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Construction and characterization of a *Yarrowia lipolytica* mutant lacking genes encoding cytochromes P450 subfamily 52

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ABSTRACT

The initial hydroxylation of *n*-alkane is catalyzed by cytochrome P450ALK of the CYP52 family in the *n*-alkane-assimilating yeast *Yarrowia lipolytica*. A mutant with a deletion of all 12 genes, *ALK1* to *ALK12*, which are deduced to encode cytochromes P450 of the CYP52 family in *Y. lipolytica*, was successfully constructed. This deletion mutant, Δ*alk1-12*, completely lost the ability to grow on *n*-alkanes of 10–16 carbons. In contrast, Δ*alk1-12* grew on the metabolite of *n*-dodecane, i.e., *n*-dodecanol, *n*-dodecanal, or *n*-dodecanoic acid, as well as the wild-type strain. In addition, production of *n*-dodecanoic acid was not observed when Δ*alk1-12* was incubated in the presence of *n*-dodecane. These results indicate the essential roles of P450ALKs in the oxidation of *n*-alkane. Δ*alk1-12* will be valuable as a host strain to express an individual *ALK* gene to elucidate the molecular function and substrate specificity of each P450ALK. Transcriptional activation of the *ALK1* promoter by *n*-alkanes was observed in Δ*alk1-12* as in the wild-type strain, implying that *n*-alkanes *per se*, but not their metabolites, trigger *n*-alkane-induced transcriptional activation in *Y. lipolytica*.

1. Introduction

A variety of yeast species have acquired the ability to assimilate *n*-alkane as a sole carbon source. In typical *n*-alkane-assimilating yeasts, utilization of *n*-alkanes starts with its terminal hydroxylation to fatty alcohols by cytochromes P450ALK (P450ALKs), which are classified into the CYP52 family (Nelson, 2009), in the endoplasmic reticulum (ER) membrane (Fig. 1). Fatty alcohols are subsequently oxidized to fatty aldehydes either by fatty alcohol dehydrogenase (FADH) in the ER or by fatty alcohol oxidase (FAOD) in peroxisome. Fatty aldehydes are further oxidized by fatty aldehyde dehydrogenase (FALDH) in the ER or peroxisome to fatty acids, which are then activated by acyl-CoA synthetase I or II (ACS I or II) and metabolized *via* β-oxidation in the peroxisome, or utilized as a part of membrane or storage lipids (Fickers et al., 2005).

Yeasts that possess the ability to assimilate *n*-alkane as a carbon source, *Candida tropicalis* (Craft et al., 2003; Eschenfeldt et al., 2003; Sanglard and Loper, 1989; Seghezzi et al., 1991, 1992), *Candida maltosa* (Ohkuma et al., 1991, 1995), *Debaryomyces hansenii* (Yadav and Loper, 1999), *Candida albicans* (Kim et al., 2007; Panwar et al., 2001), *Candida bombicola* (Van Bogaert et al., 2009), *Candida parapsilosis*, *Lodderomyces elongisporus*, *Pichia stipitis*, and *Yarrowia lipolytica* (Fickers et al., 2005; Hirakawa et al., 2009; Iida et al., 1998, 2000), have multiple paralogs of P450s belonging to the CYP52 family (Nelson, 2009). Some of the paralog genes seem to have diversified in their inducibility and the substrate specificity of their protein products (Craft et al., 2003; Eschenfeldt et al., 2003; Hirakawa et al., 2009; Iida et al., 2000; Ohkuma et al., 1995, 1998; Seghezzi et al., 1992; Zimmer et al., 1996, 1998). Genes encoding cytochromes P450 (P450s) of the CYP52 family are also present in the genomes of filamentous fungi, including those of *Aspergillus* genus and others (Nelson, 2009). However, functional analyses have been performed for only a small subset of the P450s in *C. tropicalis* (Craft et al., 2003; Eschenfeldt et al., 2003; Sanglard and Loper, 1989; Seghezzi et al., 1991, 1992), *C. maltosa* (Ohkuma et al., 1998; Scheller et al., 1996, 1998; Zimmer et al., 1996, 1998), *C. albicans* (Kim et al., 2007), and *Y. lipolytica* (see below) (Iida et al., 2000), and the functions of most P450s of the CYP52 family remain to be established.

Y. lipolytica has an outstanding ability to assimilate a wide variety of hydrophobic compounds, a characteristic feature attracting biological interest and important for biotechnological applications (Barth and Gaillardin, 1997; Fickers et al., 2005). In *Y. lipolytica*, eight genes (*ALK1* to *ALK8*) deduced to encode P450ALKs were

Abbreviations: ACS, acyl-CoA synthetase; ARE1, alkane responsive element 1; bHLH, basic helix-loop-helix; ER, endoplasmic reticulum; FADH, fatty alcohol dehydrogenase; FALDH, fatty aldehyde dehydrogenase; FAOD, fatty alcohol oxidase; 5-FOA, 5-fluoroorotic acid; GC–MS, gas chromatography–mass spectrometry; P450, cytochrome P450.

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cloned (Iida et al., 1998, 2000) and four additional genes (*ALK9* to *ALK12*) were identified on the basis of the genome sequence of this yeast (Casaregola et al., 2000; Dujon et al., 2004; Fickers et al., 2005; Hirakawa et al., 2009). Alk1p to Alk10p and Alk12p were classified into the CYP52F subfamily, while Alk11p was classified as CYP52S1 (Nelson, 2009). We constructed and characterized single or double mutants of *ALK1*, *ALK2*, *ALK3*, *ALK4*, and *ALK6*. A deletion mutant of *ALK1* exhibited a severe growth defect on *n*-decane, a shorter carbon chain *n*-alkane, while a single deletion mutant of *ALK2*, *ALK3*, *ALK4*, or *ALK6* grew normally on *n*-decane. Double deletion of *ALK1* and *ALK2* conferred a profound growth defect on *n*-hexadecane, a longer carbon chain *n*-alkane. These results indicate that *ALK1* and *ALK2* play primary roles in the metabolism of *n*-alkanes. Hanley et al. attempted to express *ALK* genes in the plant *Nicotiana benthamiana* and found that Alk3p, Alk5p, and Alk7p had ω-hydroxylation activities against *n*-dodecanoic acid, while Alk1p, Alk2p, Alk4p, or Alk6p did not (Hanley et al., 2003). These results together suggest the functional diversity of Alk proteins in *Y. lipolytica*, as observed in *Candida* species (Ohkuma et al., 1998; Seghezzi et al., 1991; Zimmer et al., 1996, 1998).

Transcription of *ALK1* is highly induced by *n*-alkanes, and repressed strongly by glycerol. The transcriptional regulation of *ALK1* is mediated by 2 basic helix-loop-helix (bHLH)-type transcription activators, Yas1p and Yas2p, through the alkane-responsive element 1 (ARE1) in the *ALK1* promoter and an Opi1-family transcription repressor, Yas3p, which binds to Yas2p (Endoh-Yamagami et al., 2007; Hirakawa et al., 2009; Sumita et al., 2002b; Yamagami et al., 2004). The expression of *ALK1* is very low even in the presence of *n*-alkanes in the Δ*yas1* or Δ*yas2* mutant, and is highly derepressed on glucose, glycerol or *n*-alkanes in the Δ*yas3* mutant. Yas1p and Yas2p are constitutively localized in the nucleus. Yas3p is localized in the nucleus in glucose-containing medium, but it changes its localization to the ER upon transfer to *n*-alkane-containing medium, suggesting that this localization change of Yas3p is critical for transcriptional activation of *ALK1* by *n*-alkanes. However, how Yas3p changes its localization is not clear.

We analyzed the expression profiles of *ALK1* to *ALK12* in wild-type, Δ*yas1*, Δ*yas2*, and Δ*yas3* cells cultured on glucose, glycerol, and *n*-alkanes by RT-PCR (Hirakawa et al., 2009). *ALK1*, *ALK2*, *ALK4*, *ALK6*, *ALK9*, and *ALK11* exhibited Yas1p- and Yas2p-dependent *n*-alkane-responsive expression, but 6 other *ALK* genes exhibited distinct expression patterns, some of which were expressed at a low level irrespective of the presence or absence of *n*-alkanes.

Despite analysis of the phenotypes of the deletion mutants and expression profiles of *ALK* genes, the molecular functions and physiological substrates of Alk proteins, particularly those from Alk3p to Alk12p, remain unclear. This is largely due to the existence of multiple paralogs of *ALK* genes in the genome of *Y. lipolytica*, which makes it difficult to establish the functions of individual *ALK* genes. Some P450ALKs of *n*-alkane-assimilating yeasts can be expressed in other organisms that do not have P450ALK, such as *Saccharomyces cerevisiae* (Sanglard and Loper, 1989; Zimmer et al., 1995), but active P450ALKs are often not produced in heterologous hosts and it is difficult to find out how individual *ALK* genes function in *Y. lipolytica*. Therefore, we constructed a series of *ALK* deletion mutants, including the one in which all *ALK* genes are deleted, and analyzed their properties.

2. Materials and methods

2.1. Yeast strains and growth conditions

Y. lipolytica strain CXAU1 strain (*ura3 ade1*) (Iida et al., 1998) was used as a wild-type strain. Deletion of 12 *ALK* genes and introduction of *ALK1* into the genome were performed using pop-in–pop-out method (described below).

An appropriate carbon source was added to YNB {0.17% yeast nitrogen base without amino acids and ammonium sulfate (Difco), 0.5% ammonium sulfate} as follows: 2% (w/v) glucose; 2% (w/v) glycerol; 2% (v/v) *n*-alkanes. Uracil (24 mg/l) and/or adenine (24 mg/l) were/was added, if necessary. For solid media, 2% agar was added. *n*-Alkanes were supplied in the vapor phase to YNB solid media. A piece of filter paper was soaked with *n*-alkanes and placed on the lid of a Petri dish, which was sealed and kept upside down. *n*-Dodecanol, *n*-dodecanal, or *n*-dodecanoic acid was added to solid medium at a concentration of 0.1% (v/v or w/v) with 0.5% (v/v) Triton X-100. Yeast cells were grown at 30 °C.

2.2. Plasmids

To obtain pBURA3, *URA3*-carrying fragment was excised from pSUT5 (Yamagami et al., 2001) with NcoI, blunted, and cloned into EcoRV site of pBluescript II SK (+).

Sequences of the primers used to construct plasmids are listed in Table 1.

The deletion cassettes for *ALK* genes were constructed as follows. The 5′- and 3′-adjacent regions of *ALK* genes were amplified by PCR using primers listed in Table 2 and cloned into pBURA3. The deletion cassettes for *ALK* genes were obtained by digestion of the plasmids with restriction enzymes shown in Table 2.

Introduction of *ALK1* into the *ura3* locus of the mutant, in which all *ALK* genes were deleted, was performed as follows. The ORF of *ALK1* with its 5′- and 3′-non-coding regions was amplified by PCR using primers ALK1P-F and ALK1T-R. The amplified fragment was cloned into pBURA3 after digestion with BamHI and XbaI yielding pBURA3-ALK1pOt. pBURA3-ALK1pOt was digested with Bsp1407I, and introduced into Δ*alk1-12*.

For the *lacZ* reporter assay, pSUT1642 (Sumita et al., 2002b) and pS3 × LZ (Yamagami et al., 2004), which contain *lacZ* under the control of 1642-bp *ALK1* promoter or *LEU2* minimal promoter with three copies of ARE1, respectively, were used.

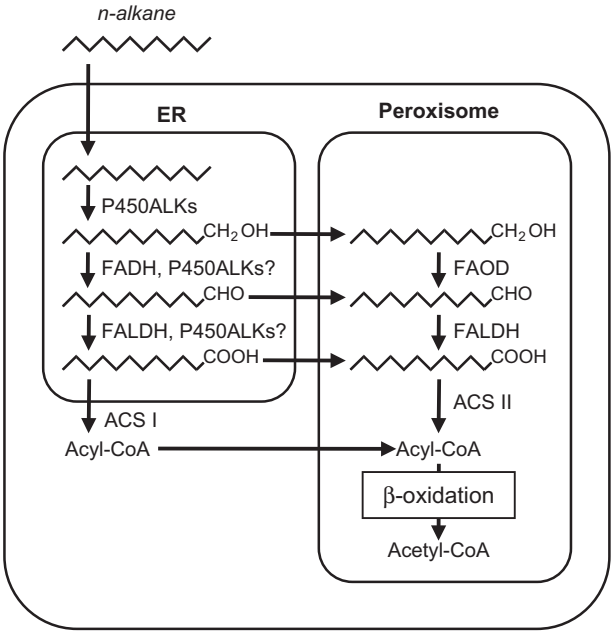

Fig. 1. Metabolism of *n*-alkane in *Y. lipolytica*. See text for detail.