Genetic Features and Regulation of *n*-Alkane Metabolism in Yeasts

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Ryouichi Fukuda and Akinori Ohta

Contents

Abstract

The yeasts *Candida tropicalis*, *Candida maltosa*, and *Yarrowia lipolytica* have an excellent ability to use *n*-alkanes as the sole carbon and energy source. Here, we summarize the current knowledge of the genetic features and regulation of *n*-alkane metabolism in these yeasts. The transcription of genes encoding the CYP52-family cytochromes P450 that catalyze the initial hydroxylation of *n*-alkanes has been shown to be activated when these yeasts are cultured in the presence of *n*-alkanes. In *Y. lipolytica*, the transcription of *ALK1*, the gene

R. Fukuda (✉)
Department of Biotechnology, The University of Tokyo, Tokyo, Japan
e-mail: afukuda@mail.ecc.u-tokyo.ac.jp

A. Ohta
Department of Biological Chemistry, College of Bioscience and Biotechnology, Chubu University, Kasugai, Aichi, Japan
e-mail: aaohta@isc.chubu.ac.jp

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encoding P450, is activated by a complex composed of two basic helix-loop-helix transcription activators Yas1p and Yas2p through a promoter element ARE1. This transcription is regulated by an Opi1-family transcriptional repressor Yas3p. In the absence of *n*-alkanes, Yas3p binds to Yas2p in the nucleus thereby repressing the transcription of *ALK1*. However, in the presence of *n*-alkanes, Yas3p is sequestered to the endoplasmic reticulum to derepress the transcription of the gene.

1 Introduction

A variety of microorganisms, including certain species of yeasts, has developed metabolic systems to assimilate *n*-alkanes containing 10–18 carbons as the sole carbon and energy source. The degradation pathway of *n*-alkanes in yeasts and the enzymes involved in it have been extensively studied in *Candida tropicalis* (Tanaka and Fukui 1989), *Candida maltosa* (Mauersberger et al. 1996), and *Yarrowia lipolytica* (Barth and Gaillardin 1996, 1997; Fickers et al. 2005; Fukuda 2013; Fukuda and Ohta 2013; Nicaud 2012) as described in Chap. 6, ▶ "Enzymes for Aerobic Degradation of Alkanes in Yeasts" by Fukuda and Ohta. In these yeasts, *n*-alkanes are first hydroxylated to fatty alcohols in the endoplasmic reticulum (ER) by cytochromes P450 belonging to the CYP52 family (Figs. 2 and 4 of the Chap. 6, ▶ "Enzymes for Aerobic Degradation of Alkanes in Yeasts" by Fukuda and Ohta). Fatty alcohols are then oxidized to fatty aldehydes by fatty alcohol dehydrogenase (FADH) in the ER or by fatty alcohol oxidase (FAO) in the peroxisome. Fatty aldehydes are further oxidized to fatty acids by fatty aldehyde dehydrogenase (FALDH) in the ER or the peroxisome, and then finally activated to acyl-CoAs by acyl-CoA synthetase (ACS). These activated fatty acids are then utilized for lipid synthesis, or degraded in the peroxisome via β-oxidation.

It is crucial for an organism to respond to and adapt rapidly to environmental changes for survival. In *C. tropicalis*, *C. maltosa*, and *Y. lipolytica*, the metabolism of *n*-alkanes was found to be regulated at the transcriptional level. The transcription of the genes encoding enzymes involved in the metabolism of *n*-alkanes was found to be activated in the presence of *n*-alkanes. The mechanism of the transcription regulation in these yeasts is a very interesting subject, particularly because the regulation of transcription by hydrophobic compounds in lower eukaryotes remains largely elusive. This chapter will focus on the molecular mechanisms of the transcriptional regulation of genes involved in the degradation of *n*-alkanes in these yeasts.

2 Response to *n*-Alkanes in *n*-Alkane-Assimilating Yeasts

Experiments conducted in the 1970s showed that the production of cytochromes P450 was induced when *C. tropicalis* was cultured in a medium containing *n*-tetradecane (Lebeault et al. 1971). Cloning and subsequent characterization of

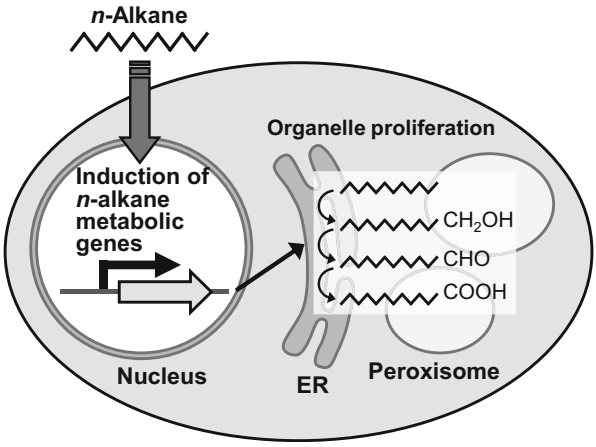

Fig. 1 Response to *n*-alkanes in *n*-alkane-assimilating yeasts. When *n*-alkane-assimilating yeasts are cultured in the medium containing *n*-alkane, the transcriptional induction of genes involved in the degradation of *n*-alkanes and the proliferation of the ER and the peroxisome, in which *n*-alkanes are degraded, are observed

the genes in *C. tropicalis* revealed that this yeast has at least eight genes encoding the CYP52-family P450s, (*ALK1–ALK7* and *CYP52D2*) and that the transcription of four of them (*ALK1–ALK3* and *ALK5*) is activated by *n*-alkanes (Fig. 1) (Craft et al. 2003; Nelson 2009; Sanglard et al. 1987;Seghezzi et al. 1991, 1992). In *C. maltosa*, of the eight genes (*ALK1–ALK8*) encoding the CYP52-family P450s, the transcription of all except *ALK4* was induced by *n*-alkanes (Ohkuma et al. 1991, 1995a). *Y. lipolytica* has 12 genes (*ALK1–ALK12*) encoding the CYP52-family P450s, transcription of nine of which (*ALK1–ALK6*, *ALK9*, *ALK11*, and *ALK12*) was induced by *n*-alkanes (Hirakawa et al. 2009; Iida et al. 1998, 2000; Iwama et al. 2016; Takai et al. 2012). The transcriptional induction of the P450 genes by *n*-alkanes was also observed in sophorolipid-producing yeast *Candida bombicola* (Van Bogaert et al. 2009).

The transcription of genes involved in the degradation of *n*-alkane metabolites was also found to be induced in the presence of *n*-alkanes in *Y. lipolytica* (Fig. 4 of the Chap. 6, ▶ "Enzymes for Aerobic Degradation of Alkanes in Yeasts" by Fukuda and Ohta). In *Y. lipolytica*, the transcription of *ADH1* and *ADH3*, encoding alcohol dehydrogenases, and *FAO1*, encoding a fatty alcohol oxidase, is upregulated in the presence of *n*-alkanes (Iwama et al. 2015). In addition, transcription of three (*HFD1–HFD3*) of the four genes (*HFD1–HFD4*) encoding fatty aldehyde dehydrogenases and *FAA1* and *FAT1* encoding ACSs was increased in the presence of *n*-alkanes (Iwama et al. 2014; Tenagy et al. 2015). The transcription of *PAT1* encoding a peroxisomal acetoacetyl-CoA thiolase involved in β-oxidation was also induced by *n*-alkanes (Yamagami et al. 2001). These results confirm that the transcription of genes important for the *n*-alkane degradation is activated in response to *n*-alkanes. Our transcriptome analysis in *Y. lipolytica* cells cultured in medium containing either glucose or *n*-decane suggested that the transcripts of approximately 500 genes were increased more than twofold in response to *n*-decane (our unpublished results).

n-Alkanes have also been reported to induce the proliferation of the ER and peroxisome in the *n*-alkane-assimilating yeasts (Fig. 1) (Mauersberger et al. 1987;

Osumi et al. 1974; Vogel et al. 1992). Proliferation of the peroxisome by fatty acids has been widely observed in various organisms, including *C. tropicalis*, *Y. lipolytica*, and *Saccharomyces cerevisiae*, and its mechanism has been extensively studied (Gurvitz and Rottensteiner 2006). In contrast, the mechanism underlying the proliferation of the ER by *n*-alkanes in *n*-alkane-assimilating yeasts remains poorly understood. Interestingly, overproduction of the CYP52-family P450 induced the proliferation of the ER in *C. maltosa* and *S. cerevisiae* (Ohkuma et al. 1995b; Schunck et al. 1991). Proliferation of the ER due to overproduction of various membrane proteins has been reported (Federovitch et al. 2005) and may be one of the quality control mechanisms of the ER to avoid over accumulation of proteins in the ER membrane.

3 Mechanism of Transcriptional Activation of Genes Responsible for *n*-Alkane Degradation in *Y. lipolytica*

The transcriptional induction of the P450 genes in response to *n*-alkane was first identified in yeasts of the *Candida* genus as described above. However, the molecular mechanism of its regulation remains unclear. This is largely due to the difficulty in obtaining mutant strains defective for transcriptional activation in the presence of *n*-alkanes, since *C. tropicalis* and *C. maltosa* are diploid or partial diploid yeasts, in which teleomorphs have not been found. Furthermore, the CUG codon has been shown to code for serine instead of leucine in *C. tropicalis* and *C. maltosa*, as well as in other *n*-alkane-assimilating yeasts phylogenetically close to them, including *Candida albicans*, *Candida dubliniensis*, *Candida parapsilosis*, *Debaryomyces hansenii*, *Lodderomyces elongisporus*, and *Meyerozyma guilliermondii* (Massey et al. 2003; Sugiyama et al. 1995; Ueda et al. 1994), and this poses an obstacle to the analysis of DNA-protein and/or protein-protein interaction using *S. cerevisiae* system.

Y. lipolytica, on the other hand, has a teleomorph and a stable haploid and diploid life cycle, and genetic methods that permit isolation and characterization of mutants as well as molecular biology methods are well established in it (Barth and Gaillardin 1996, 1997). Among the yeasts that can assimilate *n*-alkanes, the genome sequences were determined initially in *Y. lipolytica*, *D. hansenii* (Dujon et al. 2004), and *C. albicans* (Jones et al. 2004), followed by *C. tropicalis* and others (Butler et al. 2009). As a result of these advantages, the mechanism of the transcriptional regulation by *n*-alkane has been elucidated in *Y. lipolytica*.

3.1 Promoter Elements of the CYP52-Family P450 Gene Involved in *n*-Alkane Response

The transcriptional regulation in response to *n*-alkanes has been investigated by studying *ALK1* encoding the primary CYP52-family P450 in the assimilation of *n*-alkanes in *Y. lipolytica* (Iida et al. 1998, 2000; Iwama et al. 2016; Takai et al.

2012). The transcription of *ALK1* was highly activated in the presence of *n*-alkanes and the transcript level was found to be highest among the 12 *ALK* genes (Hirakawa et al. 2009). Promoter analysis of *ALK1* led to the identification of a sequence named ARR1 (alkane responsive region 1), which is involved in the transcription activation (Sumita et al. 2002). ARR1 was found to contain two elements, ARE1 (alkane responsive element 1) and ARE2. In electrophoretic mobility shift assay, specific shift bands corresponding to both ARE1 and ARE2 could be identified with cellular extracts of *Y. lipolytica* cells cultured in *n*-alkane-containing medium. ARE1 contains a sequence similar to the E-box motif, the consensus sequence to which basic helix-loop-helix (bHLH) transcription factors interact (Murre et al. 1994), and was found to play a critical role in the transcriptional activation process (see below). Interestingly, ARE1-like sequences were found in the promoter regions of various genes involved in *n*-alkane metabolism (Yamagami et al. 2004). In contrast, the role of ARE2 is still unknown.

3.2 Transcription Activators Involved in *n*-Alkane Response

A gene *YAS1* encoding a transcription factor that activates the ARE1-mediated transcription was identified by the analysis of a mutant defective in the transcriptional activation through ARE1 by *n*-alkanes and in the growth on *n*-alkanes (Yamagami et al. 2004). *YAS1* encodes a bHLH transcription factor of 137 amino acids (Fig. 2). A deletion mutant of *YAS1* showed defects in the transcription activation of *ALK1* by *n*-alkanes. However, Yas1p did not bind to ARE1 in vitro. bHLH transcription factors generally form homo- or heterodimers through their HLH regions and interact with the E-box motif through the basic regions (Murre et al. 1994). Indeed, a gene named *YAS2* encoding a 700-amino acid protein that contains a bHLH motif similar to that of Yas1 was identified from the genome database. This gene was found to be involved in the ARE1-mediated transcriptional activation (Fig. 2) (Endoh-Yamagami et al. 2007). The deletion mutant of *YAS2* was also defective in the transcription induction of *ALK1* by *n*-alkanes. Yas1p and Yas2p formed a complex in vitro and bound to ARE1 only when both proteins existed. Yas1p and Yas2p constitutively localized in the nucleus (Hirakawa et al. 2009; Yamagami et al. 2004). These results suggest that the complex of Yas1p and Yas2p binds to ARE1 and activates the transcription of *ALK1* in response to *n*-alkane. Deletion mutants of *YAS1* or *YAS2* did not have the ability to grow on *n*-alkanes, indicating the importance of Yas1p-Yas2p complex in the ARE1-mediated transcriptional activation.

3.3 Regulation of *n*-Alkane Metabolic Genes by the Opi1-Family Transcription Factor

The bHLH motifs of Yas1p and Yas2p show sequence similarities to Ino4p and Ino2p, respectively, of *S. cerevisiae*. In *S. cerevisiae*, these function as transcription

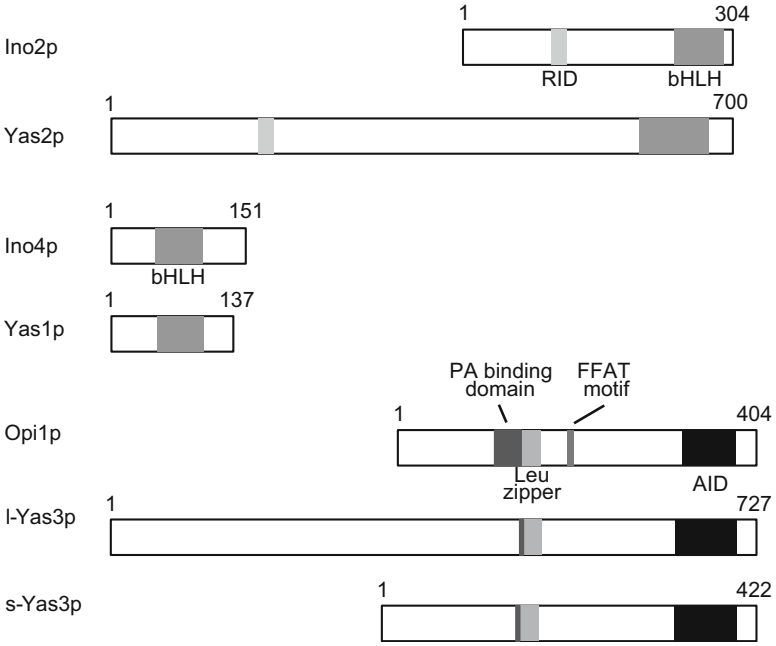

Fig. 2 Schematic diagrams of bHLH transcription activators and Opi1-family proteins in *S. cerevisiae* and *Y. lipolytica*. The bHLH motifs, repressor interaction domains (RID), PA-binding domains, FFAT motifs, leucine zipper motifs, and activator interaction domains (AID) are indicated by *grey boxes*

activators regulating genes involved in phospholipid synthesis. In this yeast, transcription of phospholipid synthetic genes, including *INO1* encoding inositol-3-phosphate synthase, is activated in the absence of *myo*-inositol and repressed in the presence of it (Henry et al. 2012). A heterodimer of Ino2p and Ino4p constitutively binds to an upstream activating element, UAS_{INO}/ICRE (inositol choline responsive element), in the promoter regions of the target genes. The transcription repressor Opi1p binds through its activator interaction domain (AID) to the repressor interaction domain (RID) of Ino2p (Heyken et al. 2005). Loewen et al. proposed a model in which Opi1p is retained to the ER by binding to phosphatidic acid (PA) using its PA-binding domain and to an ER membrane-spanning protein Scs2p through its FFAT (two phenylalanine in an acidic tract) motif in the absence of *myo*-inositol. This leads to the activation of transcription of the phospholipid synthesis genes by the Ino2-Ino4 complex (Loewen et al. 2004). However, in the presence of *myo*-inositol, PA is utilized for the synthesis of phosphatidylinositol (PI). Opi1p is then released from the ER and transported to the nucleus where it binds to Ino2p to repress the transcription. Therefore, Opi1p is the key regulator controlling transcription of genes responsible for phospholipid biosynthesis in response to *myo*-inositol.

In the *Y. lipolytica* genome database, an ortholog of *OPI1* was identified and named as *YAS3* (Hirakawa et al. 2009). Two different transcripts with different transcription initiation sites were obtained from *YAS3*. These transcripts were predicted to encode a long form of Yas3p (l-Yas3p of 727 amino acids) and a short form of Yas3p (s-Yas3p of 422 amino acids) (Fig. 2). The AID, PA-binding domain, and leucine zipper domain found in Opi1p were conserved in both forms of Yas3p, but the FFAT motif was absent in Yas3p (Fig. 2). Whether these two forms of Yas3p have different functions is still not clear, but it has been shown that the s-Yas3p is sufficient to regulate the transcription of *ALK1*. The deletion mutant of *YAS3* accumulated much more transcripts of *ALK1* than the wild-type strain, even when cells were cultured in the medium containing glucose or glycerol. Yas3p interacted with Yas2p, but not with Yas1p. In line with this observation, a RID-like sequence motif was found in Yas2p (Fig. 2). In addition, while Yas3p was localized in the nucleus when cultured in the medium containing glucose, it was sequestered to the ER in the presence of *n*-alkanes. Among the 12 *ALK* genes, the transcription of *ALK1*, *ALK2*, *ALK4*, *ALK6*, *ALK9*, and *ALK11* appeared to be regulated by the Yas1p-Yas2p-Yas3p system. In contrast, Yas3p was not involved in the transcriptional regulation of *INO1* by *myo*-inositol.

A question remained as to why Yas3p remained localized to the ER in the presence of *n*-alkanes. Yas3p was found to bind to PA and phosphoinositides (PIPs), particularly to phosphatidylinositol 4-phosphate (PI(4)P), in vitro, but not to *n*-alkanes (Kobayashi et al. 2013). In addition, the ARE1-mediated transcription was upregulated in mutants defective for an ortholog of *S. cerevisiae PAH1*, encoding PA phosphatase and an ortholog of *SAC1*, encoding PIP phosphatase in the ER. These results suggest that Yas3p is localized to the ER by binding to PA and/or PIP in the ER membrane. In contrast to *S. cerevisiae*, an ortholog of *SCS2* or its paralog, *SCS22*, was not required for the transcriptional activation of *ALK1* by *n*-alkanes in line with the absence of FFAT motif-like sequence in Yas3p, although the deletion mutant of *SCS2* exhibited a growth defect when cultured on *n*-decane (Kobayashi et al. 2008). Based on these results, a model of the transcriptional regulation of *n*-alkane metabolic genes was proposed (Fig. 3). A heterocomplex of the bHLH transcription activators, Yas1p and Yas2p, constitutively localizes in the nucleus and binds to ARE1 in the promoter regions of the genes involved in the *n*-alkane metabolism. In the absence of *n*-alkanes, Yas3p is transported to the nucleus and binds to Yas2p of the Yas1p-Yas2p complex, resulting in repression of the ARE1-dependent transcription. When the medium is supplemented with *n*-alkanes, Yas3p is retained to the ER by binding to PA and/or PIP, and the transcription is activated by Yas1p-Yas2p complex. It remains to be determined whether the amounts of PA and PIPs in the ER membrane increase in response to *n*-alkanes. Scs2p is not involved in this process, but it is possible that other ER-resident protein is involved in the localization of Yas3p to the ER. This is suggested by the fact that the C-terminal region of Yas3p was found to be localized to the ER in a PA- and PI(4)P-independent manner (Kobayashi et al. 2015).

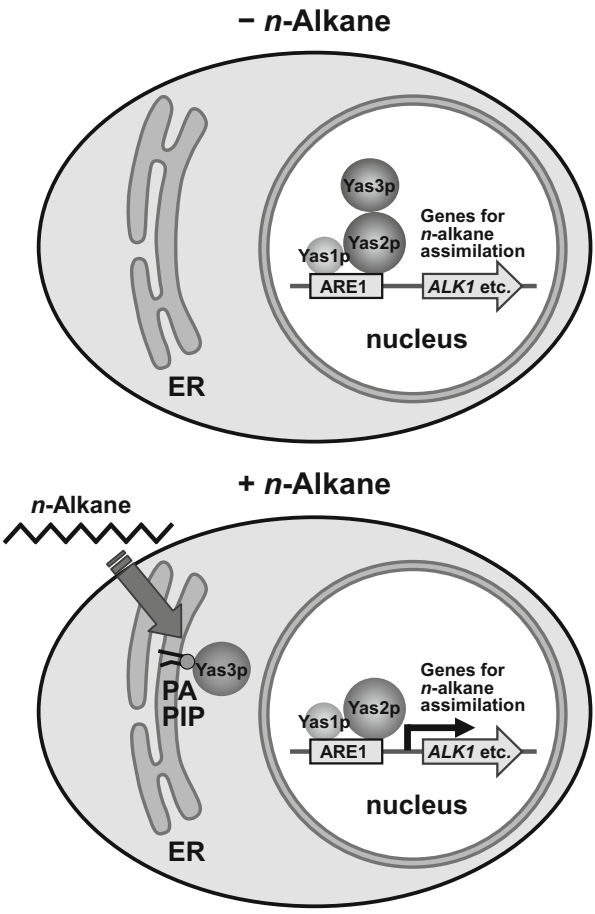

Fig. 3 Model of *n*-alkane-responsive transcriptional regulation by the transcription factors Yas1p, Yas2p, and Yas3p in *Y. lipolytica*. In the absence of *n*-alkanes (*upper*), Opi1-family transcription repressor Yas3p binds to the complex composed of bHLH transcription activators Yas1p and Yas2p in the nucleus, and ARE1-dependent transcription is repressed. In the presence of *n*-alkanes (*lower*), Yas3p is sequestered to the ER membrane through the interaction with PA and/or PIP, and ARE1-dependent transcription is activated by Yas1p-Yas2p complex

3.4 Role of the Opi1-Family Proteins in Other Yeasts

Orthologs of *OPI1* exist in the genomes of a variety of yeasts (Fig. 4), although their functions are largely unknown. In *Candida glabrata*, a yeast closely related to *S. cerevisiae* phylogenetically, a homolog of Opi1p is involved in the transcriptional regulation of an ortholog of *INO1* by *myo*-inositol (Bethea et al. 2010). In contrast, Opi1p homolog does not regulate *INO1* expression in *C. albicans*, but it controls the expression of *SAP2* encoding the secreted aspartyl protease and is involved in the filamentous growth and virulence (Chen et al. 2015). Therefore, Opi1-family proteins possibly regulate processes other than phospholipid synthesis. *n*-Alkane assimilating yeasts *C. tropicalis*, *C. maltosa*, *C. dubliniensis*, *C. parapsilosis*, *D. hansenii*, *L. elongisporus*, and *M. guilliermondii* all have Opi1-family proteins, and it would be of great interest to examine whether these orthologs are involved in the transcriptional regulation of *n*-alkane metabolism in these yeasts.

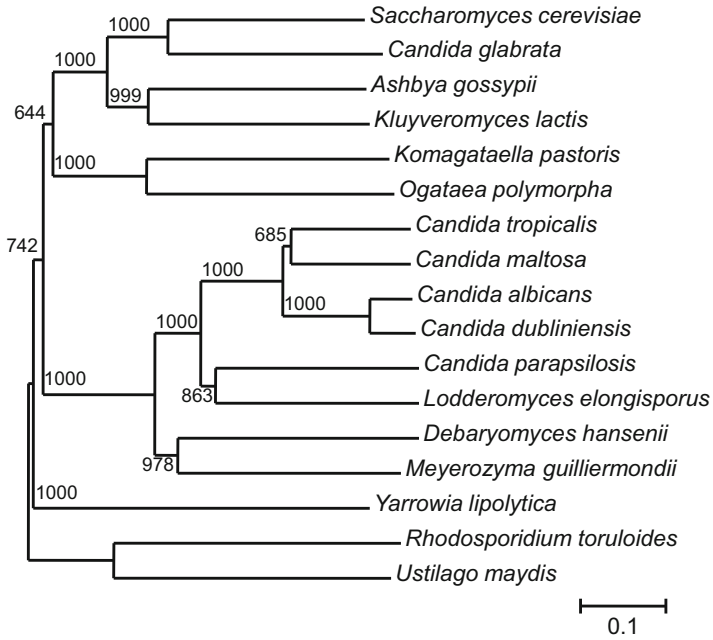

Fig. 4 Phylogenetic tree of the Opi1-family proteins in yeasts. Phylogenetic tree of the Opi1-family proteins of yeasts was constructed using ClustalW (DDBJ, v2.1) and drawn using Njplot. The *scale bar* indicates 0.1 substitutions per site. The bootstrap values by 1000 repetitions are indicated. The accession numbers of sequences from UniProtKB are as follows: *Ashbya gossypii* (Q75DH7), *Candida albicans* (Q5ALN4), *Candida dubliniensis* (B9WAR2), *Candida glabrata* (Q6FN27), *C. maltosa* (M3JFB2), *Candida parapsilosis* (G8BGL1), *C. tropicalis* (C5M6C2), *Debaryomyces hansenii* (Q6BJD0), *Kluyveromyces lactis* (Q6CIM8), *Komagataella pastoris* (A0A1B2J6H0), *Lodderomyces elongisporus* (A5DT94), *Meyerozyma guilliermondii* (A5DPS9), *Ogataea polymorpha* (A0A1B7SCJ0), *Rhodosporidium toruloides* (M7XL84), *S. cerevisiae* (P21957), *Ustilago maydis* (A0A0D1CM93), and *Y. lipolytica* (B9X0I4)

4 Repression of Transcription of *n*-Alkane Metabolic Genes

Glucose is the primary source of carbon and energy for most organisms, and the transcription of genes involved in other carbon and energy source metabolism remains repressed in the presence of glucose. Transcriptional repression of genes involved in *n*-alkane metabolism by glucose is observed in *n*-alkane-assimilating yeasts. In *C. tropicalis* and *C. bombicola*, expression of a subset of the CYP52-family P450 genes induced by *n*-alkanes is repressed by glucose (Seghezzi et al. 1992; Van Bogaert et al. 2009). In *C. maltosa*, transcription of most of the *ALK* genes is severely repressed by glucose, but not by glycerol (Ohkuma et al. 1995a). The carbon catabolite repression by glucose has been well documented in *S. cerevisiae* (Conrad et al. 2014; Kayikci and Nielsen 2015), but it remains to be elucidated

whether the transcription of the P450 genes is repressed by a similar mechanism in these *n*-alkane-assimilating yeasts.

In marked contrast to these yeasts, in *Y. lipolytica*, the transcription of genes involved in *n*-alkane metabolism is strictly repressed by glycerol, but not so much by glucose (Iida et al. 1998, 2000; Mori et al. 2013). In line with this observation, glycerol is a preferred carbon and energy source for this organism, and it shows better growth on glycerol than on glucose (Mori et al. 2013). The transcriptional repression by glycerol was also observed in *Kluyveromyces lactis*, in which the transcription of *KlICL1* encoding isocitrate lyase was repressed by glycerol (Rodicio et al. 2008). The molecular mechanisms underlying the transcriptional repression by glycerol is unclear, but it was shown that phosphorylation of glycerol is required for the glycerol repression in both these yeasts (Mori et al. 2013; Rodicio et al. 2008).

5 Research Needs

It has been revealed that, in *Y. lipolytica*, the Opi1-family protein Yas3p plays a pivotal role in the transcriptional regulation of genes involved in *n*-alkane metabolism by *n*-alkanes. However, it remains to be clarified how *n*-alkanes are recognized and how these signals are transduced retaining Yas3p to the ER. Transcriptional activation of the ARE1-containing promoter by *n*-alkane was also observed in the deletion mutant of the 12 *ALK* genes, which could not utilize *n*-alkanes owing to a defect in the hydroxylation of *n*-alkanes (Takai et al. 2012). This suggests that *n*-alkanes and not their metabolites activate the ARE1-mediated transcription. It is possible that there are proteins that sense *n*-alkanes and trigger the transcriptional response to *n*-alkanes. Alternatively, since *n*-alkanes are supposed to accumulate in the membranes, alterations in the membrane conditions may be perceived by a sensor protein.

n-Alkane-assimilating yeasts have been shown to have great potential for production of single-cell protein (SCP) as well as various useful compounds, including long-chain dicarboxylic acids, by metabolizing *n*-alkanes (Barth and Gaillardin 1996, 1997; Fickers et al. 2005; Mauersberger et al. 1996; Tanaka and Fukui 1989). Elucidation of the mechanisms underlying the regulation of *n*-alkane metabolism will contribute to the construction of efficient bioconversion systems using these *n*-alkane-assimilating yeasts.

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