrome $b_5$ was similar to those of K-3, K-4, and K-5 fragments, while the latter sequence had no identity with those of cytochrome $b_5$ from other organisms. These findings indicated that the amino acid sequence of cytochrome $b_5$ is conserved between *M. alpina* 1S-4 and *M. hygrophila* of the *Mortierella* subgenus.

The *M. alpina* 1S-4 cytochrome $b_5$ also shows sequence similarities with cytochrome $b_5$ from such organisms as rat (*5, 38*), chicken (*39*), and yeast (*13*). Two histidine residues, regarded as heme iron ligands, were conserved in all species, suggesting that the amino acid sequence of a hydrophilic heme-containing catalytic domain needs to be conserved in cytochrome $b_5$. We also found conserved amino acid residues in this catalytic domain between *M. alpina* 1S-4 and yeast cytochrome $b_5$. Four amino acid residues [*i.e.*, aspartic acid (aa 48), serine (aa 57), aspartic acid (aa 65), and glutamic acid (aa 66)] of the former sequence were coincident with those of the latter. We assume that these residues are unique and important in terms of the structure and function of cytochrome $b_5$ in these microorganisms.

The evolutionary relationship of cytochrome $b_5$s from *M. alpina* 1S-4, chicken, rat, house fly, tobacco, and yeast as determined by the UPGMA (Unweighted Pair-Group Method with Arithmetic Mean) procedure with the GENETYX-MAC 7.0 program (Software Development, Tokyo) is shown in Fig. 7. Although the microbial *Mortierella* and *Saccharomyces* cytochrome $b_5$s have sequence similarity to mammalian ones, the dendrogram revealed that they are evolutionarily remote from the mammalian ones. The plant-representative tobacco cytochrome $b_5$ exhibited highest identity to the yeast (*S. cerevisiae*) cytochrome $b_5$ in sequence similarity. The dendrogram also indicates that plant cytochrome $b_5$s are clustered with the microbial ones rather than the mammalian ones.

In general, cytochrome $b_5$ has two functional domains: a hydrophilic N-terminal domain of some 100 amino acid residues (which contain single heme group), and a hydrophobic C-terminal domain of about 30 amino acids (which anchor to the cytoplasmic face of the endoplasmic reticulum membrane) (*6*). A hydropathy plot of the rabbit liver cytochrome $b_5$ sequence (Fig. 4) demonstrates a single hydrophobic stretch of 23 amino acids at the C-terminus as the membrane anchor of the protein. The membrane-binding domain of the rat microsomal cytochrome $b_5$ has been proposed to be a conventional transmembrane $\alpha$-helix (*45–47*). However, it has also been proposed that the carboxyl and amino termini are on the cytoplasmic side due to a hairpin loop in the $\alpha$-helical hydrophobic segment (*48, 49*). The corollary is that the hydrophobic domain does not extend into the lumen of the endoplasmic reticulum. The hydropathy plot of the *M. alpina* cytochrome $b_5$ sequence showed some resemblance to that of the rat cytochrome $b_5$ sequence. These findings suggest that the *M. alpina* 1S-4 cytochrome $b_5$ is also a hemoprotein that anchors to the cytoplasmic faces of endoplasmic reticulum membranes.

We performed high-level expression and efficient purification of the soluble form of fungal cytochrome $b_5$ in *E. coli* using a T7 expression system. The high-level expressions of plant and mammalian cytochrome $b_5$ in *E. coli* by use of a T7 expression system have been reported (*50, 51*). The use of the pET-16b expression system for the production of the recombinant cytochrome $b_5$ not only has the disadvantage that the protein is produced as a fusion protein, but it resulted in a simple procedure to purify the target protein by a His-Tag resin column chromatography. The soluble form of cytochrome $b_5$ exhibits absorbance spectra identical with those of cytochrome $b_5$ from fungal microsomal preparations (*21*). Whether the purified cytochrome $b_5$ is reduced by NADH and NADH-cytochrome $b_5$ reductase in the microsomal membrane remains to be determined. In constrast to the soluble forms of cytochrome $b_5$ highly expressed in *E. coli*, the recombinant full-length form of cytochrome $b_5$ was expressed at low level in *E. coli* and was also produced as inclusion bodies. We assume that the lower expression level of the full-length form in contrast with the high expression of the soluble form, was due to the involvement of approximately 30 residues at the C-terminus in which the hydrophobic region hirders solubilization.

SDS-PAGE analysis indicated that the soluble and full-length forms of cytochrome $b_5$ had molecular masses of 18 and 22 kDa, respectively. These values, however, did not agree with the molecular masses (12,783 and 16,487 Da, respectively) deduced from the nucleotide sequence of *M. alpina* 1S-4 cytochrome $b_5$. These differences may be due to unique characteristics of the cytochrome $b_5$, such as electric charged values.

Both full-length and soluble forms of the recombinant cytochrome $b_5$ accumulated predominantly as the apo-form protein lacking the heme prosthetic group. This finding is in agreement with the previous studies on the expression of animal cytochrome $b_5$ in *E. coli*, where the rate-limiting step in cytochrome $b_5$ holoprotein assembly would be at an early stage in the *de novo* synthesis of heme (*52*). Even when the transformed cells were cultured in medium containing $Fe_2(SO_4)_3$ or bovine hemin chloride (Sigma, USA) to be 20 $\mu$g/ml of final concentration, no increase was

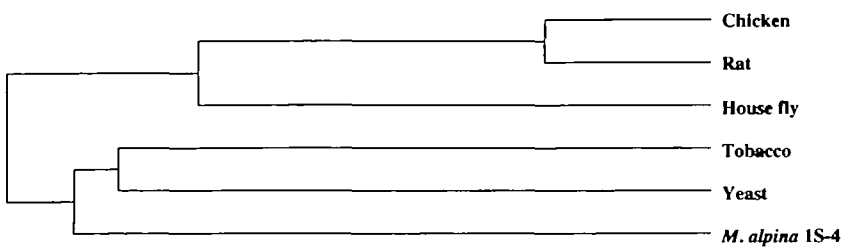


Fig. 7. **Phylogenetic tree for cytochrome $b_5$s.** This tree was constructed by the UPGMA (Unweighted Pair-Group Method with Arithmetic Mean) procedure with the GENETYX-MAC 7.0 program (Software Development). The accession numbers for each sequence are given in parentheses after each name as follows: chicken (SwissProt P00174); rat (SwissProt P00173); house fly (SwissProt P49096); tobacco (SwissProt P49098); yeast, *S. cerevisiae* (SwissProt P40312); *M. alpina* 1S-4 (DDBJ AB022443).

observed in either the cytochrome $b_5$ production or the proportion of holo-form cytochrome $b_5$ (data not shown). This observation may indicate that *E. coli* could not incorporate these factors or that factors incorporated as heme prosthetic groups were not appropriate for the production of the holo-form cytochrome $b_5$. In addition, the cultivation of cells at 28°C resulted in a significant increase of total cell mass but no increase of cytochrome $b_5$ production (data not shown). We found no effect of lower temperature on the production of the recombinant cytochrome $b_5$.

Both full-length and soluble forms of cytochrome $b_5$ were present in their reduced states in the cells, reflecting the highly reduced intracellular environment of *E. coli* (*53*). Lysis of the transformants resulted in the rapid oxidation of the cytochrome and the disappearance of the reduced form. We need to investigate further the subcellular localization of each recombinant cytochrome $b_5$ in *E. coli*.

*M. alpina* 1S-4 is a "practical fascinating microorganism" (*22*). However, genetic information available on *Mortierella* is extremly limited. Only two enzymes from this genus have been investigated at the gene level: $\alpha$-galactosidase (*54, 55*) and desaturase (*56-58*). Studies on the genetics of *Mortierella* could contributle greatly toward the production of various useful PUFAs. Studies on the fungal cytochrome $b_5$ could also provide information about the role of the hemoprotein in fungal fatty acid desaturation reactions and electron-transport processes.

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