A Consensus Sequence for Long-chain Fatty-acid Alcohol Oxidases from *Candida* Identifies a Family of Genes Involved in Lipid ω-Oxidation in Yeast with Homologues in Plants and Bacteria*

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The yeast *Candida cladoaca* is capable of growing on alkanes and fatty acids as sole carbon sources. Transfer of cultures from a glucose medium to one containing oleic acid induced seven proteins of *M*$_r$ 102,000, 73,000, 61,000, 54,000, and 46,000 and two in the region of *M*$_r$ 45,000 and repressed a protein of *M*$_r$ 64,000. The induction of the *M*$_r$ 73,000 protein reached a 7-fold maximum 24 h after induction. The protein was confirmed by its enzymatic activity to be a long-chain fatty-acid alcohol oxidase (LC-FAO) and purified to homogeneity from microsomes by a rapid procedure involving hydrophobic chromatography. An internal peptide of 30 amino acids was sequenced. A 1100-base pair cDNA fragment containing the LC-FAO peptide coding sequence was used to isolate a single exon genomic clone containing the full-length coding sequence of an LC-FAO (*fao1*). The *fao1* gene product was expressed in *Escherichia coli* and was translated as a functional long-chain alcohol oxidase, which was present in the membrane fraction. In addition, full-length coding sequences for a *Candida tropicalis* LC-FAO (*faoT*) and a second *C. cladoaca* LC-FAO (*fao2*) were isolated. The DNA sequences obtained had open reading frames of 2094 (*fao1*), 2091 (*fao2*), and 2112 (*faoT*) base pairs. The derived amino acid sequences of *fao2* and *faoT* showed 89.4 and 76.2% similarities to *fao1*. The *fao1* gene is much more highly induced on alkane than is *fao2*. Although this study describes the first known DNA sequences encoding LC-FAOs from any source, there are unassigned Arabidopsis sequences and an unassigned Mycobacterium sequence in the GenBank™ Data Bank that show strong homology to the described LC-FAO sequences. The conservation of sequence between yeast, plants, and bacteria suggests that an as yet undescribed family of long-chain fatty-acid oxidases exists in both euukaryotes and prokaryotes.

*Candida cladoaca* and *Candida tropicalis* are industrial yeast species capable of utilizing both alkanes and long-chain fatty acids as sole carbon sources for growth. These water-immiscible substrates are metabolized to carbon dioxide by two sequential oxidative pathways: (a) the membrane-bound ω-oxidation pathway and (b) the β-oxidation pathway located in peroxisomes. During ω-oxidation, the methyl end of the molecule is oxidized successively by a cytochrome P450 alkane/fatty-acid oxidase, a hydrogen peroxide-generating alcohol oxidase, and an aldehyde dehydrogenase, producing ω-alcohols, ω-aldehydes, and ω-fatty acids, respectively. Growth on both alkanes and fatty acids results in dicarboxylic acid formation as a metabolic intermediate. Such long-chain dicarboxylic acids are very versatile raw materials for the oleochemical industry and are used in the production of fragrances, polyamides, polyesters, adhesives, and macrolide antibiotics (1). The dicarboxylic acid products of ω-oxidation are further oxidized in the peroxisome by the β-oxidation pathway following activation to acyl-CoAs.

Two different biological modes of alcohol dehydrogenation have been identified. The first is a nicotinamide-dependent reaction catalyzed by alcohol dehydrogenases, and the second is a flavin-dependent reaction catalyzed by alcohol oxidase. The latter uses molecular oxygen as acceptor and generates hydrogen peroxide (2, 3). Flavin-dependent alcohol oxidases have been isolated from a number of different fungal sources (4–9). In all organisms studied to date, the enzyme is octameric, with the exception of *C. tropicalis*, where it is dimeric (7). The substrate specificity of the enzyme differs considerably depending on the source of the enzyme. In *C. tropicalis*, the enzyme is most active on long-chain fatty alcohols (7), whereas secondary alcohols such as dodecan-2-ol and long-chain ω-hydroxy fatty acids are also good substrates of the enzyme (7). Alcohol oxidases from *Kloeckera sp.*, *Hansena polymorpha*, and *Candida boidinii* are very active toward methanol and ethanol, but are inactive toward substrates of chain length longer than C$_5$ and will not oxidize secondary alcohols or ω-hydroxy fatty acids (4, 6). Substrate specificity has also been investigated for the alcohol oxidase from the filamentous fungus *Aspergillus flavipes* grown on hexadecanol. Although this enzyme will oxidize long-chain alcohols, it will not utilize ω,ω-diols, ω-hydroxy fatty acids, or secondary alcohols (9). The enzymes from *Candida maltosa* (10), *C. tropicalis* (7), and *C. cladoaca* (11) all oxidize ω-hydroxy fatty acids, whereas those from other organisms studied lack the ability to use these substrates. Long-chain alcohol oxidase has been purified from *C. tropicalis* (7), but to date, there is no gene or amino acid sequence available.

Oxidation of long-chain alcohols by alcohol oxidase has been reported in germinating seedlings of the plant *Simmondsia chinesis* (jojoba) (12), which accumulates long-chain fatty alcohol-containing waxes as its main storage product. The utilization of

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AJ242496, AJ242497, and AJ242498.

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The procedure developed involved microsomal preparation, solubilization, and \( (NH_4)_2SO_4 \) precipitation followed by chromatography on phenyl-Superose. All procedures were performed at 4 °C unless otherwise specified. Cells (132 g, wet weight) were passed through a French press cell three times at 20,000 p.s.i.; the disrupted cell extract was centrifuged at 20,000 \( \times g \) for 30 min; and the precipitate was discarded. At this and other stages, the preparation was snap-frozen in liquid \( N_2 \) and stored at − 80 °C prior to further processing. The supernatant was thawed, and 100 mM Tris-HCl (pH 7.5) was added to give a final volume of 230 ml. The microsomal fraction was pelleted by ultracentrifugation at 140,000 \( \times g \) for 1.5 h. The pellet was washed by suspending in 100 ml HEPES/NaOH (pH 8.0) containing 0.15 M KCl (final volume of 115 ml) and pelleted at 140,000 \( \times g \) for 1.5 h. The washed microsomes were resuspended in 50 mM HEPES/NaOH (pH 8.0) (final volume of 62 ml). The resuspended pellet was made up to 500 ml with 50 mM HEPES/NaOH (pH 8.0). Sodium cholate was added to 1.0%, and phenylmethylsulfonyl fluoride (in isopropyl alcohol) was added to 1 mM. \( (NH_4)_2SO_4 \) was added to 35% (w/v); the solution was stirred for 20 min and reverse primed (5'-CAC GCA GAT ATG GTG C-3' \( \times 8 \) and 5'-CAC GCA GAT ATG GTG C-3' \( \times 5 \) to the supernatant; the solution was stirred for 20 min and centrifuged at 20,000 \( \times g \) for 5 min. The resulting pellet was resuspended in 50 mM HEPES/NaOH (pH 8.0) containing 1% sodium cholate to a final volume of 52 ml, dialyzed 2 \( \times 1 \) h against two changes of 2 liters of 50 mM HEPES/NaOH (pH 8.0) and a further 500 ml of 50 mM HEPES/NaOH (pH 8.0) containing 1% CHAPS for 1 h, and centrifuged at 20,000 \( \times g \) for 5 min. The supernatant was resuspended, dialyzed, and diluted to 15 ml with the column equilibration buffer; filtered through a 0.2-μm filter; and loaded onto a phenyl-Superose HR 5/5 column (Amersham Pharmacia Biotech) equilibrated in 10 mM Tris-HCl (pH 8.5) containing 1.7 mM \( (NH_4)_2SO_4 \) and 0.5% CHAPS. After washing the column with 15 ml of buffer, a 10-ml linear gradient was run from 1.7 to 0.85 mM \( (NH_4)_2SO_4 \) followed by a 15-ml linear gradient from 0.85 to 0.5 mM \( (NH_4)_2SO_4 \). A flow rate of 0.6 ml/min was used throughout.

**PCR Isolation of a Probe for fao**

To produce a probe for the cDNA library screen, 1 μl (1.5 \( \times 10^3 \) plaque-forming units) of the *C. cloacae* oleic acid-induced cDNA (non-amplified) library was placed into a reaction mixture containing 10 pmol of M13 forward primer (5'-TTG TAA AAC GAC GGC CAG T-3') or M13 reverse primer (5'-CAC GCA GAT ATG GTG C-3') and 20 pmol of internal degenerate FAO primer (5'-ACN AAY CAR CAT CNT TTY ATG ATH GC-3', where N = AC/GT, Y = CT, R = AG, and H = ACT; corresponding to the amino acid sequence TNQQFLMIA). 2 units of *Taq* polymerase, 1.5 mM MgCl₂-containing PCR buffer, and 1.25 mM dNTPs (Roche Molecular Biochemicals). Amplification was performed under the following conditions for 35 cycles: denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, and extension at 72 °C for 30 s. Amplified products were run on 1% agarose with 0.5% agarose; DAS, dense alignment surface.

**Construction of DNA Libraries**

cDNA Libraries—Poly(A)^+^ mRNAs from 24-h oleic acid-induced *C. cloacae* and *C. tropicalis* were used for the construction of a random-primed non-directional cDNA library in EcoRI-digested alkaline phosphatase-treated Azap II vector (Stratagene). Approximately 5 μg of mRNA was used in the reverse transcriptase reaction (TimeSaver cDNA synthesis kit, Amersham Pharmacia Biotech). EcoRI/NorI adapters were added to the ends, and cDNA was ligated with EcoRI-digested Azap II. Gigapack II Gold packaging extract (Stratagene) was used to form phage particles, which were then used to transfect E. coli XLI-Blue cells, and cDNA libraries were screened.

**Genomic Libraries**—Genomic *C. cloacae* and *C. tropicalis* DNA were isolated from cells grown on 10% yeast extract, 20 g/liter peptone, and 20 g/liter glucose according to Philippens et al. (20). A Sau3A partial digest of *C. cloacae* DNA was size-fractionated to 14–23 kb on a 10–40% sucrose gradient and ligated into ABluestar BamHI arms (Novagene). Gigapack II Gold packaging particles were used to transfect E. coli ER1647 (Novagen). A Sau3A partial digest of *C. tropicalis* DNA was size-fractionated on a 10–40% sucrose gradient to 10–12 kb and ligated with kb, kilobase(s); bp, base pair(s); PAGE, polyacrylamide gel electrophoresis; DAS, dense alignment surface.

**Purification of Long-chain Fatty-acid Alcohol Oxidase from *C. cloacae***

*The abbreviations used are: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid, RT-PCR, reverse transcription-polymerase chain reaction; LC-FAO, long-chain fatty-acid alcohol oxidase;***
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into ZapExpress BamHI arms (Stratagene). PhageMaker packaging extract (Novagen) was used to form phage particles, which were then used to transfect XL1-Blue MRF” cells (Stratagene).

Hybridization Screening of DNA Libraries
cDNA Libraries—For the C. clacae cDNA screen, prehybridization was carried out at 65 °C for 2 h in 6× SSC, 1× Denhardt’s solution, 0.5% SDS, 0.05% sodium pyrophosphate, and 0.05 mg/ml herring sperm DNA, and hybridization was carried out at 65 °C overnight in essentially the same buffer but without herring sperm DNA and with 1 mM EDTA. Filters were washed at 65 °C with 2× SSC and 0.1% SDS (2× 15 min) and with 0.2× SSC and 0.1% SDS (2× 15 min). For the C. tropicalis screen, prehybridization was carried out at 55 °C for 2 h, and hybridization was carried out at 55 °C overnight in the same buffers as listed above. Filters were washed 2× 30 min at 55 °C with 2× SSC and 0.1% SDS.

Genomic Libraries—For the C. clacae screen, prehybridization was carried out for 2 h at 55 °C in 6× SSC, 5× Denhardt’s solution, 0.5% SDS, 100 µg/ml herring sperm DNA, and 50% formamide, and hybridization was carried out with the probe overnight at 55 °C in the same hybridization mixture. Filters were washed 3× 15 min in 2× SSC and 0.1% SDS at 55 °C. For the C. tropicalis screen, prehybridization was carried out for 2 h at 42 °C, and hybridization was carried out overnight at 42 °C in the same hybridization mixture as described above. Filters were washed 2× 15 min in 2× SSC and 0.1% SDS and 2× 15 min in 1× SSC and 0.1% SDS at 42 °C.

Northern Analysis
C. clacae cells were grown for 24 h in minimal medium containing either 2% glucose or 1% oleic acid as a carbon source. The cells were harvested and ground in a mortar and pestle under liquid N2, and RNA was extracted using hot SDS (21). mRNA was prepared using oligo(dT) spin columns (Pharmacia mRNA Purification kit). Northern analysis was conducted using 2 µg of mRNAs on agarose gels containing 0.66% agarose, blotting onto Hybond-N+ membrane (Amersham Pharmacia Biotech), and hybridizing according to standard methods (22) with a Rediprime random primer-labeled probe (Amersham Pharmacia Biotech).

RT-PCR Quantification of fao1 and fao2 Gene Expression
RNA samples were prepared from cells grown under identical conditions described for Northern analyses, except that samples were grown on hexadecane. They were treated with RNase-free DNAse I (Life Technologies, Inc.) at room temperature for 15 min to remove possible DNA contamination. DNase I was subsequently inactivated at 65 °C for 10 min in the same buffer. Filters were washed 2× 5 min in 2.5 mM EDTA. The resulting RNA was used as a template for RT-PCR using the CLONTECH RT-PCR kit following the manufacturer’s instructions. The oligonucleotides 5’-atgcctcatgaagtcgac-3’ and 5’-atgagctccccgcttgagc-3’ are specific to fao1 and fao2 5’-end coding sequences, respectively; the oligonucleotides 5’-cttaagtttttttttttttttttttttttttttttttttttttttttttggc-3’ are specific to fao1 and fao2 3’-end coding sequences, respectively. PCRs were performed for 30 cycles (30 s at 94 °C, 30 s at 55 °C, and 150 s at 72 °C) and were performed on a Robocycler (Stratagene). Initial experiments demonstrated that up to 30 cycles of PCR product formation was linear.

Overexpression of C. clacae Long-chain Fatty-acid Alcohol Oxidase FAO1 in E. coli
Oligonucleotide primers were designed to PCR-amplify the full-length fao1 coding sequence: 5’-CTAGCTGATCTCCATCAAGGT-GAAGACC-3’ and 5’-CCGGATCCCTAAAGTTTAGTTGTTGTTCTCAAGTC-3’. The purified PCR product was digested with NaeI and BamHI and subsequently cloned into the overexpression vector pET17b (Novagen); the resulting plasmid was designated pET17b-fao1. The sequence of the fao1 coding region was confirmed by DNA sequencing. The expression construct was transformed into E. coli BL21(DE3) cells according to the Stratagene protocol, and the cells were grown on an LB/ampicillin plate overnight. One colony was transferred into 5 ml of LB medium containing ampicillin (100 µg/ml), and the culture was grown overnight at 37 °C with shaking at 200 rpm. The cells were harvested by centrifugation (5000 rpm, 10 min) and resuspended in 250 µl of buffer containing 20 mM Tris-HCl (pH 8.0) and 2 mM EDTA. For enzyme assay, microsomal fractions were prepared from 500 ml of overnight culture grown on LB medium. The pellet was finally washed and resuspended in 1 ml of buffer containing 20 mM Tris-HCl (pH 8.0) and 2 mM EDTA.

Miscellaneous Methods
SDS-Polyacrylamide Electrophoresis—SDS-polyacrylamide gels consisted of a 5% stacking gel with a 10% running gel and were run on a Bio-Rad Mini-Protein gel kit. The buffers used were as described by Laemmli (16).

Native Gel Electrophoresis and Biological Activity Staining—Native PAGE was carried out at 200 V for 2.5 h on a 10% resolving and 5% stacking gel described above for SDS-PAGE, except that the SDS was replaced by 1 and 0.5% sodium cholate in the gel and running buffers, respectively. The sample buffer consisted of 10 mM Tris-HCl (pH 6.8), 2% glycerol, 1% sodium cholate, and sufficient bromphenol blue to make it visible. The running buffer and apparatus were chilled on ice before and during the run. The sample was 50 µl of 0.2 units of hydroxylapitate-purified C. clacae material prepared by the method of Dickinson and Wadforth (7). To stain the gel for enzyme activity, buffers ABTS and peroxidase at 10 times the concentration used in the standard LC-FAO assay and dodecanol at 2.7 mM were applied to the surface of the gel. This was incubated at room temperature for 5 min. Alcohol oxidase activity was identified as a region of green stain on the surface of the gel. The region containing the biological activity was cut out from the native gel and then subjected to SDS-PAGE.

Enzyme Assays—LC-FAO was assayed spectrophotometrically (3). The assay mixture contained 50 mM Tris-HCl (pH 8.5) 0.7 mg/ml ABTS, 7 units of horseradish peroxidase, and 50 µM dodecanol previously dissolved in Me3SO, in a final volume of 1.0 ml unless otherwise specified. Reactions were initiated by addition of enzyme, and the increase in absorbance at 405 nm was measured. The value of e for the radical cation of ABTS is 18.4 M-1 cm-1, and 1 mol of substrate gives rise to 2 mol of radical cation (17). One unit of enzyme activity catalyzes the conversion of 1 nmol of substrate to product/min.

Protein Concentration—This was determined using the dye binding method of Bradford (18) with bovine serum albumin as a standard.

Generation and Sequencing of Long-chain Fatty-acid Alcohol Oxidase Peptides—This was performed using the Promega procedure with Chromomaph green to detect the protein in the first gel and Endoproteinase Glu-C for digestion (19). For N-terminal amino acid sequence determination, proteins were transferred onto Problot membrane (Applied Biosystems, Inc.) and visualized by Coomassie Blue staining as directed in the Problot manual. Amino acid sequencing was performed with an ABI Model 477 sequencer.

DNA Sequencing—Sequencing was carried out using an Applied Biosystems Model 373 DNA sequencer. Computer analysis of DNA sequences was carried out using DNA Strider (23) and Fasta alignments.

Chemicals—All biochemical reagents were obtained from Sigma and were highest purity available. Reagents for electrophoresis were from Bio-Rad.

RESULTS
Alterations in the Protein Profile upon Oleic Acid Induction—Both LC-FAO enzyme activity and SDS-PAGE protein profiles were analyzed in cell extracts of C. clacae following transfer from a sucrose-containing growth medium to one containing oleic acid as the sole carbon source. The induction of LC-FAO enzyme activity increased 4-fold in 6 h and reached a maximum of 7-fold at 24 h; similar results were obtained using the alkane hexadecane as an inducer (data not shown).

C. clacae cell extracts from cultures grown for 0, 6, and 125 h after addition of oleic acid were run on SDS-polyacrylamide gel and stained for protein with Coomassie Blue. Growth on oleic acid resulted in the induction of proteins of Mr 102,000, 73,000, 61,000, 54,000, and 46,000 and two in the region of Mr 45,000 and the disappearance of a protein of Mr 64,000 (Fig. 1).

Purification of Long-chain Fatty-acid Alcohol Oxidase from C. clacae—We initially adopted a procedure similar to that used for the purification of LC-FAO from C. tropicalis (7). The C. tropicalis procedure did not, however, result in a homogeneous preparation with C. clacae extracts. Accordingly, we developed a procedure with hydrophobic chromatography on phenyl-Superose as a key step in the purification, taking advantage of the hydrophobic nature of the protein. It was essential to prepare the microsomes from alkane-induced cells as oleic acid-induced cells accumulated fatty acids, which interfere with membrane pelleting.
during the ultracentrifugation step. By directly applying the resuspended (NH₄)₂SO₄ precipitate to a phenyl-Superose column and eluting it with a reverse gradient of (NH₄)₂SO₄, it was possible to obtain a homogeneous preparation. The fractions with highest activity eluted at 0.7 M (NH₄)₂SO₄ (Fig. 2A). Analysis of alcohol oxidase activity and protein profiles by SDS-PAGE demonstrated that the activity coincided with the elution of the Mᵣ 73,000 protein (Fig. 2B). The enzyme was purified 230-fold with a 10.7% recovery of biological activity (Table I). Further evidence that LC-FAO is an Mᵣ 73,000 protein was obtained from native gels. The enzyme activity was visualized directly on the gel as a green band when the gel was incubated with enzyme assay reagents. This band was excised and subjected to SDS-PAGE, and a major band at Mᵣ 73,000 was seen, confirming assignment of this band as LC-FAO. The molecular weight of the alcohol oxidase from C. cloacae is the same as that reported for the LC-FAO enzyme from C. tropicalis (7). The pH optimum of the enzyme from C. cloacae was between 8.5 and 9.0, and the apparent Kₘ for dodecanol was between 4.0 and 5.0 μM (data not shown).

**Amino Acid Sequence of Long-chain Fatty-acid Alcohol Oxidase**—N-terminal amino acid sequencing was performed three times on purified LC-FAO following transfer to Problot. No amino acid sequence data were obtained, indicating that the alcohol oxidase from C. cloacae had a blocked N terminus. When protein bound to a polyvinylidene difluoride membrane was treated with CNBr to cleave methionine residues, amino acid sequence data were obtained at the 50–100-pmol level, indicating that protein was present on the sequencing disc at approximately the expected levels given the typical recoveries for these processes. In-gel digestion of the Mᵣ 73,000 protein with Glu-C resulted in three major peptides, which were separated electrophoretically and blotted onto Problot. One of these gave an unambiguous sequence of 30 amino acids (Table II), which was suitable for degenerate primer design.

**Isolation of LC-FAO-encoding cDNAs from C. cloacae**—The cDNA library and a degenerate primer based on the internal amino acid sequence SGGTPSTNQQLFMIAGSTFGGGSTVNW from C. cloacae LC-FAO were used in combination with library vector-based M13 forward and T3 primers to create a probe for screening the cDNA library. PCR yielded products of 1100, 800, 600, 550, and 300 bp, which were directly subcloned into pGEM®-T vector. Sequencing of these clones showed that they all carried the same translated LC-FAO-derived partial amino acid sequence, TNQQLFMIAGSTFGGGSTVNW (data not shown). Screening of the C. cloacae cDNA library with the insert from the subcloned 1100-bp PCR product (pAX17) resulted in four independent clones containing inserts of 2.4, 1.0, 0.6, and 0.6 kb. It was clear from sequencing analysis that the four clones represented two classes of coding sequence. One cDNA of 600 bp had an internal translated amino acid sequence identical to the sequenced peptide and was designated as fao1 class. Three cDNAs with 2400-, 1000-, and 600-bp inserts with identical overlapping sequences were grouped as fao2 class. The fao2 cDNAs encoded a 2-amino acid variant of the amino acid sequence obtained by direct protein sequencing. The other factor determining fao classification was the polymorphism in restriction endonuclease target sites. The fao1 class of LC-FAO carried an XbaI site not present in fao2; additionally, fao2 carried a HindIII site not present in fao1.

**Northern Analysis**—To verify the full-length size of LC-FAO-encoding cDNA, the mRNA from oleic acid-induced C. cloacae was probed in a Northern analysis with the insert from clone Fig. 1. SDS-PAGE analysis of protein profiles of C. cloacae cells prior to and after induction by oleic acid. Samples are at 0, 6, and 125 h after induction. Induced proteins are shown by arrows. Arrow a depicts a protein of Mᵣ 64,000, which is reduced in quantity upon induction.

Fig. 2. A, phenyl-Superose chromatography of partially purified alcohol oxidase-containing extract. Biological activity was detected only in fractions 1–6. B, SDS-PAGE analysis of fractions 3–6 from phenyl-Superose chromatography of alcohol oxidase. Lanes 1–4 correspond to fractions 3–6, respectively, from A. Molecular mass markers are indicated on the left. The arrow corresponds to a protein of Mᵣ 73,000.
pAX17 (representative of the fao1 class gene). A single dominant mRNA species of 2.4 kb hybridized to the probe. The sequence similarity between fao1 and fao2 meant that the probe hybridized with both species of mRNA, confirming that the fao2 2400-bp fragment was full-length. A classical 5’-ATG start codon and 3’-poly(A) tail also supported this assumption. The size of the hybridizing species of mRNA also predicts an Mr of ~75,000 for the LC-FAO protein. This confirmed the estimate of Mr, 73,000 for the LC-FAO protein by SDS-PAGE electrophoresis and the in-gel enzyme assay described earlier. As in the case of LC-FAO, fao mRNA was induced five to seven times on transfer of the organism to an oleic acid medium (Fig. 3A). It should be borne in mind that this probe will hybridize to fao1 and fao2 as described below.

Isolation of the fao1 Class Full-length Coding Sequence—The only representative of the fao1 class was not full-length, and we required the promoter sequence driving the oleic acid induction for further studies. To obtain the full-length coding sequence, a genomic library was screened with the fao1 600-bp fragment of cDNA already isolated. Twenty hybridizing clones were purified, and after PCR isolation of the complementary 600-bp fragment, XbaI/HindIII restriction enzyme analysis was carried out to compare them with fao1 class genes. Plasmids were rescued from λ clones, and plasmid pgfAO14, which carried an insert of 18 kb containing the full-length fao1 class gene, was further characterized. Sequencing of the 4.3-kb region of the genomic clone containing the LC-FAO gene identified a single exon with an open reading frame (2094 bp) in the same order as that predicted by Northern analysis (2.4 kb). The 3’-end of the fao1 gene derived from genomic DNA contained a typical eukaryotic polyadenylation signal, AAATAAA (24), as well as a Saccharomyces cerevisiae consensus sequence for transcription termination, TAG . . . TA/T/AGT . . . TTT (25). The derived protein of Mr, 77,300 of both fao1 and fao2 sequences (698 and 697 amino acids, respectively) is also consistent with LC-FAO protein analysis by SDS-PAGE.

The deduced amino acid sequence of fao2 contained a carboxyl-terminal peroxisome-targeting sequence, SKL (26). However, the corresponding fao1 carboxyl-terminal sequence was TKL. It is possible that in fao1 the corresponding sequence may not function in targeting the protein into peroxisomes, and thus, the two gene products would be located in different cell compartments. Both fao1 and fao2 contain the consensus sequence Cys-X-X-Cys-His for a cytochrome c family heme-binding signature (27). The fao1 and fao2 sequences showed 80.0% sequence identity at the nucleotide level, and the deduced amino acid similarity was 89.4%. The FASTA alignment of the deduced fao1 and fao2 amino acid sequences is shown in Fig. 4.

RT-PCR Quantification of fao1 and fao2 Gene Expression—Using gene-specific primers for fao1 and fao2, we used RT-PCR to investigate the expression of both the fao1 and fao2 genes following alkane induction (Fig. 3B). fao1 gene expression could be detected in the alkane-free medium, whereas fao2 could not. Following induction, fao1 was elevated ~6-fold, and fao2 could be detected, but was the minor species. From a mRNA abundance perspective, fao1 would seem to be the dominant form of alcohol oxidase in C. cloacae and is 10 times more highly expressed than fao2 following alkane induction.

Overexpression of C. cloacae Long-chain Fatty-acid Alcohol Oxidase FAO1 in E. coli—The fao1 gene product was expressed in E. coli using the pET17b expression system. Both membrane
Fig. 4. Comparison of deduced amino acid sequences of fatty-acid alcohol oxidases from *Candida* species (*fao1* and *fao2* of *C. clacae* and *faoT* of *C. tropicalis*) and unassigned *Arabidopsis* sequences. Amino acid sequence data obtained by direct sequencing of the *C. clacae* alcohol oxidase peptide are shown above *fao1* at amino acid 250 (*Peptide Seq*), and the amino acid data used to design the gene-specific degenerate PCR primer are flanked by brackets. The PCR product obtained using this primer is underlined in the *fao1* sequence. The identical amino acids in all three *Candida* alcohol oxidase sequences are indicated by asterisks. The closed circles indicate identical amino acids in the *Candida* sequence and the unassigned *Arabidopsis* sequence (accession number AL022580) with its intron removed. The open circles indicate identical amino acids in the *Candida* sequence and the unassigned genomic fragment from *M. tuberculosis* (accession number Z77162). The sequences run consecutively from A–D.
fractions and soluble proteins were analyzed by SDS-PAGE. Membranes isolated from the fao1-containing vector showed an elevated level of a protein of $M_r \approx 73,000$, consistent with expression of the fao1 gene (Fig. 5). Membranes were prepared from BL21 cells transformed with either the empty vector or one containing the fao1 gene and assayed for alcohol oxidase activity. Using 144 $\mu$g of membrane protein/incubation, the pET17b-fao1 membranes had 2000 units of enzyme activity with juniperic acid ($\omega$-hydroxy-C16:0) and 10,000 units with 1-dodecanol. No activity was detected with membranes isolated from BL21 cells transformed with the empty vector sequence even after a 2-day incubation. In contrast, the reaction with membranes from BL21 cells transformed with the vector sequence virtually went to completion within 5 min. The specific activity was five times higher with 1-dodecanol (0.069) than with juniperic acid (0.013). This difference in specific activity between the two substrates is very similar to that reported for the purified enzyme from C. tropicalis (7). The fao2 gene would not express in E. coli, and the reason for this is unknown.

Cloning and Sequencing of Fatty-acid Alcohol Oxidase from C. tropicalis—Much of our current knowledge about yeast species that are able to utilize alkanes and long-chain fatty acids comes from C. tropicalis. To expand and complement this knowledge, we isolated fao1-like clones from a C. tropicalis cDNA library using the C. cloaece fao1 fragment from pgFAO14 as a probe. One of the cDNA clones isolated contained an open reading frame of 617 amino acids. Comparison with the corresponding derived 679-residue-long C. cloaece fao1 gene sequence suggested that the 3'-end of this cDNA clone was missing. To obtain the rest of the sequence, we generated a product of $\approx 750$ bp with identical overlapping sequence from the C. tropicalis genomic library by PCR. This fragment contained the 3'-end of the C. tropicalis fao1T gene. The combined faoT sequence of 4233 bp has an open reading frame of 2112 bp, which corresponds to 704 amino acid residues (Fig. 4). faoT shares 60.6 and 61.7% nucleotide identities and 74.8 and 76.2% amino acid sequence similarities with C. cloaece fao1 and fao2, respectively (Fig. 4).

Identification of Unassigned Sequences Homologous to Candida fao Genes—Alignment comparison of the three Candida LC-FAOs revealed large regions of homologous domains throughout the full length of the derived sequences (Fig. 4). Further analysis of the GenBank™ Data Bank with the FASTA-generated Candida fao consensus sequence showed that there were two high-scoring unassigned Arabidopsis sequences (accession numbers AB015474 and AL022580) and an unassigned genomic fragment from Mycobacterium tuberculo-

FIG. 5. Analysis of expression of fao1 in membranes isolated from E. coli BL21(DE3) cells using 10% SDS-PAGE and Coomassie Blue staining. Lane A, E. coli cells transformed with pET17b-fao1; lane B, E. coli cells transformed with pET17b empty vector; lane C, molecular mass markers expressed in kilodaltons (KD). The position of the protein corresponding to FAO1 is indicated by the arrow.

FIG. 6. Transmembrane domain prediction by the DAS method (27). A–C show the DAS analysis results for FAO1, FAO2, and FAOT, respectively. The $x$ axis indicates the DAS score, and the $y$ axis indicates the amino acid locality within the derived sequences. The hit at a DAS score of 2.2 (indicated by the solid lines) is informative in terms of the number of matching segments, whereas the hit at a DAS score of 1.7 (indicated by the dashed lines) gives the actual location of the transmembrane segment.
ing a transmembrane α-helix prediction model, of the translated gene sequences (28) showed that both C. cloacae fao genes contained three and C. tropicalis faoT contained four putative transmembrane-spanning regions within the first 200 amino acids. In addition, all of them have a putative transmembrane-spanning region in the carboxyl-terminal region around amino acids –550–600 (Fig. 6).

DISCUSSION

Transfer of C. cloacae from a sucrose-containing medium to one containing alkane or fatty acid as the carbon source results in a distinct alteration in the protein profile. Seven new proteins were present in the lipid-containing medium, and one protein disappeared. When C. tropicalis and C. cloacae cells are grown on lipid-containing medium, there is a strong induction of both the ω- and β-oxidation pathways together with peroxisomal proliferation. Proteins that are likely to be up-regulated are (a) those involved in import of the substrate into the cells such as alkane/fatty acid transporters; (b) ω-oxidation pathway enzymes including cytochrome P450 reductase, alkane cytochrome P450, alcohol oxidase, and aldehyde dehydrogenase; (c) components of the β-oxidation pathway including acyl-CoA synthetase, acyl-CoA oxidase, acetoacyl-CoA thiolase, 3-ketoacyl-CoA thiolase, and the trifunctional enzyme containing acyl-CoA hydrolase, 3-hydroxyacyl-CoA dehydrogenase, and 3-hydroxyacyl-CoA epimerase activities; and (d) peroxisomal components such as catalase and appropriate transporters. From molecular weight assignments, the Mₐ 54,000 protein is possibly the alkane-induced cytochrome P450. Assignment of functions of the other proteins presented here will have to await further protein sequencing and protein function studies. Sequencing of these induced proteins in Candida will provide valuable information about the pathway of lipid degradation in this organism. The identity of the Mₐ 73,000 protein has been confirmed in this paper as long-chain fatty-acid alcohol oxidase following cloning and expression in E. coli.

The highest sequence homologies of fao genes determined in this study are to genes in Arabidopsis and Mycobacterium of unknown function/assignment. There is also high homology in domain V to other known oxidoreductases, in particular cholesterol oxidase (29), choline oxidase (30), and cellulose oxidase (31). These three enzymes are flavoproteins that utilize molecular oxygen in their reactions and that generate hydrogen peroxide, characteristics shared with the alcohol oxidase from Candida species. The purified cellulose oxidase protein has been studied spectroscopically and has a heme and flavin domain (31). It is of interest that domain III present in all three of the Candida species contains a heme-binding motif.

Alcohol oxidases from both C. cloacae and C. tropicalis have wide substrate specificity, oxidizing both straight-chain alcohols and ω-hydroxy fatty acids. The kinetic constants and the pH optimum of the enzyme from C. cloacae are similar to those reported for C. tropicalis (7). It is known that C. cloacae can utilize a wide range of fatty acids as sole carbon sources (11). The two C. cloacae genes identified may code for proteins of slightly different substrate specificity or cellular location. In C. maltosa, direct gene disruption has been used to elucidate the function of the n-alkane-inducible cytochrome P450 genes ALK1, ALK2, ALK3, and ALK5 (14). Each of the four individual genes is sufficient for growth on n-alkanes, but disruption of all four genes prevents growth on alkanes. The ALK5 gene exhibits narrower chain length specificity and cannot support growth on short-chain n-alkanes (14). The next metabolic step in ω-oxidation involves an LC-FAO enzyme. C. cloacae contains two LC-FAO genes, fao1 and fao2, cloned as part of this study. The fao1 gene is much more highly expressed than the fao2 gene both in the absence of alkane and following induction. Gene disruption studies should permit the generation of a strain of C. cloacae that can utilize fatty acids as substrates and produce ω-hydroxy fatty acids. This creates the possibility of making a wide range of ω-hydroxy fatty acids, which are at present unavailable in either natural or synthetic form, by growing C. cloacae on a variety of both saturated and unsaturated fatty acids (11). The limiting factor is the availability of a transformation system. There is one for C. tropicalis based on URA3 selection (32), but at present, none is available for C. cloacae.

Availability of a Candida strain disrupted in the fao genes would also allow complementation studies with the unassigned Arabidopsis and Mycobacterium genes identified in this paper. Their similarity to the data presented in this study suggests they are part of an ω-oxidation pathway. Complementation could confirm their proposed cellular function as LC-FAOs. Such complementation studies using plant genes in both yeast and bacterial systems have been successful (33, 34). Since there are no identified long-chain alcohol oxidase genes in Saccharomyces or bacteria, generation of a null mutant in Candida would provide an ideal test system for the direct isolation of alcohol oxidases from diverse species, as has been used for acyltransferases (34, 35).

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