Fatty Aldehyde Dehydrogenase Multigene Family Involved in the Assimilation of n-Alkanes in Yarrowia lipolytica*

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Background: Fatty aldehydes are converted to fatty acids in the metabolism of n-alkanes of Yarrowia lipolytica.

Results: Four fatty aldehyde dehydrogenases (FALDHs) are required for the assimilation of long-chain n-alkanes.

Conclusion: FALDHs oxidize fatty aldehydes to fatty acids in the peroxisome and/or the endoplasmic reticulum in n-alkane metabolism.

Significance: FALDH genes may have been multiplicated and functionally diversified in Y. lipolytica.

In the n-alkane assimilating yeast Yarrowia lipolytica, n-alkanes are oxidized to fatty acids via fatty alcohols and fatty aldehydes, after which they are utilized as carbon sources. Here, we show that four genes (HFD1–HFD4) encoding fatty aldehyde dehydrogenases (FALDHs) are involved in the metabolism of n-alkanes in Y. lipolytica. A mutant, in which all of four HFD genes are deleted (Δhfdl–4 strain), could not grow on n-alkanes of 12–18 carbons; however, the expression of one of those HFD genes restored its growth on n-alkanes. Production of Hfd2Ap or Hfd2Bp, translation products of transcript variants generated from HFD2 by the absence or presence of splicing, also supported the growth of the Δhfdl–4 strain on n-alkanes. The FALDH activity in the extract of the wild-type strain was observed in the absence or presence of splicing, whereas Hfd1p and Hfd2Ap are localized predominantly in the peroxisome, whereas Hfd1p and Hfd2Ap are localized in both the endoplasmic reticulum and the peroxisome. These results suggest that the HFD multigene family is responsible for the oxidation of fatty aldehydes to fatty acids in the metabolism of n-alkanes, and raise the possibility that Hfd proteins have diversified by gene multiplication and RNA splicing to efficiently assimilate or detoxify fatty aldehydes in Y. lipolytica.

A variety of yeasts have acquired the ability to assimilate n-alkanes, some of the most hydrophobic compounds, as sole carbon and energy sources. In n-alkane assimilating yeasts, n-alkanes are incorporated into cells and hydroxylated to fatty alcohols by cytochromes P450ALK (P450ALKs), which are classified into the CYP52 family of P450 (1) present in the endoplasmic reticulum (ER) membrane (2–7). Fatty alcohols are oxidized to fatty aldehydes by fatty alcohol dehydrogenase (FADH) in the ER or fatty alcohol oxidase (FAOD) in the peroxisome. Fatty aldehydes are oxidized to fatty acids by fatty aldehyde dehydrogenase (FALDH) in the ER or in the peroxisome. Fatty acids are finally activated to fatty acyl-CoA and metabolized through β-oxidation in the peroxisome, or are used in lipid synthesis.

n-Alkane assimilating yeasts, including Candida tropicalis (8–10), Candida maltosa (11–13), Debaryomyces Hansenii, Candida albicans (14), Candida bombicola, Candida parapsilosis, Lodderomyces elongisporus, Pichia stipitis, and Yarrowia lipolytica (15–17), have multiple paralogs of P450ALKs (1). Y. lipolytica, one of the most well studied n-alkane-assimilating yeasts, has 12 genes (ALK1–ALK12) that are predicted to encode P450ALKs (15–18). A deletion mutant of all of 12 ALK genes was unable to grow on n-alkanes of 10–18 carbons, indicating the critical role of ALK genes in the assimilation of n-alkanes (19). Among the 12 ALK genes, ALK1 and ALK2 encode pivotal P450ALKs. Deletion of ALK1 confers severely defective growth on n-decane and double deletion of ALK1 and ALK2 leads to a profound growth defect on n-hexadecane (15, 16). Transcription of ALK1 and a subset of ALK genes is up-regulated by the presence of n-alkanes (15, 18). A heterocomplex of two basic helix-loop-helix transcription factors, Yas1p and Yas2p, binds to a promoter element alkane-responsive element 1 in the ALK1 promoter and activates transcription of ALK1 in response to n-alkanes (20–22). An Opil family transcription repressor, Yas3p, interacts with Yas2p and represses the transcription of ALK1 in the absence of n-alkane (18, 23).

In marked contrast to P450ALKs, FADH, FAOD, and FALDH that are involved in the metabolism of n-alkanes have remained elusive. FADH that is involved in the oxidation of fatty alcohols of more than 10 carbons has remained unidentified in all n-alkane-assimilating yeasts. FAOD activity has been

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3 The abbreviations used are: ER, endoplasmic reticulum; EGFP, enhanced green fluorescent protein; FADH, fatty alcohol dehydrogenase; FALDH, fatty aldehyde dehydrogenase; FAOD, fatty alcohol oxidase; qRT-PCR, quantitative real-time PCR; PTS1, peroxisomal targeting signal 1.
detected in cell extracts of a subset of n-alkane-assimilating yeasts, including C. maltosa, C. bombicola, C. parapsilosis, C. tropicalis, and D. hansenii, and coding genes have been characterized in some of these yeasts (24, 25). An orthologous gene, however, has not been identified in the genome sequence of Y. lipolytica. It was recently shown that alcohol dehydrogenase genes, FADH and ADH1–ADH7, and a fatty alcohol oxidase gene, FAO1, are involved in the oxidation of ω-hydroxy fatty acids in Y. lipolytica; however, the deletion mutant strain lacking all these genes is able to oxidize dodecanol (26). FALDH activities have been reported in C. tropicalis, Y. lipolytica, and Candida intermedia (27–29), but the enzymes catalyzing the reactions remain unidentified. It has been reported that C. maltosa P450 52A3 expressed heterologously in Saccharomyces cerevisiae oxidizes n-alkane of 16 carbons to the corresponding fatty alcohol, fatty aldehyde, and fatty acid in vitro (30).

ALDH3A2, one of the mammalian FALDH, belongs to a superfamily of NAD(P)⁺-dependent aldehyde dehydrogenases (31). Human ALDH3A2 have high activities toward saturated and unsaturated fatty aldehydes of 6–24 carbons in length (32). ALDH3A2 is involved in various cellular processes: it has been suggested that ALDH3A2 is involved in the breakdown of phytic acid in the peroxisome (33, 34), and ALDH3A2 is further reported to have a protective role against oxidative stress associated with lipid peroxidation (35). Mutations in the ALDH3A2 gene have been shown to result in Sjögren–Larsson syndrome (36). S. cerevisiae has an ortholog of the ALDH3A2 gene, HFD1, and S. cerevisiae Hfd1p and mammalian ALDH3A2 are responsible for the conversion of hexadecenal to hexadecenoic acid in the degradation process of sphingosine 1-phosphate (37).

The focus of this study was four genes predicted to encode FALDHs, whose amino acid sequences exhibit similarities to those of FALDHs of S. cerevisiae (Hfd1p) and mammals (ALDH3A2), in the Y. lipolytica genome. These four genes were named HFD1, HFD2, HFD3, and HFD4 and were analyzed in terms of the roles they play in the n-alkane assimilation. Our data suggest that Hfd proteins are involved in the oxidation of fatty aldehydes in the n-alkane metabolism process in Y. lipolytica.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Growth Conditions**—Yeast strains used in this study are shown in Table 1. Y. lipolytica strains CXAU1 and CXAU/A1 were used as wild-type strains (15, 21).

| Strain | Genotype | Source or ref |
|--------|----------|---------------|
| CXAU1 | MATA ade1 | 15 |
| CXAU/A1 | CXAU1 ade1::ADE1 | 21 |
| REA1 | CXAU1 sec61::SEC61-DsRed ade1::ADE1 | 23 |
| POC | CXAU1 poc1::POT1-mCherry-ADE1 | This study |
| Δhfd1 | CXAU1 hfd1 | This study |
| Δhfd2 | CXAU1 hfd2 | This study |
| Δhfd3 | CXAU1 hfd3 | This study |
| Δhfd4 | CXAU1 hfd4 | This study |
| Δhfd1,2 | CXAU1 hfd1 hfd2 | This study |
| Δhfd1,3 | CXAU1 hfd1 hfd3 | This study |
| Δhfd1,4 | CXAU1 hfd1 hfd4 | This study |
| Δhfd2,3 | CXAU1 hfd2 hfd3 | This study |
| Δhfd2,4 | CXAU1 hfd2 hfd4 | This study |
| Δhfd3,4 | CXAU1 hfd3 hfd4 | This study |
| Δhfd1,2,3 | CXAU1 hfd1 hfd2 hfd3 | This study |
| Δhfd1,2,4 | CXAU1 hfd1 hfd2 hfd4 | This study |
| Δhfd1,3,4 | CXAU1 hfd1 hfd3 hfd4 | This study |
| Δhfd2,3,4 | CXAU1 hfd2 hfd3 hfd4 | This study |
| Δhfd1–4 | CXAU1 hfd1 hfd2 hfd3 hfd4 | This study |

The deletion cassettes for HFD genes were constructed as follows. The 5’- and 3’-adjacent regions of the HFD genes were amplified from CXAU1 total DNA by PCR using the primers listed in Table 4 and cloned into pURA3 (19). The deletion cassettes for HFD genes were obtained by digestion of the plasmids with restriction enzymes shown in Table 4.

The coding regions of HFD genes with the 5’- and 3’-adjacent regions were amplified from CXAU1 total DNA by PCR using primers HFD1P-F2 and HFD1T-R2 for HFD1, HFD2P-F2 and HFD2T-R2 for HFD2, HFD3P-F2 and HFD3T-R2 for HFD3, and HFD4P-F2 and HFD4T-R2 for HFD4. Obtained fragments were digested with EcoRI and Apal, BamHI and Apal, EcoRI and Apal, and BamHI and Apal, and cloned into pSU5 digested with same restriction enzymes (38), resulting in pSHFD1, pSHFD2, pSHFD3, and pSHFD4, respectively.

Plasmids, pSEGFP-HFD1, pSEGFP-HFD2, pSEGFP-HFD3, and pSEGFP-HFD4, to express Hfd1p, Hfd2p, Hfd3p, and Hfd4p fused with enhanced green fluorescent protein (EGFP) at their N termini, respectively, from their own promoters, were constructed as follows. The 5’-adjacent regions with their start codons and the ORFs with the 3’-adjacent regions of HFD genes were amplified from CXAU1 total DNA by PCR using primers HFD1P-F3, HFD1P-R2, HFD1T-R2, and HFD1T-R2 for HFD1, HFD2P-F2 and HFD2T-R2 for HFD2, HFD3P-F2 and HFD3T-R2 for HFD3, and HFD4P-F2 and HFD4T-R2 for HFD4. Obtained fragments were digested with EcoRI and Apal, BamHI and Apal, BamHI and Apal, and cloned into pSU5 digested with same restriction enzymes (38), resulting in pSHFD1, pSHFD2, pSHFD3, and pSHFD4, respectively.

Plasmids, pSEGFP-HFD1, pSEGFP-HFD2, pSEGFP-HFD3, and pSEGFP-HFD4, to express Hfd1p, Hfd2p, Hfd3p, and Hfd4p fused with enhanced green fluorescent protein (EGFP) at their N termini, respectively, from their own promoters, were constructed as follows. The 5’-adjacent regions with their start codons and the ORFs with the 3’-adjacent regions of HFD genes were amplified from CXAU1 total DNA by PCR using primers HFD1P-F3, HFD1P-R2, HFD1T-R2, and HFD1T-R2 for HFD1, HFD2P-F2 and HFD2T-R2 for HFD2, HFD3P-F2 and HFD3T-R2 for HFD3, and HFD4P-F2 and HFD4T-R2 for HFD4. Obtained fragments were digested with EcoRI and Apal, BamHI and Apal, BamHI and Apal, and BamHI and Apal, and cloned into pSU5 digested with same restriction enzymes (38), resulting in pSHFD1, pSHFD2, pSHFD3, and pSHFD4, respectively.

The EGFP ORF was amplified from pEGFP by PCR using primers EcoRVStul-EGFP-F and EGFP-R-EcoRI. The amplified fragment was digested with EcoRV and EcoRI, and cloned into the EcoRV-EcoRI sites of pBluescript II SK⁺ to obtain pBS-EGFP. The fragments carrying EGFP ORF obtained by digestion of pBS-EGFP with HindIII and EcoRI, Stul and BamHI, EcoRV and EcoRI, and HindIII and BamHI were cloned into the HindIII-EcoRI sites of pSHFD1pROt, the Stul-BamHI sites of

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**TABLE 1**

| Yeast strains used in this study |
|---------------------------------|
| Strain | Genotype | Source or ref |
| CXAU1 | MATA ade1 | 15 |
| CXAU/A1 | CXAU1 ade1::ADE1 | 21 |
| REA1 | CXAU1 sec61::SEC61-DsRed ade1::ADE1 | 23 |
| POC | CXAU1 poc1::POT1-mCherry-ADE1 | This study |
| Δhfd1 | CXAU1 hfd1 | This study |
| Δhfd2 | CXAU1 hfd2 | This study |
| Δhfd3 | CXAU1 hfd3 | This study |
| Δhfd4 | CXAU1 hfd4 | This study |
| Δhfd1,2 | CXAU1 hfd1 hfd2 | This study |
| Δhfd1,3 | CXAU1 hfd1 hfd3 | This study |
| Δhfd1,4 | CXAU1 hfd1 hfd4 | This study |
| Δhfd2,3 | CXAU1 hfd2 hfd3 | This study |
| Δhfd2,4 | CXAU1 hfd2 hfd4 | This study |
| Δhfd3,4 | CXAU1 hfd3 hfd4 | This study |
| Δhfd1,2,3 | CXAU1 hfd1 hfd2 hfd3 | This study |
| Δhfd1,2,4 | CXAU1 hfd1 hfd2 hfd4 | This study |
| Δhfd1,3,4 | CXAU1 hfd1 hfd3 hfd4 | This study |
| Δhfd2,3,4 | CXAU1 hfd2 hfd3 hfd4 | This study |
| Δhfd1–4 | CXAU1 hfd1 hfd2 hfd3 hfd4 | This study |
pSHFD2pROt, the EcoRV-EcoRI sites of pSHFD3pROt, and the HindIII-BamHI sites of pSHFD4pROt, to obtain pSEGFP-HFD1, pSEGFP-HFD2, pSEGFP-HFD3, and pSEGFP-HFD4, respectively.

The plasmid pBPOT1-mCherry-ADE1 carrying a cassette to express Pot1p fused with mCherry at its C terminus in its chromosomal location was constructed as follows. The coding region of the C-terminal half of Pot1p was amplified from CXAU1 total DNA by PCR using primers EcoRI-POT1-F and POT1T-R-XbaI and the amplified fragment was digested with EcoRI and EcoT22I. The ORF of mCherry was amplified from pmCherry-N1 by PCR using primers EcoT22I-mCherry-F and mCherry-R-NotI and the amplified fragment was digested with NotI and BamHI, and cloned into the NotI-BamHI sites of pSUT5 to obtain pSHFD2A-EGFP, pSHFD2A and pSEGFP-HFD2B, respectively.

The plasmids, pSEGFP-HFD2A and pSEGFP-HFD2B, were constructed as follows. The DNA fragments containing HFD2 ORF were amplified from CXAU1 total DNA by PCR using primers HFD2-F and HFD2A-R for pSEGFP-HFD2A and HFD2-F and HFD2B-R for pSEGFP-HFD2B, digested with BamHI and ApaI, and cloned into the BamHI-ApaI sites of pBS-EGFP to obtain pSHFD2AORt, the EcoRV-EcoRI sites of pSHFD3pROt, to obtain pSHFD2AORt2 to obtain pBPOT1-mCherry-ADE1.

The plasmids, pSHFD2A and pSHFD2B, were constructed as follows. The DNA fragments containing HFD2 ORF with the 5′-adjacent region were amplified from CXAU1 total DNA by PCR using primers HFD2P-F2 and HFD2A-R, and HFD2P-F2 and HFD2B-R, and cloned into the BamHI-Apal sites of pSUT5, to obtain pSHFD2A and pSHFD2B, respectively.

The plasmids, pSEGFP-HFD2A and pSEGFP-HFD2B, were constructed as follows. The DNA fragments containing HFD2 ORF were amplified from CXAU1 total DNA by PCR using primers HFD2-F and HFD2A-R for pSEGFP-HFD2A and HFD2-F and HFD2B-R for pSEGFP-HFD2B, digested with BamHI and ApaI, and cloned into the BamHI-Apal sites of pSHFD2pROt. The fragment carrying EGFP ORF obtained by digestion of pBS-EGFP with Stul and BamHI was cloned into the Stul-BamHI sites of these plasmids, resulting in pSEGFP-HFD2A and pSEGFP-HFD2B.

The plasmid pSHFD2A-EGFP to express Hfd2Ap fused with EGFP at its C terminus was constructed as follows. The DNA fragment containing HFD2A with the 5′-non-coding region was amplified from CXAU1 total DNA by PCR using primers EcoT22I-HFD2A-F and HFD2A-R for pSEGFP-HFD2A, digested with BamHI and ApaI, and cloned into the BamHI-ApaI sites of pSUT5 to obtain pSHFD2AORt. The fragment carrying EGFP ORF obtained by digestion of pBS-EGFP with Stul and BamHI was cloned into the Stul-BamHI sites of these plasmids, resulting in pSEGFP-HFD2A and pSEGFP-HFD2B.

To obtain pET3a-HFD1, pET3a-HFD2A, pET3a-HFD3, and pET3a-HFD4, ORFs of HFD1, HFD2A, HFD3, and HFD4 were amplified from CXAU1 total DNA by PCR using primers HFD1-F2 and HFD1-R for HFD1, HFD2F2 and HFD2B-R for HFD2A, HFD3-F2 and HFD3-R for HFD3, and HFD4-F2 and HFD4-R for HFD4, and cloned into pET-3a (Novagen), resulting in pET3a-HFD1, pET3a-HFD2A, pET3a-HFD3, and pET3a-HFD4, respectively.
Fatty Aldehyde Dehydrogenases in Y. lipolytica

TABLE 3

| Name               | Primer sequence (5’-3’)                                      |
|--------------------|--------------------------------------------------------------|
| EcoRI-POT1-F       | TTGAATTCGTTGCCGCCAAGTACAACGTGTC                              |
| POT1-R-EcoT22I     | TTGAATTCGTTGCCGCCAAGTACAACGTGTC                              |
| BamHI-POT1-F       | TTGAATTCGTTGCCGCCAAGTACAACGTGTC                              |
| POT1-R-Xbal        | TTGAATTCGTTGCCGCCAAGTACAACGTGTC                              |
| EcoT22I-mCherry-F  | TTGAATTCGTTGCCGCCAAGTACAACGTGTC                              |
| mCherry-R-NotI     | TTGAATTCGTTGCCGCCAAGTACAACGTGTC                              |

TABLE 4

| Primers used for amplification of adjacent regions | Restriction enzymes |
|--------------------------------------------------|---------------------|
| HFD1     | HFD1P-F, HFD1P-R, HFD1T-F, HFD1T-R | PsbAl               |
| HFD2     | HFD2P-F, HFD2P-R, HFD2T-F, HFD2T-R | EcoT22I             |
| HFD3     | HFD3P-F, HFD3P-R, HFD3T-F, HFD3T-R | BspT104I            |
| HFD4     | HFD4P-F, HFD4P-R, HFD4T-F, HFD4T-R | Mun1                |

Northern Blot Analysis—Northern blot analysis was performed as described previously (39). DNA probes were amplified using genomic DNA of CXA1U as a template with primers Northern(HFD1-F) and Northern(HFD1-R) for HFD1, Northern(HFD2-F) and Northern(HFD2-R) for HFD2, Northern(HFD3-F) and Northern(HFD3-R) for HFD3, and Northern(HFD4-F) and Northern(HFD4-R) for HFD4 (Table 3).

Quantitative Real-time PCR (qRT-PCR) —Quantitative real-time PCR was performed as described previously (18). The gene-specific primers are RT-PCR(HFD1-F) and RT-PCR(HFD1-R) for HFD1, RT-PCR(HFD2-F) and RT-PCR(HFD2-R) for HFD2, RT-PCR(HFD3-F) and RT-PCR(HFD3-R) for HFD3, and RT-PCR(HFD4-F) and RT-PCR(HFD4-R) for HFD4 (Table 3).

Rapid Amplification of cDNA Ends (RACE) —Rapid amplification of cDNA ends was performed using the SMARTer™ RACE cDNA Amplification Kit (Clontech), according to the manufacturer’s instructions, with primers HFD1_GSP1 and HFD1_NGSP1 for 5’-RACE of HFD1, HFD1_GSP2 and HFD1_NGSP2 for 3’-RACE of HFD1, HFD2_GSP1 and HFD2_NGSP1 for 5’-RACE of HFD2, HFD2_GSP2 and HFD2_NGSP2 for 3’-RACE of HFD2, HFD3_GSP1 and HFD3_NGSP1 for 5’-RACE of HFD3, HFD3_GSP2 and HFD3_NGSP2 for 3’-RACE of HFD3, HFD4_GSP1 and HFD4_NGSP1 for 5’-RACE of HFD4, and HFD4_GSP2 and HFD4_NGSP2 for 3’-RACE of HFD4 (Table 5).

Fatty Aldehyde Dehydrogenase Activity Assay—Yeast cells were cultured in the SD medium for 24 h. n-Decane was added into the medium to a final concentration of 1%, and cells were further incubated for 6 h. Cells were collected by centrifugation, washed with phosphate-buffered saline, suspended in homogenization buffer (25 mM HEPES-NaOH (pH 7.3), 100 mM KCl, 10% glycerol, 1 mM dithiothreitol, and 1% Protease inhibitor mixture (Sigma)), and crushed by glass beads of 0.45–0.5-mm diameter for 3 min using Multi-Beads Shocker (YASUI KIKAI). The homogenates were centrifuged twice at 1,000 g for 10 min at 4 °C. The lysates were centrifuged twice at 10,000 × g for 10 min at 4 °C. The supernatants were used as cell extracts.

Escherichia coli BL21(DE3) cells harboring pET3a-HFD1, pET3a-HFD2, pET3a-HFD3, or pET3a-HFD4, were cultured to an 900D of 0.5 at 37 °C in LB Broth (Lenox) (1% tryptone, 0.5% yeast extract, 0.5% NaCl). Following 4 h of incubation with 0.1 mM isopropyl 1-thio-β-D-galactopyranoside, cells were harvested and broken using Multi-Beads Shocker with glass beads of 0.3 mm diameter for 5 min in the homogenization buffer. Cell lysate was centrifuged twice at 1,000 × g for 10 min at 4 °C. Tween 80 was added to the supernatants at a final concentra-

Transformation of Y. lipolytica—Y. lipolytica was transformed by electroporation as described previously (15).

Southern Blot Analysis—Southern blot analysis was performed as described previously (19).
tion of 1% (v/v), and the mixtures were placed on ice for 20 min and centrifuged at 13,000 × g for 10 min at 4 °C. The supernatants were used as cell extracts.

Dodecanal or tetradecanal was mixed with M-buffer (25 mM HEPE–NaOH (pH 7.3), 100 mM glycine, 0.25% (v/v) Tween 80) at a final concentration of 1 mM and the mixture was sonicated at 30 °C, and the absorbance at 340 nm was measured.

One unit of activity was defined as the amount of enzyme that catalyzes the production of 1 μmol of cell extracts, and 50 μl of 4 mM NAD⁺ in M-buffer were mixed and incubated at 30 °C, and the absorbance at 340 nm was measured using Microplate Reader SH-8000 (CORONA ELECTRIC Co., Ltd.). The extinction coefficient of NADH was 6.22 mM⁻¹ cm⁻¹.

The phylogenetic tree of aldehyde dehydrogenases in S. cerevisiae shows that Hfd proteins are in a clade distinct from that of S. cerevisiae Ald proteins, which catalyze the conversion of acetaldehyde to acetate (41), and their orthologs in Y. lipolytica (Fig. 1A).

A RACE analysis of these HFD genes suggested that the open reading frames (ORFs) of HFD1, HFD3, and HFD4 are consistent with those predicted by the Génolevures project and that they encode proteins of 519, 533, and 529 amino acids, respectively. The result of the analysis also suggested that two variants of HFD2 transcripts, named variant A and B, exist (Fig. 1B).

As predicted by the Génolevures project, variant A encodes a protein of 521 amino acids, which was named Hfd2Ap. Variant B was generated by splicing at nucleotide 1494 and encodes a 503-amino acid protein with the peroxisomal targeting signal 1 (PTS1)-like sequence at its C terminus. This protein was named Hfd2Bp. The ratio of DNA clones of these two variants obtained in a 3′-RACE analysis of cDNA from cells cultured in medium containing glucose was ~1:1 (Table 6). Significant differences in this ratio of variants were not observed in cells cultured in the absence of glucose (n = 24). The extinction coefficient of NADH was 6.22 mM⁻¹ cm⁻¹. One unit of activity was defined as the amount of enzyme that catalyzes the production of 1 μmol of cell extracts.

Fluorescence Microscopy—Fluorescence microscopic observation was performed as described previously (23).

## RESULTS

### Four Genes Encoding Fatty Aldehyde Dehydrogenases

We searched the genome database of Y. lipolytica for orthologs of Hfd1p and ALDH3A2 using the BLAST program provided by the Génolevures project with the amino acid sequences of S. cerevisiae Hfd1p and ALDH3A2 as queries. The trans-

| Name       | Primer 5′-3′                          | Primer 3′-5′                          |
|------------|--------------------------------------|--------------------------------------|
| HFD1_GSP1  | GCTTGAGCTCCGCCGGGGGTGGGGTGGCTCAAG   | GCTTGAGCTCCGCCGGGGGTGGGGTGGCTCAAG   |
| HFD1_NGSP1 | GCAGGGAGGCGTTCCGGTAGTGTCGGAG        | GCAGGGAGGCGTTCCGGTAGTGTCGGAG        |
| HFD1_GSP2  | GCTTGAGCTCCGCCGGGGGTGGGGTGGCTCAAG   | GCTTGAGCTCCGCCGGGGGTGGGGTGGCTCAAG   |
| HFD1_NGSP2 | GCAGGGAGGCGTTCCGGTAGTGTCGGAG        | GCAGGGAGGCGTTCCGGTAGTGTCGGAG        |

FIGURE 1. Fatty aldehyde dehydrogenases of Y. lipolytica. A, phylogenetic tree of aldehyde dehydrogenases of S. cerevisiae and Y. lipolytica. The phylogenetic tree of aldehyde dehydrogenases of S. cerevisiae (Sc) and Y. lipolytica (Yl) was constructed using ClustalW and drawn using NJplot. The scale bar denotes 0.1 substitutions per site. The accession numbers of sequences from UniProtKB are as follows: Ald1p/Alld6p (P54115), Ald2p (P46537), Ald3p (P46537), Ald4p (P46537), Ald5p (P46537), Ald6p (P46537), Ald7p (P46537), Ald8p (P46537), and Ald9p (P46537). B, nucleotide sequences of two variants of the HFD2 transcripts, variant A and variant B, and deduced amino acid sequences of their products. The nucleotides underlined in variant A are removed in variant B. PTS1-like sequence was highlighted with thick underline. Asterisks indicate stop codons.

## TABLE 5

Primer used in RACE analysis

| Name       | Primer 5′-3′                          | Primer 3′-5′                          |
|------------|--------------------------------------|--------------------------------------|
| HFD1_GSP1  | GCTTGAGCTCCGCCGGGGGTGGGGTGGCTCAAG   | GCTTGAGCTCCGCCGGGGGTGGGGTGGCTCAAG   |
| HFD1_NGSP1 | GCAGGGAGGCGTTCCGGTAGTGTCGGAG        | GCAGGGAGGCGTTCCGGTAGTGTCGGAG        |
| HFD1_GSP2  | GCTTGAGCTCCGCCGGGGGTGGGGTGGCTCAAG   | GCTTGAGCTCCGCCGGGGGTGGGGTGGCTCAAG   |
| HFD1_NGSP2 | GCAGGGAGGCGTTCCGGTAGTGTCGGAG        | GCAGGGAGGCGTTCCGGTAGTGTCGGAG        |

### Fluorescence Microscopy—Fluorescence microscopic observation was performed as described previously (23).
aldehyde dehydrogenase family in the Pfam database. A glutamic acid residue that coordinates nicotinamide ribose, and a cysteine residue that provides the catalytic thiol in the active sites of aldehyde dehydrogenases, were found to be conserved in all Hfd proteins (42). Using TMHMM, single transmembrane helices were predicted at the C termini of Hfd1p and Hfd2Ap.

**Transcription of the HFD Genes**—The transcriptional profiles of HFD1–HFD4 were assessed by Northern blot analysis and qRT-PCR (Fig. 2). To confirm the specificities of the probes used for Northern blot analysis, a deletion mutant strain of each HFD gene was constructed using the pop-in/pop-out method (19, 43). The wild-type *Y. lipolytica* strain and the deletion mutant strains of each HFD gene were cultured in long chain n-Alkanes with glucose for 3 days, on and the deletion mutants of the pop-in/pop-out method. These strains were analyzed in terms of their growth on various carbon sources. The wild-type strain and the deletion mutants of the HFD genes were incubated on glucose for 3 days, on n-alkanes of 10–18 carbons for 7 days, or on dodecanal for 10 days (Fig. 3A and Table 7). The single deletion mutants exhibited no growth defects on the medium containing glucose or n-alkane. In contrast, the deletion mutant lacking all four HFD genes (Δhfd1–4 strain) grew normally on glucose, but did not grow on n-alkanes of 12–18 carbons (Fig. 3A and Table 7). The Δhfd1–4 strain was found to grow on shorter chain n-alkanes, n-decane and n-undecane, but showed defective growth compared with the wild-type strain. Of the triple deletion mutants, the Δhfd1,2,3 and Δhfd1,3,4 strains exhibited slower growth on n-alkanes and formed smaller colonies on n-hexadecane, whereas the Δhfd1,2,4 and Δhfd2,3,4 strains grew normally on n-alkanes (Fig. 3B and Table 7). The growth of the Δhfd1–4 strains harboring a plasmid to express a HFD gene was comparable with that of the corresponding triple mutant (data not shown). These findings suggest that the HFD genes share an essential function in the assimilation of n-alkanes of 12–18 carbons. The relative growth of the triple deletion mutants also suggests that HFD1 and HFD3 play major roles in n-alkane assimilation. Consistent with this, the double deletion mutant of HFD1 and HFD3 grew slowly on longer-chain n-alkanes (Table 7).

On dodecanal, the growth of Δhfd1–4 strain was found to be comparable with the growth of the wild-type strain (Fig. 3A).

**TABLE 6**

| Carbon sources | Variant A | Variant B | n |
|----------------|-----------|-----------|---|
| Glucose        | 8         | 8         | 16|
| Glucose and n-dodecane | 8 | 10 | 18|
| n-Decane       | 6         | 7         | 13|

**FIGURE 2.** The transcript levels of HFD1–HFD4. A, Northern blot analysis using total RNAs extracted from the wild-type, Δhfd1, Δhfd2, Δhfd3, and Δhfd4 strains. Cells were grown to logarithmic phase in the glucose-containing medium, after which they were cultured in fresh medium containing glucose or n-decane for 1 h. Ribosomal RNAs stained with ethidium bromide are shown as loading controls. To detect HFD mRNA, the probe specific for HFD1, HFD2, HFD3, or HFD4 was used. B, qRT-PCR analysis. The wild-type strain was cultured to logarithmic phase in the glucose-containing medium, after which they were cultured in fresh medium containing glucose (Glc), n-decane (C10), n-dodecane (C12), n-tetradecane (C14), n-hexadecane (C16), or oleic acid (Ole) for 1 h. Total RNAs were extracted from the cells and reverse-transcribed to cDNAs. cDNAs were detected using primers specific for HFD1, HFD2, HFD3, or HFD4. Each result represents an average of three independent experiments ± S.E.
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Because the Δhfd1–4 strain exhibited severely defective growth on the n-alkanes, it is possible that fatty aldehydes incorporated from the culture medium were oxidized by enzyme(s) different from those involved in the oxidation of fatty aldehydes produced in the process of n-alkane metabolism. The Δhfd1–4 strain also exhibited comparable growth to the wild-type strain on 1-dodecanol (Fig. 3A).

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**TABLE 7**

Growth of deletion mutants on n-alkanes of various carbon lengths

Deletion mutants were cultivated on indicated n-alkanes for 1 week. Growth equivalent to the wild-type cells was indicated as “+++.”

| Strain   | Glc | C10 | C11 | C12 | C13 | C14 | C15 | C16 | C17 | C18 |
|----------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| WT       | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +   | +   | +   |
| Δhfd1    | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +   | +   |
| Δhfd2    | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +   | +   |
| Δhfd3    | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +   | +   |
| Δhfd4    | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +   | +   |
| Δhfd1,2  | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +   | +   |
| Δhfd1,3  | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +   | +   |
| Δhfd1,4  | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +   | +   |
| Δhfd2,3  | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +   | +   |
| Δhfd2,4  | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +   | +   |
| Δhfd3,4  | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +   | +   |
| Δhfd1,2,3| +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +   | +   |
| Δhfd1,2,4| +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +   | +   |
| Δhfd1–4  | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +   | +   |

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**FALDH Activities of Hfd Proteins**—Cell extracts were prepared from wild-type and Δhfd1–4 strains cultured in medium containing glucose alone or n-decane and glucose. FALDH activity in these cell extracts was assessed using either dodecanal or tetradecanal as a substrate (Fig. 4A). The n-decane-containing medium was supplemented with glucose to support growth of the Δhfd1–4 strain, because glucose does not significantly affect the n-decane-induced growth of Hfd proteins have FALDH activity. Therefore, it is possible that Hfd1p is degraded in the presence of n-decane and tetradecanal were detected in the Hfd3p-producing cell extract. These results suggest that Hfd proteins have FALDH activity. HFD1 is predicted to encode a protein of 519 amino acids, whereas Hfd2p produced in E. coli is predicted to be a protein of 521 amino acids. However, the mobility of Hfd1p produced in E. coli is much higher than that of Hfd2p (Fig. 4B). Therefore, it is possible that Hfd1p is degraded in the E. coli lysate and this affects the activity of Hfd1p in vitro.

**Localization of Hfd Proteins**—To investigate the subcellular localization of Hfd proteins in Y. lipolytica, we constructed plasmids pSEGFp-HFD1, pSEGFp-HFD2, pSEGFp-HFD3, and pSEGFp-HFD4 that were constructed to express Hfd1p, Hfd2p, Hfd3p, and Hfd4p, respectively, fused with EGFP at their N termini (EGFP-Hfd1p, EGFP-Hfd2p, EGFP-Hfd3p, and EGFP-
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A

![Graph showing FALDH activities in Y. lipolytica wild-type and Δhfd1-4 strains.](image)

B

![Graph showing FALDH activities in Y. lipolytica wild-type and Δhfd1-4 strains.](image)

**FIGURE 4. FALDH activities of the Hfd proteins.** A, FALDH activities in the Y. lipolytica wild-type and Δhfd1-4 strains. The FALDH activities were measured with cell extracts prepared from wild-type and Δhfd1-4 strains incubated in medium containing glucose (white bars) or glucose with n-dodecane (black bars) for 6 h using dodecanal or tetradecanal as a substrate as described under “Experimental Procedures.” Each result represents an average of three independent experiments ± S.E. *, p < 0.05; **, p < 0.0005 (t test, two-tailed). B, FALDH activity in E. coli cells carrying pET3a, pET3a-HFD1, pET3a-HFD2A, pET3a-HFD3, or pET3a-HFD4. Upper panel, total proteins of these strains were separated by 8% SDS-PAGE and visualized by Coomassie staining. White arrowheads indicate the bands, intensities of which were specifically increased. Lower panels, the FALDH activities were measured with extracts of the cells carrying pET3a (no Hfd), pET3a-HFD1 (Hfd1p), pET3a-HFD2A (Hfd2Ap), pET3a-HFD3 (Hfd3p), or pET3a-HFD4 (Hfd4p), as described under “Experimental Procedures.” Each result represents an average of three independent experiments ± S.E. * and **, statistically significant difference (*, p < 0.05; **, p < 5 × 10⁻³) relative to the result of pET3a-induced cells.

Hfd4p) under control of their native promoters. The introduction of these plasmids into the Δhfd1-4 strain restored the growth on n-alkanes, indicating that these proteins are functional in Y. lipolytica (data not shown). These plasmids were introduced into the REA1 strain, which produces Sec61p tagged with DsRed as an ER marker (23), and into the POC strain, which produces Pot1p tagged with mCherry as a peroxisome marker. These cells were then cultured to logarithmic phase in medium containing glucose. n-Dodecane was added to the culture medium at a final concentration of 2%, after which the cells were further incubated for 1 h. The fluorescent signals of EGFP-Hfd1p and EGFP-Hfd2p were found to be partially colocalized with those of Sec61p-DsRed and Pot1p-mCherry (Fig. 5), suggesting that Hfd1p and Hfd2p are localized in both the ER and peroxisome. The fluorescent signal of EGFP-Hfd3p was colocalized exclusively with that of Pot1p-mCherry (Fig. 5), indicative of a predominant peroxisomal localization for Hfd3p. The fluorescent signal of EGFP-Hfd4p was weak compared with that of the other Hfd proteins, and they exhibited a punctate pattern distinct from the signals of Sec61p-DsRed and Pot1p-mCherry (Fig. 5). Similar localization patterns were observed for EGFP-Hfd1p, EGFP-Hfd2p, EGFP-Hfd3p, and EGFP-Hfd4p when the cells were cultured in glucose-containing medium (data not shown).

**Roles of Two Hfd2p Variants**—To determine which variant of Hfd2p contributes to n-alkane assimilation, plasmids pSHFD2A and pSHFD2B were constructed to express either Hfd2Ap or Hfd2Bp alone, respectively, under the native promoter, and were introduced into the Δhfd1-4 strain. These strains were incubated on n-dodecane or n-hexadecane for 7 days, and their growth was observed (Fig. 6A). The growth of both strains was found to be similar to that of the Δhfd1-4 strain containing the wild-type HFD2, which produces both variants.

Next, the localization of Hfd2Ap and Hfd2Bp was investigated. Plasmids pSEGFP-HFD2A and pSEGFP-HFD2B were constructed to express Hfd2Ap and Hfd2Bp, respectively, fused with EGFP at their N termini (EGFP-Hfd2Ap and EGFP-Hfd2Bp). These fusion proteins were shown to complement the defective growth of the Δhfd1-4 strain on n-alkanes, indicating that they are functional in vivo (data not shown). The pSEGFP-HFD2A and pSEGFP-HFD2B plasmids were then introduced into REA1 and POC strains to allow for subcellular localization of EGFP-Hfd2Ap and EGFP-Hfd2Bp to be assessed. The cells were cultured to logarithmic phase in glucose-containing medium, after which n-dodecane was added to the culture medium at a final concentration of 2%. The cells were further incubated for 1 h. The fluorescent signal of EGFP-Hfd2Ap was colocalized with that of Sec61p-DsRed and Pot1p-mCherry (Fig. 6B). Similar results were obtained when localization of Hfd2Ap tagged with EGFP at its C terminus (Hfd2Ap-EGFP), which was indicated to be functional in Y. lipolytica (data not shown), was observed (Fig. 6B). These results imply that Hfd2Ap is localized in both the ER and the peroxisome. In contrast, the fluorescent signal of EGFP-Hfd2Bp was colocalized with the signal of Pot1p-mCherry, but not with that of Sec61p-DsRed, as expected from the presence of the PTS1-like sequence. These results suggest that Y. lipolytica utilizes FALDH in the peroxisome to assimilate n-alkanes.

**DISCUSSION**

In this study, the four HFD genes encoding FALDHs in Y. lipolytica were characterized. The Δhfd1-4 strain did not grow on n-alkanes of 12–18 carbons and exhibited partial growth defects on n-alkanes of 10–11 carbons, whereas Y. lipolytica mutant strains expressing one of four HFD genes exhibited substantial growth on n-alkanes. n-Alkane-induced FALDH activity was not detected in the Δhfd1-4 strain extract. Significant FALDH activity was, furthermore, detected in extracts of bac-
Transcription of HFD Genes—Transcription of HFD1, HFD2, and HFD3 was notably induced by the presence of n-alkanes, whereas that of HFD4 was not significantly induced by n-alkanes (Fig. 2). Transcription of ALK1 and a subset of ALK genes is known to be regulated by transcription activators Yas1p and Yas2p and repressor Yas3p (18, 21–23). It is thus possible that transcription of HFD1–HFD3 is regulated by the Yas1p-Yas2p-Yas3p system.

qRT-PCR analysis revealed that HFD1 transcription is highly up-regulated by oleic acid (Fig. 2B). In Y. lipolytica, the transcription of genes involved in the metabolism of fatty acids is activated in the presence of fatty acids, and Por1p, a Zn$_2$Cys$_6$ transcription factor, is involved in the transcriptional activation of a subset of these genes by fatty acids (44). Whether or not Por1p is involved in the regulation of HFD1 transcription in response to fatty acids remains to be addressed. MTLY 37, a Y. lipolytica strain, in which POX2, POX3, POX4, and POX5 encoding acyl-CoA oxidases involved in fatty acid β-oxidation are deleted, was reported to accumulate hexadecanedioic acid when fed hexadecanoic acid (45). HFD1 may be involved in the oxidation cascade of the ω-terminal end of fatty acids.

Oxidation of Fatty Aldehydes in n-Alkane Assimilation—According to the model of n-alkane assimilation in yeasts, fatty aldehydes are oxidized to fatty acids in the ER or in the peroxisome (4). The localization of Hfd1p, Hfd2p, and Hfd3p to the ER and/or the peroxisome observed in this study strongly supports this model. Because the cells expressing only Hfd2Bp or Hfd3p, which were suggested to be localized exclusively in the peroxisome (Figs. 5 and 6B), were found to grow on n-alkanes, it can be concluded that fatty aldehydes are oxidized to fatty acids in the peroxisome in the process of n-alkane assimilation. Considering that n-alkanes are hydroxylated to fatty alcohols by P450ALKs in the ER, our findings imply that fatty alcohols and/or fatty aldehydes are transported from the ER to the peroxisome in these strains. It remains to be elucidated how Hfd1p, Hfd2Ap, and Hfd3p lacking typical PTS1 sequences are targeted to the peroxisome. It would be of interest to assess whether the C-terminal sequences of Hfd1p and Hfd2Ap, ALL and IFL, respectively, function as peroxisome targeting signals.

It is not clear whether n-alkanes can be assimilated by the ER-localized FALDH. In mice, variants of FALDH (FALDH-N, -V, -V2, and -V3) are generated by alternative splicing from the ALDH3A2 gene (34, 46). Among these variants, FALDH-N, V2, and V3 are localized in the ER and FALDH-V is localized in the peroxisome (34, 47). Interestingly, overproduction of
FALDH-V or FALDH-N was reported to confer resistance to dodecanal on HEK293 cells, suggesting that FALDH variants are involved in protection against the cytotoxicity of dodecanal in the peroxisome or the ER. Fatty aldehydes derived from \( n \)-alkanes may thus be converted to fatty acids by the ER-localized FALDH in \( Y. \) lipolytica. EGFP-Hfd4p did not appear to be localized in the peroxisome in this study; however, the expression of \( HFD4 \) in the \( \Delta hfd1–4 \) strain grown on \( n \)-alkanes was found to restore the growth partially. Although subcellular localization of Hfd4p was not determined in this study, the fluorescent signal of EGFP-Hfd4p might be partially colocalized with that of the Sec61p-DsRed signal. Further detailed analysis of the localization of Hfd4p would provide important information on whether or not fatty aldehydes are oxidized to fatty acids in the ER or in other organelle(s) in the process of \( n \)-alkane assimilation. \( S. \) cerevisiae Hfd1p and mammalian ALDH3A2 are known to catalyze the conversion of 1-hexadecenal to hexadecenoic acid in the metabolism of sphingosine 1-phosphate probably in the ER (37). The ER-localized Hfd proteins of \( Y. \) lipolytica may thus be involved in the metabolism of sphingosine 1-phosphate.

The \( \Delta hfd1–4 \) strain was found to grow on the medium containing dodecanal as the sole carbon source (Fig. 3A), whereas it could not grow on \( n \)-alkanes of more than 12 carbons. One potential reason for this is that the enzymes oxidizing fatty aldehydes incorporated from culture medium could be different from those involved in the oxidation of fatty aldehydes produced in the metabolism of \( n \)-alkanes. It is possible that there is channeling of \( n \)-alkane metabolites, perhaps in the peroxisome. Fatty aldehyde dehydrogenase activity was still detected in the \( \Delta hfd1–4 \) strain extract (Fig. 4C), and the \( \Delta hfd1–4 \) strain might grow on dodecanal by oxidizing it with this residual activity. \( Y. \) lipolytica has four orthologs of the \( S. \) cerevisiae ALD genes encoding cytosolic or mitochondrial aldehyde dehydrogenases (Fig. 1A). However, in our preliminary results, a mutant deleted for all of four \( HFD \) genes and four \( ALD \) orthologs was able to grow on dodecanal. Thus, other dehydrogenase(s) could be involved in the oxidation of incorporated fatty aldehydes in \( Y. \) lipolytica. Cytochrome P450 52A3 of \( C. \) maltosa was suggested to catalyze the oxidation of fatty aldehydes (30), and thus P450ALKs represent another candidate group for the oxidation.

FIGURE 6. Functions and subcellular localization of Hfd2Ap and Hfd2Bp. A, the wild-type strain carrying pSUT5 (vector) and the \( \Delta hfd1–4 \) strain carrying pSUT5, pSHFD2, pSHFD2A, or pSHFD2B were cultivated on glucose for 3 days or on \( n \)-dodecane or \( n \)-hexadecane for 7 days. B, cells co-expressing Hfd2Ap or Hfd2Bp fused with EGFP at its N or C terminus and Sec61p-DsRed or Pot1p-mCherry were grown in glucose-containing medium to logarithmic phase. \( n \)-Dodecane was added, after which the cells were further incubated for 1 h. Localization of Hfd fusion proteins and Sec61p-DsRed or Pot1p-mCherry was observed as described under “Experimental Procedures.” Bars, 3 \( \mu \)m.
of short chain fatty aldehydes or incorporated fatty aldehydes in *Y. lipolytica*.

Alternatively, Hfd proteins may function upstream of fatty aldehyde oxidation in the n-alkane metabolism pathway. The initial hydroxylation of n-alkanes was shown to be catalyzed by P450ALKs, and the mutant deleted for all of 12 ALK genes are unable to grow on n-alkanes (19). Therefore, it is less probable that Hfd proteins play critical roles in the hydroxylation of n-alkanes. The Δhfd1–4 strain appears not to be defective in the oxidation of incorporated fatty alcohols, because it grew on 1-dodecanol comparably to the wild-type strain (Fig. 3A). However, it is not clear whether Hfd proteins are involved in the oxidation of fatty alcohols produced by the hydroxylation of n-alkanes. Thus, it remains to be determined whether Hfd proteins play additional roles in the n-alkane metabolism pathway.

The Δhfd1–4 strain exhibited slight growth on n-decane or n-undecane (Table 7). Because the mutant deleted for all four HFD genes and four ALD orthologs also could grow on these n-alkanes in our preliminary results, other dehydrogenase(s) or P450ALKs might be involved in the oxidation of shorter chain fatty aldehydes, as discussed above.

**Transcript Variants of HFD2**—Two HFD2 transcript variants, which encode Hfd2Ap without PTS1 and Hfd2Bp with a PTS1-like sequence, were generated from *HFD2*. Our results suggest that Hfd2Ap is localized in both the ER and the peroxisome, whereas Hfd2Bp is localized exclusively in the peroxisome. The distinct production of two FALDH isoforms localized in the ER or the peroxisome is achieved by alternative splicing in mice and humans (34). Our findings indicate that the determination of FALDH localization in the ER and/or the peroxisome by RNA splicing is conserved from yeasts to mammals. Overproduction of the peroxisome-localized FALDH-V, but not the ER-localized FALDH-N, was reported to protect HEK293 cells from damage caused by phytanic acid, implying an involvement of FALDH-V in the metabolism of phytanic acid in the peroxisome (34). The generation of the transcript variants by RNA splicing could thus contribute to diversification of FALDH proteins. In *Y. lipolytica*, significant differences were not observed between the ratios of the two HFD2 transcript variants in the cells cultured in the presence or absence of n-alkanes. In mouse liver, the relative mRNA ratios of the transcript variants of the ALDH3A2 gene were not altered significantly in the presence or absence of Wv14,643, an agonist of PPARα, which activates transcription of the ALDH3A2 gene (34). The functional differences between Hfd2Ap and Hfd2Bp remain unclear.

**Chain-length Specificity of Hfd Proteins**—The transcript levels of HFD1 and HFD2 were found to be higher in the presence of n-decane compared with longer chain n-alkanes. In contrast, the transcript level of HFD3 was higher in cells grown on n-hexadecane compared with cells grown on shorter chain n-alkanes. The correlation between the transcript profile of each HFD gene in response to n-alkanes of various carbon numbers and substrate specificities of its product is unclear, because we were unable to measure FALDH activity using fatty aldehydes of 10 or 16 carbons as a substrate. It is possible that the solubility of hexadecanal is too low to be used as a substrate in our FALDH activity assay system.

**Pathway of n-Alkane Assimilation**—In n-alkane-assimilating yeasts, n-alkanes are converted to fatty acids via a three-step oxidation process. It has been shown that the initial oxidation of n-alkanes to fatty alcohols is catalyzed by P450ALKs (2–7). The results of this study suggest that Hfd proteins are responsible for the oxidation of fatty aldehydes to fatty acids in *Y. lipolytica*. The missing link in the n-alkane assimilation pathway is the enzyme(s) that catalyze(s) the oxidation of fatty acids to fatty aldehydes. It has been reported that a deletion mutant lacking the alcohol dehydrogenase genes, *FADH* and *ADH1–ADH7*, and a fatty alcohol oxidase gene, *FAO1*, exhibited defects in the oxidation of long chain ω-hydroxy fatty acids, but was still able to metabolize n-dodecanol and oxidize 1-dodecanol (26). It is thus possible that multiple genes are involved in the oxidation of fatty acids, as is the case for n-alkanes and fatty aldehydes. Alternatively, fatty aldehydes may be oxidized by P450ALKs as reported for *C. maltosa* P450 S2A3 (30). Our findings provide a crucial step toward elucidating the n-alkane metabolism in yeasts, and will contribute to our deeper understanding of the metabolism of hydrophobic compounds in lower eukaryotes.

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