Enzymes for Aerobic Degradation of Alkanes in Yeasts

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Contents

Abstract

A wide variety of yeasts can utilize *n*-alkanes as sole carbon and energy sources. The degradation pathways of *n*-alkanes in yeasts and the enzymes associated with these pathways have been studied intensively in the ascomycetous yeasts, *Candida tropicalis*, *Candida maltosa*, and *Yarrowia lipolytica*, for biotechnological applications, such as conversion of *n*-alkanes to proteins or useful compounds, as well as for elucidating the metabolism of hydrophobic substrates by fungi. Here, we describe the aerobic degradation pathway of *n*-alkanes in yeasts and the enzymes that catalyze the reactions involved in the degradation. In *n*-alkane-assimilating yeasts, incorporated *n*-alkanes are hydroxylated to fatty alcohols by cytochromes P450 of the CYP52 family in the endoplasmic reticulum (ER). Fatty

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alcohols are oxidized in the ER or the peroxisome to fatty aldehydes and finally to fatty acids, which are then activated to acyl-CoAs and metabolized by β-oxidation or used for lipid synthesis.

1 Introduction

n-Alkanes are common compounds in nature, and a variety of microorganisms, including bacteria, yeasts, and filamentous fungi, can utilize these compounds as their sole carbon and energy sources. The assimilation of *n*-hexadecane has been assessed in ~700 yeast species among the 1,270 yeasts listed in *The Yeasts: A Taxonomic Study*, and ~180 of these 700 species have been shown to have the ability to assimilate *n*-hexadecane (Kurtzman et al. 2011). These ~180 yeasts belong to 28 genera of ascomycetous and basidiomycetous yeasts including *Candida*, *Debaryomyces*, *Metschnikowia*, *Yarrowia*, and *Cryptococcus* (Fig. 1). Neither the

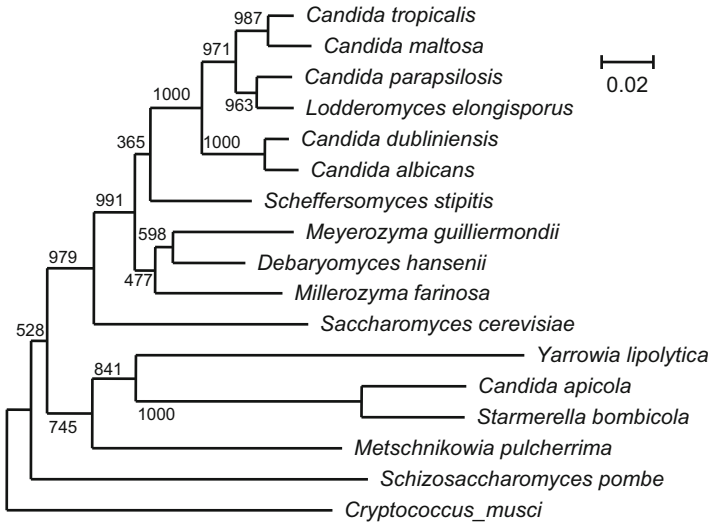

Fig. 1 Phylogenetic tree of *n*-alkane-assimilating yeasts. Phylogenetic tree of D1/D2 regions of 26S ribosomal DNA of the yeasts that can assimilate *n*-alkanes and the model yeasts, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, was constructed using ClustalW (DDBJ, v2.1) and drawn using NJplot. The scale bar indicates 0.02 substitutions per site. The bootstrap values by 1000 repetitions are indicated. The accession numbers of sequences of D1/D2 regions from GenBank are as follows: *Candida albicans* (U45776), *Candida apicola* (U45703), *Candida dubliniensis* (U57685), *C. maltosa* (U45745), *Candida parapsilosis* (U45754), *Candida tropicalis* (U45749), *Cryptococcus musci* (KC585415), *Debaryomyces hansenii* (U45808), *Lodderomyces elongisporus* (U45763), *Metschnikowia pulcherrima* (U45736), *Meyerozyma guilliermondii* (U45709), *Millerozyma farinosa* (U45739), *Saccharomyces cerevisiae* (U44806), *Scheffersomyces stipitis* (U45741), *Schizosaccharomyces pombe* (U40085), *Starmerella bombicola* (U45705), and *Yarrowia lipolytica* (U40080)

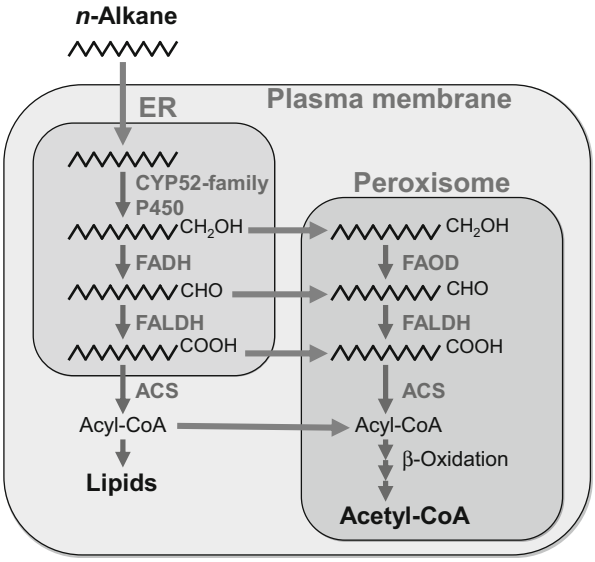

Fig. 2 Proposed pathway of *n*-alkane metabolism in yeasts. Incorporated *n*-alkanes are hydroxylated to fatty alcohols by cytochromes P450 of the CYP52 family. Fatty alcohols are oxidized to fatty aldehydes by fatty alcohol dehydrogenase (FADH) in the ER or fatty alcohol oxidase (FAOD) in the peroxisome. Fatty aldehydes are oxidized to fatty acids by fatty aldehyde dehydrogenase (FALDH) in the ER or the peroxisome. Fatty acids are activated to acyl-CoAs by acyl-CoA synthetase (ACS) and are metabolized through β-oxidation pathway in the peroxisome or utilized for membrane or storage lipid synthesis

model yeast *Saccharomyces cerevisiae* nor *Schizosaccharomyces pombe*, however, can assimilate *n*-alkanes.

The metabolic pathway of *n*-alkanes has been studied intensively in *Candida tropicalis* (Tanaka and Fukui 1989), *Candida maltosa* (Mauersberger et al. 1996), and *Yarrowia lipolytica* (Barth and Gaillardin 1996; Barth and Gaillardin 1997; Fickers et al. 2005; Fukuda 2013; Fukuda and Ohta 2013; Nicaud 2012) and has attracted considerable attentions owing to its involvement in the production of single-cell protein (SCP) and other useful materials, including long-chain dicarboxylic acids and tricarboxylic acid (TCA) cycle intermediates, from *n*-alkanes (Fickers et al. 2005; Tanaka and Fukui 1989). In order to establish and improve systems for the production of useful materials from *n*-alkanes using yeasts, the *n*-alkane metabolic pathway and associated enzymes must be understood in detail. In *C. tropicalis*, *C. maltosa*, and *Y. lipolytica*, incorporated *n*-alkanes are sequentially oxidized to fatty acids, which are then metabolized via the β-oxidation pathway in the peroxisome or utilized for the synthesis of membrane or storage lipids (Fig. 2).

This chapter discusses the pathway of *n*-alkane oxidation to fatty acids and the subsequent activation of fatty acids to acyl-CoAs, as well as the enzymes catalyzing these reactions in yeasts. The metabolism and utilization of fatty acids in yeasts will be described in another chapter.

2 Uptake of *n*-Alkanes by Yeasts

n-Alkanes exhibit poor solubility in water, and it has been proposed that yeasts facilitate the uptake of *n*-alkanes by two mechanisms, which are not mutually exclusive. In the first mechanism, reported for some species of *n*-alkane-assimilating yeasts, bioemulsifiers or biosurfactants are secreted by yeasts to solubilize *n*-alkanes (Barth and Gaillardin 1996; Tanaka and Fukui 1989): *Y. lipolytica* secretes a 28-kDa emulsifier, named liposan, composed of 83% carbohydrate and 17% protein (Cirigliano and Carman 1984, 1985), while *Starmerella bombicola* and *Candida apicola* produce the biosurfactant, sophorolipid (Van Bogaert et al. 2007). The physiological roles of these bioemulsifiers and biosurfactants in the uptake of *n*-alkanes, however, remain to be examined. In the second mechanism, *n*-alkane-assimilating yeasts adhere to *n*-alkane droplets to enhance the uptake of *n*-alkanes. Protrusions or slime-like outgrowths have been observed on the cell surface of *C. tropicalis*, *C. maltosa*, and *Y. lipolytica* cultured in the presence of *n*-alkanes (Kim et al. 2000; Mauersberger et al. 1996; Osumi et al. 1975; Tanaka and Fukui 1989). The slime-like outgrowths have been shown to reach the cell membrane through electron-dense channels in *C. tropicalis*, while the endoplasmic reticulum (ER) was found close to the cell membrane beneath such channels (Osumi et al. 1975). From these observations, it was suggested that *n*-alkanes are attached to the protrusions or slime-like outgrowths and are thereby transported through the channels to the ER, where they are hydroxylated to fatty alcohols (Sect. 3). The molecular structures of these protrusions or slime-like outgrowths and their roles in *n*-alkane assimilation, however, are not well understood.

Two models have been proposed for the uptake of *n*-alkanes by yeasts: one is a passive, diffusion-like mechanism facilitated by the hydrophobic properties of *n*-alkanes, while the other is an active, energy-dependent mechanism mediated by transporter proteins. Although the molecular mechanism of *n*-alkane uptake by yeasts is still unclear, the uptake of ^{14}C-labeled *n*-hexadecane by *Y. lipolytica* was shown to be upregulated by the incubation with *n*-decane and inhibited by KCN and 2,4-dinitrophenol (Bassel and Mortimer 1985). Mutations in the 16 loci were also shown to significantly reduce *n*-hexadecane uptake in *Y. lipolytica* (Bassel and Mortimer 1985). These results support the model of *n*-alkane incorporation across the plasma membrane by one or more energy-dependent transporters. A *Y. lipolytica* strain with an insertion mutation in *ABC1* encoding an ATP-binding cassette (ABC) transporter was shown to exhibit defective growth on *n*-hexadecane, but not on *n*-decane; however, the involvement of Abc1p in the uptake of *n*-hexadecane remains to be investigated (Thevenieau et al. 2007).

3 Terminal Hydroxylation of *n*-Alkanes by Cytochrome P450

Incorporated *n*-alkanes are transported from the plasma membrane to the ER, where they are hydroxylated to fatty alcohols by cytochromes P450 (P450s) belonging to the CYP52 family using molecular oxygen (Fig. 2) (Nelson 2009). Genes encoding

CYP52-family P450s have been identified in various *n*-alkane-assimilating yeasts, including *C. tropicalis* (Sanglard et al. 1987; Seghezzi et al. 1992; Seghezzi et al. 1991), *C. maltosa* (Ohkuma et al. 1991, 1995), *Candida albicans* (Kim et al. 2007; Panwar et al. 2001), *Candida dubliniensis*, *Candida parapsilosis*, *Debaryomyces hansenii* (Yadav and Loper 1999), *Lodderomyces elongisporus*, *Meyerozyma guilliermondii*, *Scheffersomyces stipitis*, *Starmerella bombicola* (Van Bogaert et al. 2009), and *Y. lipolytica* (Fig. 3) (Fickers et al. 2005; Hirakawa et al. 2009; Iida et al. 1998, 2000). A striking feature of the CYP52-family P450s in these yeasts is that they exist as multiple paralogs of the CYP52-family P450 gene. Eight genes encoding CYP52-family P450s have been identified in *C. tropicalis* and *C. maltosa*, and *Y. lipolytica* has twelve CYP52-family P450 genes. Furthermore, *C. albicans*, *C. dubliniensis*, *C. parapsilosis*, *D. hansenii*, *L. elongisporus*, *M. guilliermondii*, and *S. stipitis* have more than four genes encoding CYP52-family P450s. During the evolution of *n*-alkane-assimilating yeasts, the CYP52-family P450 genes may have multiplicated and functionally diversified to efficiently metabolize *n*-alkanes and their metabolites or to detoxify them (see following paragraph).

The CYP52-family P450s of *C. maltosa* are encoded by *ALK1–ALK8*, and quadruple deletion of *ALK1*, *ALK2*, *ALK3*, and *ALK5* was shown to cause defects in the utilization of *n*-alkanes for growth (Ohkuma et al. 1998). In *Y. lipolytica*, the CYP52-family P450s are encoded by *ALK1–ALK12*, and a mutant in which all twelve *ALK* genes are deleted completely lost the ability to grow on *n*-alkanes (Takai et al. 2012). These results clearly indicate the essential roles of the CYP52-family P450s in the assimilation of *n*-alkanes (Fig. 4). The *Y. lipolytica* deletion mutant of twelve *ALK* genes was furthermore shown not to be able to grow on *n*-decane even in the presence of glucose, suggesting that *n*-decane is toxic to the yeast cells and that the CYP52-family P450s are involved in the detoxification of *n*-decane (Takai et al. 2012).

Substrate specificities have been studied in a subset of the CYP52-family P450s of *C. tropicalis*, *C. maltosa*, *C. albicans*, and *Y. lipolytica*, and it has been shown that they have distinct substrate preferences (Fig. 3) (Eschenfeldt et al. 2003; Iwama et al. 2016; Kim et al. 2007; Ohkuma et al. 1998; Zimmer et al. 1996). In accordance with their involvement in *n*-alkane metabolism, some CYP52-family P450s were shown to preferentially hydroxylate *n*-alkanes, while, in contrast, a subset of CYP52-family P450s preferred hydroxylation of the ω-terminal end (ω-hydroxylation) of fatty acids. Some CYP52-family P450s hydroxylated both *n*-alkanes and the ω-terminal end of fatty acids. The amino acid sequences of Alk4 (CYP52A7) and Alk5 (CYP52A8) of *C. tropicalis* (Seghezzi et al. 1992); Alk5 (CYP52A9), Alk7 (CYP52A10), and Alk8 (CYP52A11) of *C. maltosa* (Zimmer et al. 1996, 1998); and CYP52A21 of *C. albicans* (Kim et al. 2007) – all of which were shown to preferentially hydroxylate the ω-terminal ends of fatty acids – showed significant sequence similarities (Fig. 3). Interestingly, it has been reported that CYP52A3 of *C. maltosa* catalyzes the oxidation of fatty alcohol and fatty aldehyde in addition to hydroxylating *n*-alkane and the ω-terminus of fatty acids (Scheller et al. 1998).

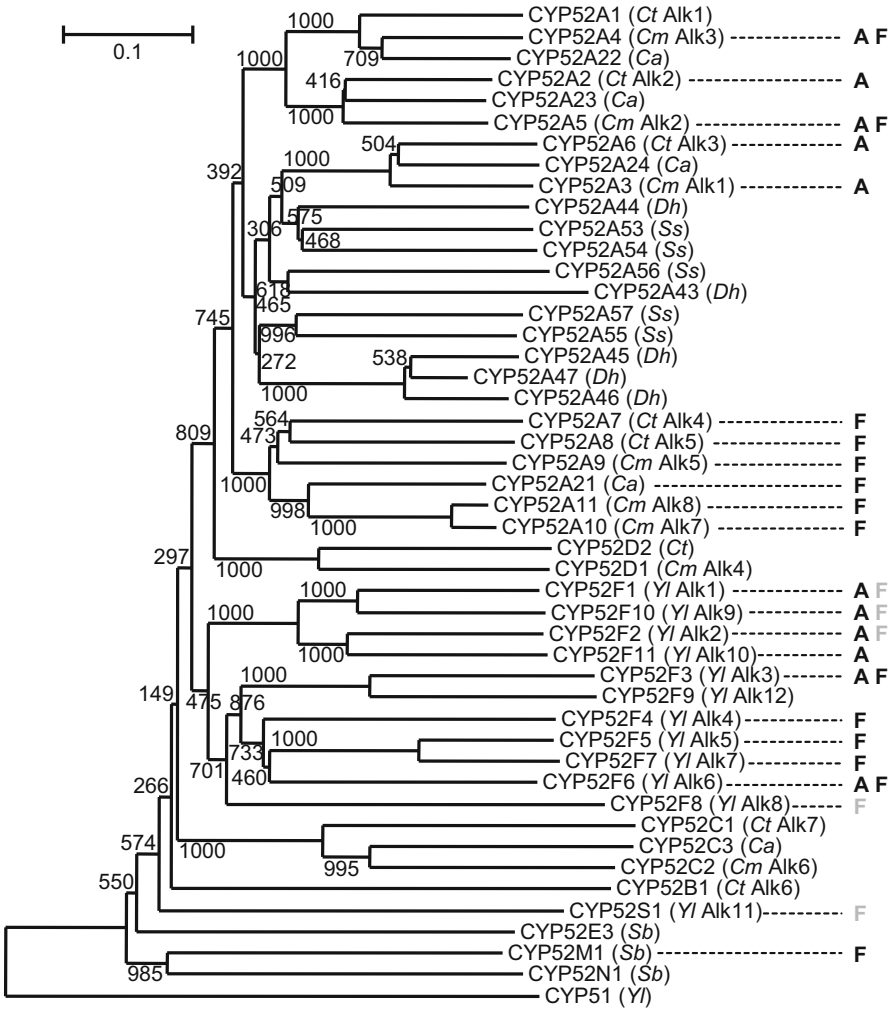

Fig. 3 Phylogenetic tree of the CYP52-family P450s in yeasts. Phylogenetic tree of the CYP52-family P450s of *n*-alkane-assimilating yeasts was constructed using ClustalW (DDBJ, v2.1) and drawn using NJplot. CYP51 of *Y. lipolytica* was used as an out-group. The scale bar indicates 0.05 substitutions per site. The bootstrap values by 1000 repetitions are indicated. The accession numbers of sequences from UniProtKB are as follows: CYP52A21 (Q59K96), CYP52A22 (Q5AAH7), CYP52A23 (Q5AAH6), CYP52A24 (Q5A8M1), CYP52C3 (Q5AGW4), CYP52A3 (P16496), CYP52A5 (Q12581), CYP52A4 (P16141), CYP52A9 (Q12586), CYP52A10 (Q12588), CYP52A11 (Q12589), CYP52C2 (Q12587), CYP52D1 (Q12585), CYP52A1 (P10615), CYP52A2 (P30607), CYP52A6 (P30608), CYP52A7 (P30609), CYP52A8 (P30610), CYP52B1 (P30611), CYP52C1 (P30612), CYP52D2 (Q874J0), CYP52A43 (Q6BVP2), CYP52A44 (Q6BVH7), CYP52A45 (Q6BNW0), CYP52A46 (Q6BNV9), CYP52A47 (Q6BNV8), CYP52A53 (A3LRT5), CYP52A54 (A3LR60), CYP52A55 (A3LS01), CYP52A56 (A3LZV9), CYP52A57 (A3LSP0), CYP52E3 (B8QHP3), CYP52M1 (B8QHP1), CYP52N1 (B8QHP5), CYP52F1 (O74127), CYP52F2 (O74128), CYP52F3 (O74129), CYP52F4 (O74130), CYP52F5 (O74131),

In *Y. lipolytica*, eleven CYP52-family P450s belong to the CYP52F subfamily and one belongs to the CYP52S subfamily. The CYP52F-subfamily P450s, Alk1p–Alk10p and Alk12p, appear to constitute a monophyletic clade in the phylogenetic tree of the CYP52-family P450s (Fig. 3). The CYP52F-subfamily P450s of *Y. lipolytica* can also be classified into four groups: P450s with significant *n*-alkane-hydroxylating activity, P450s with significant hydroxylating activity for the ω-terminus of dodecanoic acid, P450s with significant hydroxylating activity for both *n*-alkanes and dodecanoic acid, and P450s with faint or no oxidizing activity for these substrates. Alk1p, Alk9p, Alk2p, and Alk10p, which have been shown to exhibit substrate preferences for *n*-alkanes, share significant sequence similarities, while Alk5p and Alk7p, which have ω-hydroxylation activities to dodecanoic acid, are structurally similar (Iwama et al. 2016). Alk proteins that were shown to catalyze the oxidation of *n*-alkanes showed distinct preferences for different *n*-alkane chain lengths. Alk1p and Alk3p were shown to hydroxylate *n*-alkanes of various carbon numbers. Alk10p, too, oxidized *n*-alkanes of a wide range of lengths, but was shown to preferentially oxidize shorter-chain *n*-alkanes. Alk2p, Alk6p, and Alk9p, on the

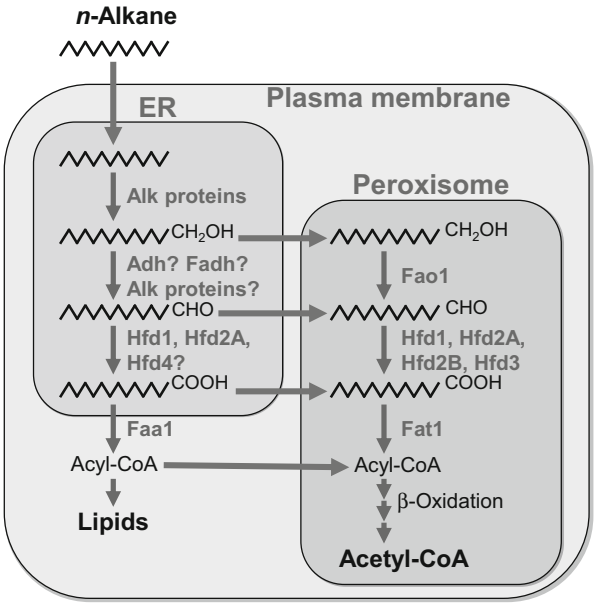

Fig. 4 *n*-Alkane metabolism in *Y. lipolytica*. See text for details

Fig. 3 (continued) CYP52F6 (O74132), CYP52F7 (O74133), CYP52F8 (O74134), CYP52F10 (A0A0K2S2A7), CYP52F11 (Q6CDW4), CYP52S1 (Q6CCE5), CYP52F9 (Q6CGD9), and CYP51 of *Y. lipolytica* (Q6CFP4). Species are indicated in parentheses as follows: *C. albicans* (*Ca*), *C. maltosa* (*Cm*), *C. tropicalis* (*Ct*), *D. hansenii* (*Dh*), *S. stipitis* (*Ss*), *S. bombicola* (*Sb*), and *Y. lipolytica* (*Yl*). P450s that were shown to catalyze the oxidation of *n*-alkanes or ω-termini of fatty acids are indicated as **A** or **F**, respectively

other hand, preferred longer-chain *n*-alkanes. In *Y. lipolytica*, the genes encoding CYP52-family P450s are likely to have multiplicated after diverging from ancestral *n*-alkane-assimilating yeasts carrying one or a small number of genes encoding CYP52-family P450s.

4 Oxidation of Fatty Alcohols to Fatty Aldehydes

In *n*-alkane-assimilating yeasts, fatty alcohols are thought to be oxidized to fatty aldehydes by NAD^+- or $NADP^+$-dependent fatty alcohol dehydrogenase (FADH) in the ER or by H_2O_2-producing fatty alcohol oxidase (FAOD) in the peroxisome (Fig. 2) (Barth and Gaillardin 1996; Fickers et al. 2005; Fukuda 2013; Fukuda and Ohta 2013; Mauersberger et al. 1996; Tanaka and Fukui 1989). Such FAODs have been detected and characterized in several *n*-alkane-assimilating yeasts including *C. tropicalis*, *C. maltosa*, *C. parapsilosis*, and *Y. lipolytica* (Dickinson and Wadforth 1992; Kemp et al. 1994; Mauersberger et al. 1992). The FAOD-coding genes *FAOT*, *FAO1*, and *FAO2* have been identified in *C. tropicalis* (Cheng et al. 2005; Eirich et al. 2004; Vanhanen et al. 2000). A *FAOT* deletion mutant exhibited defective growth on *n*-octadecane but not on shorter-chain *n*-alkanes or fatty acids, suggesting that FAOD encoded by *FAOT* is involved in the oxidation of fatty alcohol that is produced in the metabolism of *n*-octadecane and that one or more other enzymes are involved in the oxidation of shorter-chain *n*-alkanes (Cheng et al. 2005).

In the genome sequence of *Y. lipolytica*, eight alcohol dehydrogenase genes, *ADH1–ADH7* and *FADH*, and a fatty alcohol oxidase gene, *FAO1*, were identified (Gatter et al. 2014). Among these genes, a triple deletion mutant of *ADH1*, *ADH3*, and *FAO1* showed severely defective growth on 1-dodecanol and 1-tetradecanol, but not on dodecanoic acid or tetradecanoic acid, suggesting that Adh1p, Adh3p, and Fao1p are involved in the assimilation of exogenous fatty alcohols (Iwama et al. 2015). Microscopic observation suggested that Fao1p localizes in the peroxisome. Adh1p and Adh3p are cytosolic proteins, but substantial amounts of these proteins were recovered in the membrane fraction of cell extracts, raising the possibility that Adh1p and Adh3p transiently localize to the membranes, possibly to the ER. A deletion mutant of *ADH1–ADH7*, *FADH*, and *FAO1* exhibited slightly defective growth on *n*-decane and *n*-dodecane, but not on longer-chain *n*-alkanes. These results imply that any one or more of the enzymes encoded by these genes catalyze the oxidation of fatty aldehydes produced in the metabolism of shorter-chain *n*-alkanes (Fig. 4), but that one or more other enzymes are also involved in the oxidation of fatty alcohols derived from *n*-alkanes.

5 Oxidation of Fatty Aldehydes to Fatty Acids

In *n*-alkane-assimilating yeasts, fatty aldehydes generated during *n*-alkane metabolism are oxidized to fatty acids by fatty aldehyde dehydrogenase (FALDH) in the ER or in the peroxisome (Fig. 2). The model yeast *S. cerevisiae* has a single

FALDH-coding gene, *HFD1*. *S. cerevisiae* Hfd1 and its mammalian ortholog ALDH3A2 are involved in the conversion of hexadecenal to hexadecenoic acid during the degradation of sphingosine-1-phosphate, a metabolite of sphingolipids and a second messenger involved in various cellular processes (Nakahara et al. 2012). ALDH3A2 also plays a role in the degradation of phytanic acid in the peroxisome (Ashibe et al. 2007; Verhoeven et al. 1998) as well as in protection from oxidative stress associated with lipid peroxidation (Demozay et al. 2004). Mutations in *ALDH3A2* have been shown to cause Sjögren-Larsson syndrome (De Laurenzi et al. 1996). Hfd1 and ALDH3A2 belong to a superfamily of NAD(P)$^+$-dependent aldehyde dehydrogenases (Sophos et al. 2001).

In *n*-alkane-assimilating yeasts, FALDH activities have been reported in *Candida intermedia*, *C. tropicalis*, and *Y. lipolytica* (Liu and Johnson 1971; Ueda and Tanaka 1990; Yamada et al. 1980). Genes encoding FALDHs that are involved in the assimilation of *n*-alkanes were identified in *Y. lipolytica* (Iwama et al. 2014). The genome of *Y. lipolytica* contains four orthologs, *HFD1–HFD4*, of *S. cerevisiae HFD1* and mammalian *ALDH3A2*. A *Y. lipolytica* mutant lacking all four *HFD* genes did not grow on *n*-alkanes of 12–18 carbons and showed severe growth defects on *n*-alkanes of 10 and 11 carbons. The expression of any one of these genes, however, restored the growth of the deletion mutant on *n*-alkanes. Furthermore, bacterially produced Hfd proteins exhibited dehydrogenase activities to dodecanal and tetradecanal *in vitro*. Fluorescence microscopic analysis suggested that Hfd1p localizes to the ER and the peroxisome and that Hfd3p localizes to the peroxisome. Two *HFD2* transcript variants, which encode Hfd2Bp containing a peroxisomal targeting signal 1 (PTS1)-like sequence at its C-terminus and Hfd2Ap without PTS1, were generated from *HFD2*. Hfd2Ap has been suggested to localize in the ER and the peroxisome, while Hfd2Bp localizes to the peroxisome. These results imply that Hfd proteins are involved in the oxidation of fatty aldehydes produced during metabolism of *n*-alkanes in the ER and the peroxisome (Fig. 4) (Iwama et al. 2014); however, growth of the quadruple deletion mutant of *HFD* genes on dodecanal or tetradecanal indicated that one or more other enzymes are involved in the assimilation of exogenous fatty aldehydes.

The *n*-alkane-assimilating yeasts, *C. tropicalis*, *C. albicans*, *C. dubliniensis*, *C. parapsilosis*, *L. elongisporus*, and *M. guilliermondii*, have multiple FALDH genes. Although involvement of these FALDH genes in the assimilation of *n*-alkanes remains to be examined, it is possible that they were multiplicated to efficiently degrade fatty aldehydes produced during the metabolism of *n*-alkanes.

6 Activation and Utilization of Fatty Acids

Fatty acids play a critical role as hydrophobic moieties in lipid molecules that constitute biological membranes or are used as energy and carbon sources via β-oxidation. Fatty acids are also involved in a variety of cellular processes as precursors of signaling molecules and hormones as well as by acylation of proteins. Fatty acids are used for these processes in the forms of acyl-CoAs, which are

synthesized from fatty acids and coenzyme A by acyl-CoA synthetase (ACS). Multiple ACS isozymes are encoded by the genomes of eukaryotes including yeasts, and some ACS isozymes exhibit distinct substrate specificities and subcellular distributions (Black and DiRusso 2007; Soupene and Kuypers 2008; Watkins and Ellis 2012). *Y. lipolytica* has five ACSs, Faa1p and Fat1p–Fat4p, and ten ACS-like enzymes, Aa11p–Aal10p (Dulermo et al. 2014, 2016; Tenagy et al. 2015; Wang et al. 2011). A deletion mutant of *FAT1* exhibited severely defective growth on *n*-decane of 10 and 12 carbons and partially defective growth on 14 and 16 carbons. The *FAA1* deletion mutant exhibited retarded growth on *n*-alkane of 16 carbons, while deletion mutants of other ACS genes did not show any defects on *n*-alkanes. In addition, a double deletion mutant of *FAT1* and *FAA1* showed severe growth defects on *n*-alkanes of 10–18 carbons (Tenagy et al. 2015). These results suggest that Faa1p and Fat1p play critical roles in the activation of fatty acids produced during *n*-alkane assimilation (Fig. 4). The wild-type strain of *Y. lipolytica* was shown to grow in the presence of cerulenin, an inhibitor of fatty acid synthesis, when *n*-octadecane was supplemented, suggesting that the stearic acid produced by the oxidation of *n*-octadecane is activated to stearoyl-CoA and that stearoyl-CoA or its derivatives support the growth of *Y. lipolytica*. The *FAA1* deletion mutant, however, did not grow in the presence of cerulenin and *n*-octadecane, suggesting that *FAA1* is involved in the activation of fatty acids derived from *n*-alkanes for essential cellular processes including membrane lipid synthesis. Fluorescent microscopic observation and fractionation analysis of cell extracts suggested that Fat1p localizes in the peroxisome, in agreement with the presence of a PTS1-like sequence at its C-terminus, while Faa1p localizes in the cytosol and to membranes. Roles of ACS isozymes of other *n*-alkane-assimilating yeasts in the metabolism of *n*-alkanes remain to be elucidated.

7 Research Needs

The oxidation pathway of *n*-alkanes to fatty acids in yeasts has been studied primarily in *C. tropicalis*, *C. maltosa*, and *Y. lipolytica*. Apart from the enzymes that are involved in the oxidation of fatty alcohols produced through the hydroxylation of *n*-alkanes, the enzymes that catalyze the steps in the oxidation of *n*-alkanes to fatty acids have been identified. As mentioned already, CYP52A3 of *C. maltosa* reportedly catalyzes the cascade of the sequential oxidation of *n*-hexadecane to hexadecanoic acid (Scheller et al. 1998). Accordingly, the CYP52-family P450s may catalyze the sequential oxidation of fatty alcohols to fatty acids in other *n*-alkane-assimilating yeasts. The aerobic degradation of *n*-alkanes and the enzymes involved in this degradation have been studied in only a limited subset of ascomycetous yeasts (*C. tropicalis*, *C. tropicalis*, and *Y. lipolytica*), while this process in other yeasts, particularly basidiomycetous yeasts, remains to be characterized.

The metabolism of *n*-alkanes is regulated at the transcriptional level in *C. tropicalis*, *C. maltosa*, and *Y. lipolytica*. Transcription of genes encoding a subset of enzymes involved in the *n*-alkane metabolism is upregulated in the presence of

n-alkanes in these yeasts (Endoh-Yamagami et al. 2007; Hirakawa et al. 2009; Iida et al. 1998, 2000; Kobayashi et al. 2013, 2015; Mori et al. 2013; Ohkuma et al. 1991, 1995; Sanglard et al. 1987; Yamagami et al. 2004). Details of the transcriptional regulation of *n*-alkane metabolic genes in yeasts are described and discussed in other chapters.

Two organelles, the ER and the peroxisome, are involved in the metabolism of *n*-alkanes in yeasts. Fundamental and critical questions that remain to be answered about these organelles in the context of *n*-alkane metabolism in yeasts are (1) how *n*-alkanes are imported into cells and transported to the ER and (2) how hydrophobic metabolites, fatty alcohols, fatty aldehydes, and fatty acids are transported from the ER to the peroxisome during *n*-alkane metabolism. *C. maltosa* and *C. tropicalis* produce dicarboxylic acids from *n*-alkanes and excrete them into culture medium (Arie et al. 2000; Tanaka and Fukui 1989). In a dicarboxylic acid-hyperproducing mutant of *C. maltosa*, the transcription of a *CmCDR1* gene encoding an ABC transporter is highly activated in the later phase of culture on *n*-dodecane (Sagehashi et al. 2013). It would be of interest to test whether overexpression of *CmCDR1* improves the efficiency of dicarboxylic acid production from *n*-alkane. The uptake, intracellular transport, and excretion of lipophilic compounds in eukaryotic cells are important and fundamental issues of basic cell biology. The elucidation and subsequent optimization of the mechanisms of those processes will contribute to improvements in efficiency of the production of useful compounds from *n*-alkanes.

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