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Cloning and characterization of the *POX2* gene in *Candida maltosa*

(Acyl-CoA oxidase; β-oxidation; *n*-alkane; gene disruption; yeast)

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SUMMARY

To study the function of acyl-CoA oxidase in an *n*-alkane-assimilating yeast, *Candida maltosa*, we isolated the *POX2* gene which is a member of the acyl-CoA oxidase gene family. *POX2* had a 2172-bp open reading frame (ORF) encoding an approx. 84-kDa polypeptide (724 amino acids (aa)) and was contiguous to *POX4*, another member of the acyl-CoA oxidase gene family on the same chromosomal DNA in a convergent arrangement. Northern blot analysis revealed that the expression of *POX2* was induced in cells grown on oleic acid, *n*-tetradecanol and *n*-tetradecane. By using a gene-disruption technique, we constructed strains (termed P2DD and P4DD) in which both alleles of *POX2* and *POX4* were disrupted. The P2DD strain was normal in assimilation of various hydrophobic carbon sources, such as *n*-tetradecane, *n*-tetradecanol and oleic acid. In contrast, the P4DD strain was defective in its ability to grow on such hydrophobic carbon sources.

INTRODUCTION

The transfer of *Candida maltosa* (*Cm*) cells from glucose-medium to *n*-alkane medium causes the induction of various enzymes and proteins (Gallo et al., 1973; Sunairi et al., 1984) and alteration in the amounts of endoplasmic reticulum and peroxisomes in which these enzymes are located. This yeast provides a good model to investigate the mechanism of regulation of expression of genes involved in assimilation of hydrophobic compounds. We have already cloned several genes of *Cm* involved in the initial step of *n*-alkane assimilation such as cytochrome *P*-450 species (Takagi et al., 1989; Ohkuma et al., 1991).

Peroxisomes of the yeast *C. tropicalis* (*Ct*) are responsible for β-oxidation of fatty acids (Kawamoto et al., 1978) and more than 18 different proteins (PXPs) are induced in the presence of oleic acid (Kamiryo et al., 1982). Among these polypeptides, PXP2, PXP4 and PXP5 were identified as distinct long-chain acyl-CoA oxidases and the genes encoding these polypeptides were also cloned (Okazaki et al., 1986; 1987). Recently, Picataggio et al. (1991) described the sequential disruption of acyl-CoA oxidase genes (*POX4* and *POX5*) in *Ct* to show the genes are essential to utilize alkanes or fatty acids as a carbon sources. They also reported an efficient accumulation of dicarboxylic acid from a substrate (alkane or fatty acid) in these disruptants (Picataggio et al., 1992). In contrast to the importance of *POX4* and *POX5* in alkane assimilation, the properties and function of *POX2* gene are largely unknown. The present study describes the molecular cloning and characterization of *Cm POX2* and *POX4* genes. We also examined the importance of these genes in *n*-tetradecane, *n*-tetradecanol and oleic acid assimilation in *Cm* by using a gene-disruption technique.

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Abbreviations: aa, amino acid(s); bp, base pair(s); *C.*, *Candida*; *Cm*, *C. maltosa*; *Ct*, *C. tropicalis*; DD, double disruptant; *E.*, *Escherichia*; kb, kilobase(s) or 1000 bp; nt, nucleotide(s); ORF, open reading frame; *POX*, gene(s) encoding PXP; PXP, peroxisomal polypeptide(s); *S.*, *Saccharomyces*; wt, wild type; ::, novel junction (fusion or insertion).

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EXPERIMENTAL AND DISCUSSION

(a) Cloning and nt sequence of *Cm POX2*

We screened the genomic library of *Cm* using the 1.5-kb *Bam*HI-*Sal*I fragment which contains the 3′ half of *Ct POX4* gene as a hybridization probe (Okazaki et al., 1986). From a positive clone, we identified 2.4-kb and 4.4-kb *Dra*I genomic DNA fragments that had high similarity to the *Ct POX2* and *POX4* genes, respectively.

The complete nt sequence of *Cm POX4* gene was already reported by Hill et al. (1988), so we performed complete sequencing of the *POX2* gene of *Cm* (Fig. 1). A typical TATA consensus sequence, TATAAA, was present around nt −120 relative to the ATG start codon. A single 2172-nt ORF was shown and a polypeptide of 84 kDa (724 aa) was predicted. The *POX2* structural gene had 82% identity on the nt level and 85% identity on the aa level to its *Ct* counterpart. The *POX4* and *POX5* genes of *Ct* exhibited 52% and 48% identity on the deduced amino acid level to the *Cm POX2* gene.

(b) Arrangement of *POX* genes and expression on various carbon sources

In *Ct*, there were three genes (*POX2*, *POX4* and *POX5*) encoding acyl-CoA oxidase and they formed a multigene family (Okazaki et al., 1986; 1987). As shown in Fig. 2A, the sequence analysis revealed that the *Cm POX2* gene was linked to *Cm POX4* gene in a convergent arrangement like those of *Ct*.

We carried out Northern blot analysis of *Cm POX* mRNAs. As shown in Fig. 2B, the *POX2* mRNA was expressed in *n*-tetradecane-, *n*-tetradecanol- and oleic acid-grown cells (lanes 2, 3 and 4), while it was not expressed in glucose- and glycerol-grown cells (lane 1 and 5). Similar results were also obtained with *POX4* gene (data not shown).

(c) Disruption of *POX* genes

Since *Cm* has a diploid genome, two alleles of each gene should be disrupted to construct the strain that lost the function of the gene. A *Cm* adenine and histidine

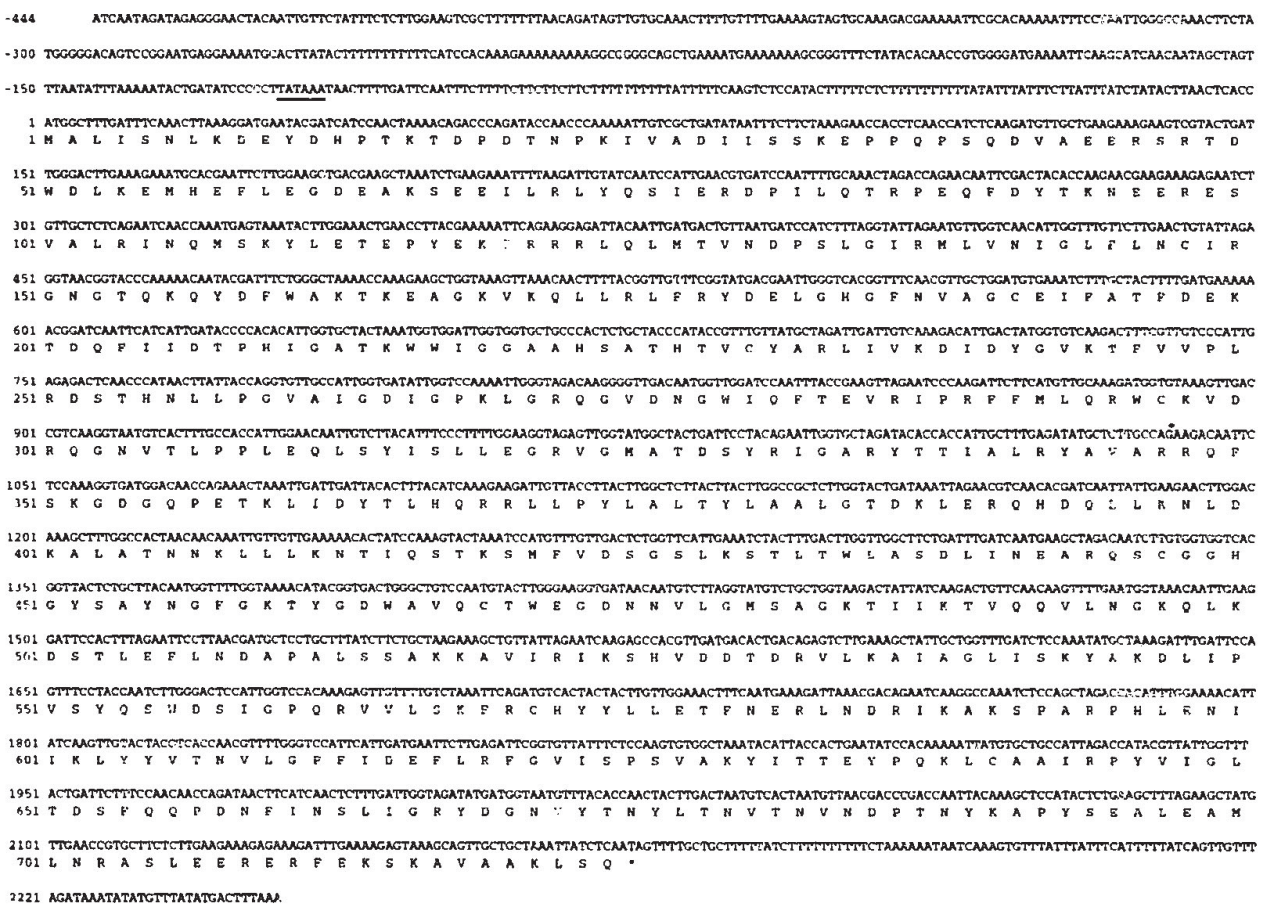

Fig. 1. Nucleotide sequence of the *Cm POX2* gene and deduced aa sequence. A potential TATA box around nt −120 upstream from the start codon is underlined. The ORF encodes a 724-aa polypeptide. The sequence data of this gene has appeared in the GenBank/EMBL/DDBJ database under accession No. D21228. **Methods:** Construction of a gene library was performed by the previously described procedure (Masuda et al., 1994).

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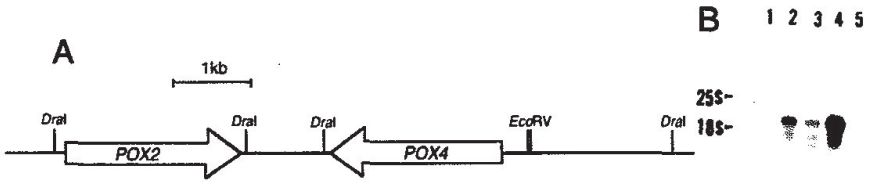

Fig. 2. Arrangement of *POX* genes and Northern blot analysis. (**A**) Arrangement of the *POX2* and *POX4* genes. ORF of the respective genes are indicated by open arrows. (**B**) Northern blot analysis of the *POX2* gene. *Cm* wt strain IAM12247 was grown on glucose (lane 1), *n*-tetradecane (lane 2), *n*-tetradecanol (lane 3), oleic acid (lane 4) and glycerol (lane 5). **Methods:** The strains and media used in this study were *C. maltosa* IAM12247 (a wt strain) and SD (0.67% Yeast Nitrogen Base w/o amino acids (Difco)/2% glucose). Glucose in SD was replaced by 1% *n*-tetradecane, *n*-tetradecanol or oleic acid when necessary. Northern blot analysis was performed by the previously described procedure (Masuda et al., 1994).

double auxotroph, CHA1, was transformed with the 4.7-kb *Pst*I-*Sac*I fragment of pPD2H (Fig. 3A) and histidine prototrophs were selected on adenine-supplemented SD plates. Southern analysis of total DNA from a His^+ transformant revealed a 1.6-kb disrupted *Dra*I band and the 2.4-kb band of the undisrupted allele (Fig. 4A, lanes 1 and 2). This *POX2* single gene disruptant was designated P2D (*POX2/pox2::C-HIS5*). Then, the P2D was transformed with the 4.0-kb *Pst*I-*Sac*I fragment of pPD2A. Southern analysis of total DNA from a His^+Ade^+ transformant revealed only a 1.6-kb *Dra*I band (Fig. 4A, lane 3), indicating that both alleles of the *POX2* gene were disrupted. This *POX2* double disruptant was termed P2DD (*pox2::C-HIS5/pox2::C-ADE1*).

The *POX4* disruptants P4D (*POX4/pox4::C-HIS5*) and P4DD (*pox4::C-HIS5/pox4::C-ADE1*) were constructed using the same procedure by using two fragments, the 5.7-kb *Xba*I fragment derived from pPD4H plasmid for construction of the P4D strain and the 5.1-kb *Xba*I fragment derived from pPD4A plasmid for P4DD (Fig. 4D). Southern analysis of P4DD strain showed the appearance of 4.4 and 3.3-kb *Dra*I-*Eco*RI disruptant bands and the disappearance of the 2.9-kb band of the undisrupted allele (Fig. 4B, lane 3).

(d) Effect of *POX2* disruption on assimilation of various carbon sources

Picataggio et al. (1991) reported the sequential disruption of the *POX4* and *POX5* genes in *C. tropicalis* and functional block of the β-oxidation pathway. In contrast to these results, the importance of *POX2* gene in assimilation of hydrophobic carbon sources is not clear. As shown in Table I, the P2DD strain grew on *n*-tetradecane, *n*-tetradecanol and oleic acid. In contrast, the P4DD strain was not able to grow on *n*-tetradecane, *n*-tetradecanol and oleic acid but able to grow in media containing glucose or glycerol. Considering the growth phenotype of the P2DD strain, the function of the *POX2* gene on assimilation of *n*-tetradecane, *n*-tetradecanol or oleic acid appears not to be important. Okazaki et al. (1987) described that the cell extract preparation containing both products of *C. tropicalis POX4* and *POX2* showed only half of the acyl-CoA oxidase activity exhibited by the preparation that was composed of the product of *POX4* gene alone. The possible explanation is that the *POX2* gene product may regulate the activity of the *POX4* gene.

(e) Conclusions

(*1*) *Cm POX2* encoded an ORF encompassing 2172 nt and a 84-kDa polypeptide (724 aa) was predicted.

TABLE I

Growth of the wt and CHA1 strains, and of *POX* gene disruptants on various carbon sources

Strain	Genotype[a]	Growth[b]				
		Glucose	*n*-tetradecane	*n*-tetradecanol	Oleic acid	Glycerol
IAM12247	wt	+	+	+	+	+
CHA1	*his5, ade1*	+	+	+	+	+
P2D	*pox2::HIS5/PO*[illegible]	+	+	+	+	+
P2DD	*pox2::HIS5/pox2::ADE1*	+	+	+	+	+
P4D	*pox4::HIS5/POX4*	+	+	+	+	+
P4DD	*pox4::HIS5/pox4::ADE1*	+	−	−	−	+

[a] Slash (/) specifies 'diploid' genotype. The DD strains are double disruptants.

[b] Growth activity was determined by colony formation on SD plate and liquid culture in SD media. Glucose in SD was replaced by other carbon sources as specified.

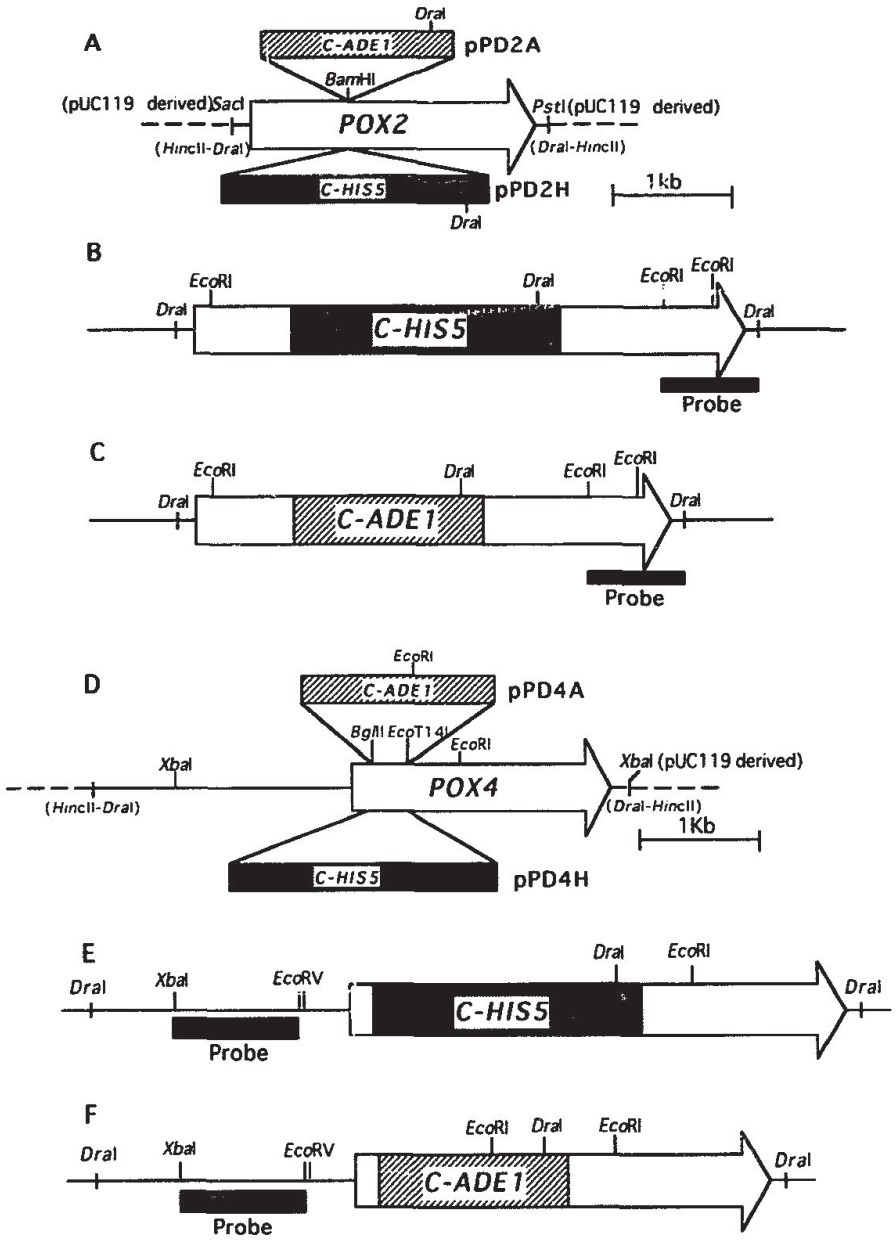

Fig. 3. Structures of the plasmids used to disrupt the *POX* genes and predicted restriction maps of the disrupted genes. (**A** and **D**) Structures of the plasmids. ORF of the *POX* genes are represented by big open arrows. The dotted line indicates pUC119 sequence. Predicted restriction maps of the *POX2* gene disruptants (**B** and **C**) and of the *POX4* gene disruptants (**E** and **F**). Blackend bars represent the region used as probe. The symbols are: ▨, *C-ADE1* gene; ■, *C-HIS5* gene. Construction of plasmids: A 2.4-kb *Dra*I fragment containing the *POX2* gene was inserted into the *Hin*cII site of pUC119. The resulting plasmid was partially digested with *Bam*HI, and 1.6 kb of *C-ADE1* gene (for pPD2A) (Kawai et al., 1991) or the 2.3-kb fragment of the *C-HIS5* gene (for pPD2H) (Hikiji et al., 1989) was inserted into the middle of the ORF of *POX2* through a *Sal*I linker (see panel **A**). The resulting plasmids were digested with *Sac*I + *Pst*I and then used for the transformation. A 4.4-kb *Dra*I fragment of the *POX4* gene was inserted into *Hin*cII site of pUC119. From the resulting plasmid, a 0.3-kb *Bgl*II-*Eco*T14I fragment was removed. Then, a 1.6-kb fragment of the *C-ADE1* gene (for pPD4A) or a 2.3-kb fragment of the *C-HIS5* gene (for pPD4H), was inserted through a *Sal*I linker. The resulting plasmids, pPD4A and pPD4H, were digested with *Xba*I and then used for the transformation. Transformation of *Cm* was performed by a modified Li-acetate method (Takagi et al., 1986).

(*2*) Northern hybridization analysis revealed that the *POX2* mRNA was expressed in *n*-tetradecane-, *n*-tetradecanol- and oleic-acid-grown cells, while it was not expressed in glucose- and glycerol-grown cells.

(*3*) The *POX2* gene was not essential for growth on such hydrophobic compounds. The *POX2* gene product may regulate the function of the *POX4* gene product or may be involved in assimilation of other carbon sources.

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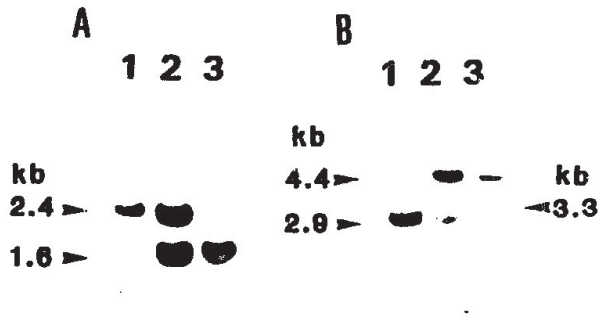

Fig. 4. Southern analysis of the genome DNA isolated from *POX2* and *POX4* gene disruptants. (**A**) Analysis of the *POX2* gene disruptants. Total DNA isolated from CHA1 (lane 1), P2D (lane 2) or P2DD (lane 3) was digested with *Dra*I. Hybridization probe was the 0.8-kb *Eco*RI-*Dra*I region containing the 3′ region of the *POX2* gene. (**B**) Analysis of the *POX4* gene disruptants. *Dra*I + *Eco*RI-digested total DNA isolated from CHA1 (lane 1), P4D (lane 2) or P4DD (lane 3) was used (Table I) and probed with the *Eco*RV-*Xba*I fragment containing the 5′ flanking region of the *POX4* gene. **Methods:** Southern blot hybridization was performed with an ECL gene detection system (Amersham) according to the instructions of supplier.

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