MOLECULAR AND CELLULAR BIOLOGY, Sept. 1991, p. 4333–4339
0270-7306/91/094333-07$02.00/0

Vol. 11, No. 9

Determination of *Candida tropicalis* Acyl Coenzyme A Oxidase Isozyme Function by Sequential Gene Disruption

STEPHEN PICATAGGIO,* KRISTINE DEANDA, AND JONATHAN MIELENZ
Department of Microbial Technology, Cognis Inc., Santa Rosa, California 95407

Received 7 February 1991/Accepted 5 June 1991

A recently developed transformation system has been used to facilitate the sequential disruption of the *Candida tropicalis* chromosomal *POX4* and *POX5* genes, encoding distinct isozymes of the acyl coenzyme A (acyl-CoA) oxidase which catalyzes the first reaction in the β-oxidation pathway. The *URA3*-based transformation system was repeatedly regenerated by restoring the uracil requirement to transformed strains, either through selection for spontaneous mutations or by directed deletion within the *URA3* coding sequence, to permit sequential gene disruptions within a single strain of *C. tropicalis*. These gene disruptions revealed the diploid nature of this alkane- and fatty acid-utilizing yeast by showing that it contains two copies of each gene. A comparison of mutants in which both *POX4* or both *POX5* genes were disrupted revealed that the two isozymes were differentially regulated and displayed unique substrate profiles and kinetic properties. *POX4* was constitutively expressed during growth on glucose and was strongly induced by either dodecane or methyl laurate and to a greater extent than *POX5*, which was induced primarily by dodecane. The *POX4*-encoded isozyme demonstrated a broad substrate spectrum in comparison with the narrow-spectrum, long-chain oxidase encoded by *POX5*. The absence of detectable acyl-CoA oxidase activity in the strain in which all *POX4* and *POX5* genes had been disrupted confirmed that all functional acyl-CoA oxidase genes had been inactivated. This strain cannot utilize alkanes or fatty acids for growth, indicating that the β-oxidation pathway has been functionally blocked.

Peroxisomes represent a class of organelles that are ubiquitous among eukaryotic organisms and are involved in a variety of metabolic processes such as β-oxidation and the degradation of hydrogen peroxide. The importance of these organelles to cellular metabolism has been made clear by the recent discovery of a new class of inherited peroxisomal disorders such as Zellweger syndrome, which has been reported to be due to a recessive mutation that abolishes the import of peroxisomal matrix proteins (11). However, the molecular events leading to compartmentalization in eukaryotic cells and to the localization of peroxisomal proteins are still largely unknown and are the subject of intense study.

The yeast *Candida tropicalis* is an important organism for the study of peroxisome biogenesis and the localization of peroxisomal proteins, since these organelles are rapidly and abundantly induced following growth on either alkane or fatty acid substrates (10). At least 18 different peroxisomal proteins have been detected in oleic acid-induced cells, and several of these have been identified as enzymes associated with the β-oxidation pathway (4). Localization of acyl coenzyme A (acyl-CoA) oxidase, enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, and 3-ketoacyl-CoA thiolase activities has shown that β-oxidation takes place solely in the peroxisomes of this yeast (14). Peroxisomal acyl-CoA oxidase is an octomeric flavoprotein (molecular weight 600,000) which catalyzes the first reaction in the β-oxidation pathway by the stoichiometric conversion of acyl-CoA to enoyl-CoA for substrates with chain lengths from 4 to 20 carbons (12). The genes encoding the subunits of distinct acyl-CoA oxidase isozymes (7–9), designated *POX4* and *POX5*, have been cloned and sequenced. These genes encode polypeptides (designated PXP4 and PXP5) composed of 708 and 661 amino acids, respectively, following the removal of the initiating methionine during maturation (8). Since these polypeptides were found to share 63% homology, it was suggested that the two acyl-CoA oxidase isozymes arose by a gene duplication event followed by a localized exchange or replacement of functional domains. However, the functional differences between these isozymes, if any, have not been determined. A third member of the acyl-CoA oxidase gene family has been identified on the basis of sequence homology as *POX2*, but its enzymatic activity and function also remain unknown (9).

The primary structure of these isozymes is of particular interest since internal topogenic signals postulated to direct their localization to the peroxisome have been identified. Two regions of PXP4 sufficient to target acyl-CoA oxidase fragments to the peroxisomes have been identified by in vitro import assays (13) and by expression of truncated *POX4* gene fragments in a related yeast, *Candida maltosa* (5). These and other studies suggest that the pathway for the localization of peroxisomal proteins is distinct from that of other organelles in that they are synthesized devoid of any discernible amino-terminal signal sequence on free polysomes and are imported directly and without posttranslational modification into the peroxisomes. However, until now it has not been possible to study either the function or the localization of these isozymes in *C. tropicalis* because of the lack of both suitable mutants and a gene transfer system for this yeast.

Recently, a transformation system for *C. tropicalis* has been described that is based on the complementation of a uracil auxotroph with the *C. tropicalis URA3A* gene (2). We report here a method for the highly specific modification of the *C. tropicalis* genome by sequential gene disruption, in which the sequence homology at the ends of a linear DNA fragment dictates the site of integration by homologous recombination into the *C. tropicalis* genome. This method has been used for the sequential disruption of the *C. tropi-*

* Corresponding author.

TABLE 1. *C. tropicalis* strains and genotypes

Strain	Genotype
SU-2	*ura3A/ura3B*
H41	*ura3A/ura3B pox4A::URA3A/POX4B*
H41B	*ura3A/ura3B POX4A/pox4B::URA3A*
H51	*ura3A/ura3B pox5::URA3A/POX5*
H51ΔKpn	*ura3A/ura3B pox5::Δura3A/POX5*
H45	*ura3A/ura3B pox4A::ura3A/POX4B pox5::URA3A/POX5*
H43	*ura3A/ura3B pox4A::URA3A/pox4B::ura3A*
H53	*ura3A/ura3B pox5:ura3A/pox5::URA3A*
H534	*ura3A/ura3B pox4A::URA3A/POX4B pox5::ura3A/pox5::ura3A*
H534B	*ura3A/ura3B POX4A/pox4B::URA3A pox5::ura3A/pox5::ura3A*
H435	*ura3A/ura3B pox4A::ura3A/pox4B:ura3A pox5::URA3A/POX5*
H5343	*ura3A/ura3B pox4A::ura3A/pox4B::ura3A pox5::ura3A/pox5::URA3A*

calis POX4 and *POX5* genes. While gene disruption has been demonstrated in other yeasts, this is the first description of its use in *C. tropicalis*. Mutants lacking either one or both isozymes have been developed and used to determine their differential regulation and function. In addition, mutants completely lacking acyl-CoA oxidase activity have been developed to allow the in vivo study of peroxisomal import in *C. tropicalis*.

MATERIALS AND METHODS

Strains. The *C. tropicalis* strains developed and their genotypes are listed in Table 1.

Plasmids. The *C. tropicalis POX4* gene was first subcloned on a 6.6-kb *Hin*dIII restriction fragment from pC1 (8) into pBR322 to construct plasmid pKD3. The *C. tropicalis POX5* gene was first subcloned on a 3.9-kb *Eco*RI restriction fragment from pC50 (8) into pBR322 to construct plasmid pKD1. To facilitate the in vitro disruption of these genes, the *Bam*HI site located within the tetracycline resistance gene was destroyed by standard methods to generate plasmids pKD1Δ*Bam*HI and pKD3Δ*Bam*HI. The *C. tropicalis URA3A* gene was obtained on a 2.2-kb *Nru*I restriction fragment from pCU2Δ*Sac*I (2) and, following the addition of *Bam*HI linkers, was ligated to *Bam*HI-linearized, dephosphorylated pKD1Δ*Bam*HI or pKD3Δ*Bam*HI. Plasmid pKD3-URA3A contains the *URA3A* gene cloned into the unique *POX4 Bam*HI site (position 2101) and is flanked by 2.1 kb of 5′ and 4.5 kb of 3′ *POX4* sequence. Digestion of this plasmid with *Eco*RI liberated a linear 5′-*pox4-URA3-pox*4-3′ cassette suitable for disruption of the *C. tropicalis* chromosomal *POX4* gene (Fig. 1A). Plasmid pKD1-URA3A contains the *URA3A* gene cloned into the unique *POX5 Bam*HI restriction site (position 1178) and is flanked by 1.2 kb of 5′ and 2.7 kb of 3′ *POX5* sequence. Digestion of the plasmid with *Eco*RI liberated a linear 5′-*pox5-URA3A-pox5*-3′ cassette suitable for disruption of the *C. tropicalis* chromosomal *POX5* gene (Fig. 1B).

Plasmid pCU3Δ*Kpn*I was constructed as a *URA3A* deletion vector by a *Bal*31-generated deletion spanning the unique *URA3A Kpn*I site in pCU3 (2). Following treatment with DNA polymerase Klenow fragment, the deleted plasmids were recircularized, digested with *Kpn*I, and transformed into *Escherichia coli* HB101. pCU3Δ*Kpn*I was found

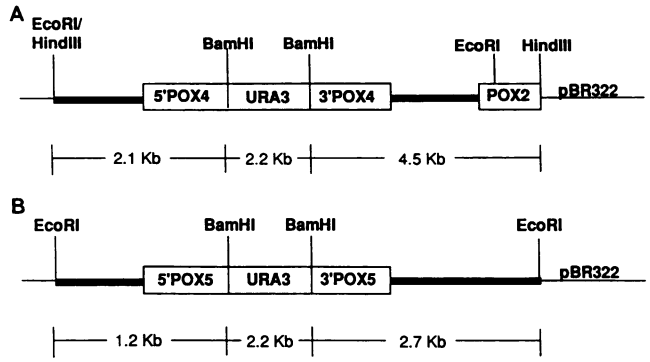

FIG. 1. *POX* gene disruption cassettes. (A) *POX4* gene disruption cassette released by digestion of plasmid pKD3-URA3A with *Eco*RI restriction endonuclease. The cassette contains the *C. tropicalis URA3A* gene cloned on a 2.2-kb *Bam*HI fragment into the unique *POX4 Bam*HI site (position 2101) and is flanked by 2.1 kb of 5′ *POX4* sequence and 4.5 kb of 3′ *POX4* sequence. (B) *POX5* gene disruption cassette released by digestion of plasmid pKD1-URA3A with *Eco*RI restriction endonuclease. The cassette contains the *C. tropicalis URA3A* gene cloned on a 2.2-kb *Bam*HI fragment into the unique *POX5 Bam*HI site (position 1178) and is flanked by 1.2 kb of 5′ *POX5* sequence and 2.7 kb of 3′ *POX5* sequence.

to contain an approximately 50-bp deletion spanning the original *Kpn*I site.

Transformation. *C. tropicalis* was transformed to uracil prototrophy as described previously (2) with *Eco*RI-digested pKD3-URA3A or pKD1-URA3A for the disruption of the *POX4* or *POX5* gene, respectively. For selected *POX4* disruptions, as indicated, spheroplasts were transformed with a truncated *POX4* disruption cassette, obtained by digestion of the 8.3-kb *Eco*RI fragment from pKD3-URA3A (Fig. 1A) with *Bal*31 and *Sal*I to generate fragments of approximately 5 kb. Transformants were characterized by hybridization of genomic DNA, digested with the appropriate restriction endonucleases (New England BioLabs), to radiolabelled probes as described previously (3). Radiolabelled pKD1, pKD3, and pCU3 were used as hybridization probes for the chromosomal *POX5*, *POX4*, and *URA3A* genes, respectively, and were prepared by using a nick translation kit (New England Nuclear) according to the manufacturer's recommendations.

Enrichment for uracil auxotrophs. The cells from the surface of transformation plates were pooled and cultured in 100 ml of YEPD (20 g of glucose, 20 g of peptone, and 10 g of yeast extract per liter) from an A_{600} of 0.1 to 0.4. All incubations were carried out at 30°C with shaking (200 rpm). The cells were harvested (5,000 × *g*, 5 min) and cultured in 100 ml of yeast carbon base (Difco) for 21 h. The cells were collected (5,000 × *g*, 5 min), washed once with sterile distilled water, and cultured in 100 ml of yeast nitrogen base (Difco) containing dextrose (20 g/liter) for 7 h. Then nystatin (Sigma) was added to a final concentration of 35 U/ml, and the culture was incubated for an additional 35 min. The cells were washed twice and resuspended in 10 ml of sterile distilled water. Nystatin-treated cells were plated onto selection medium (containing, per liter, yeast nitrogen base, 6.7 g, dextrose, 20 g; agar, 20 g; uracil, 50 mg; uridine, 150 mg; uridine 5-phosphate, 150 mg; and 5-fluoro-orotic acid [5-FOA], 750 mg) and were incubated for up to 2 weeks at 30°C. 5-FOA-resistant isolates that could not grow in the absence of uracil were selected for further analysis.

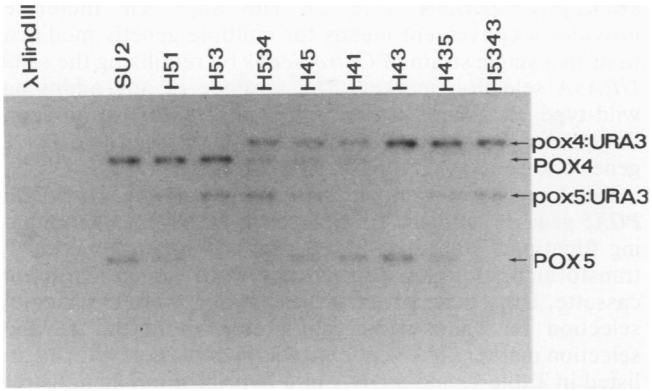

FIG. 2. Hybridization profiles of *pox4* and *pox5* mutants; DNA blot hybridization of *Eco*RI-digested genomic DNAs (6 μg per lane) probed with *POX4* (pKD3-URA3A) and *POX5* (pKD1-URA3A) gene probes. The strains from which genomic DNA was obtained are indicated above the lanes. The leftmost lane contained a *Hin*dIII digest of lambda DNA, used as size standards.

Acyl-CoA oxidase assays. Strains were cultured for 30 h in YEPD and then induced for 40 h in yeast nitrogen base containing yeast extract (3 g/liter) and either glucose, dodecane, or methyl laurate (15 g/liter). Extracts were prepared by repeated passage of washed cell suspensions through a French pressure cell (1,260 lb/in^2), and cell debris was removed by centrifugation (13,000 × *g*). Activity was measured independently on C_4-CoA through C_{18}-CoA substrates as described previously (12) and normalized to the protein concentration.

RESULTS

Disruption of the *C. tropicalis POX5* gene. To disrupt the chromosomal *POX5* locus by a gene replacement with the *POX5-URA3A* disruption cassette, *C. tropicalis* SU-2 (2) spheroplasts were transformed to uracil prototrophy with *Eco*RI-digested pKD1-URA3A (Fig. 1B), and approximately 200 mitotically stable Ura$^+$ transformants were recovered at a frequency of 70/μg of DNA. Hybridization of *Eco*RI-digested genomic DNA from 11 transformants to a *POX5* probe revealed the presence of a 6.1-kb *Eco*RI fragment (Fig. 2, strain H51) not present in the wild-type (Fig. 2, strain SU-2). This fragment, which is 2.2 kb larger than the wild-type 3.9-kb *POX5 Eco*RI fragment, was also detected with a *URA3A* probe and represents replacement of the wild-type *POX5* locus with the *POX5-URA3A* disruption cassette. However, hybridization to the *POX5* probe also revealed the presence of an additional wild-type copy of the *POX5* gene (3.9 kb) in each of these transformants. The equal hybridization intensities of the two restriction fragments detected in strain H51 compared with the more intense hybridization of the 3.9-kb wild-type *POX5 Eco*RI fragment detected in strain SU-2 suggested that there are normally two copies of the *POX5* gene in the genome.

To confirm that this *Eco*RI hybridization pattern represented a disruption of a single *POX5* gene at an apparently diploid locus, genomic DNA from strain H51 was digested with *Nco*I and analyzed by DNA blot hybridization. Since there are no internal *Nco*I sites within the *POX5-URA3A* disruption cassette, the restriction fragments generated by *Nco*I digestion depend on the chromosomal location of the *Nco*I sites nearest the site of integration. Thus, a *POX5* gene disruption at a diploid locus would yield two fragments, one of which would retain the mobility of the wild-type fragment. The hybridization to a *POX5* gene probe demonstrated the presence of two *Nco*I fragments in each of the transformants, whereas only one was detected, with greater hybridization intensity, in the wild type (results not shown). Only the larger of the two fragments in strain H51 was detected by hybridization to a *URA3A* gene probe and corresponded in size to that expected for the replacement of the chromosomal *POX5* gene with the *POX5-URA3A* disruption cassette. Thus, the *Nco*I hybridization pattern confirmed the disruption of a single copy of the *POX5* gene at an apparently diploid locus. Accordingly, selective disruption of the remaining *POX5* gene was necessary to functionally inactivate *POX5* activity.

Disruption of the *C. tropicalis POX4* gene. Similarly, to disrupt the chromosomal *POX4* locus by a gene replacement with the *POX4-URA3A* disruption cassette, *C. tropicalis* SU-2 spheroplasts were transformed to uracil prototrophy with *Eco*RI-digested pKD3-URA3A (Fig. 1A), and approximately 160 mitotically stable Ura$^+$ transformants were recovered at frequency of 25/μg of DNA. DNA blot hybridization of *Eco*RI-digested genomic DNA from selected transformants to a *POX4* probe revealed the presence of a 12-kb *Eco*RI fragment (Fig. 2, strain H41) not present in the wild type (Fig. 2, strain SU-2). This fragment, which is 2.2 kb larger than the 9.8-kb wild-type *POX4 Eco*RI fragment, was also detected by hybridization to a *URA3A* probe and corresponded in size to that expected for the replacement of the wild-type *POX4* locus with the *POX4-URA3A* disruption cassette. Hybridization to the *POX4* probe also revealed the presence of an additional wild-type copy of the *POX4* gene in each of these transformants. The hybridization intensities of these two fragments compared with the more intense hybridization of the wild-type fragment detected in strain SU-2 suggested that *C. tropicalis* is diploid at the *POX4* locus as well.

The increased size of the disrupted *POX4* gene in H41 allowed easy identification of both *POX4* homologs. By a comparison of *C. tropicalis* SU-2 and H41 digestion patterns, restriction site polymorphisms were observed for endonuclease *Hpa*I, *Hin*dIII, *Sac*I, *Bgl*II, *Bam*HI, *Xho*I, or *Sal*I, indicating significant divergence between the two homologs. For convenience in distinguishing these alleles, we arbitrarily designated the cloned *POX4* gene contained on pKD3, corresponding to the gene described by Okazaki et al. (8), as *POX4A* and the other as *POX4B*. When restriction fragments derived from the 5′ and 3′ regions of the cloned *POX4A* gene were used to probe restriction digests of H41 DNA, the downstream sequences detected both *POX4* homologs but the upstream sequence detected only the disrupted *POX4A* gene. This finding suggested that the divergence lies immediately upstream of the *POX4* genes but certainly does not rule out downstream divergence beyond the regions tested. The slightly greater hybridization intensity of the disrupted *POX4* fragment in strain H41 (Fig. 2) is believed to be due to the preferential hybridization of the *POX4A* gene contained on the pKD3 probe to the homologous copy in the genome.

While initial experiments demonstrated the tandem integration of pKD3-URA3A by a single crossover recombination event into either homolog, a disruption of the second homolog, designated *POX4B*, was not initially obtained. Since the two genes could be distinguished by restriction site polymorphism and apparently contain different flanking sequences, it seemed likely that the *POX4A* disruption cassette

would preferentially disrupt only the homologous chromosomal copy. Therefore, to disrupt the *POX4B* locus, *C. tropicalis* SU-2 was transformed to Ura$^+$ with a truncated *POX4A* disruption cassette, previously trimmed of its flanking sequences by successive *Bal*31 and *Sal*I digestions, thus depending primarily on homologous sequences within the structural genes to direct the chromosomal replacement. One of the 20 Ura$^+$ transformants screened by DNA blot hybridization of *Eco*RI- or *Hpa*I-digested genomic DNA to *POX4A* and *URA3A* probes had the expected *POX4B* disruption hybridization pattern (strain H41B; Table 1). The selective disruption of the remaining wild-type *POX4* gene in either strain H41 or H41B with the *POX4-URA3* cassette was therefore necessary to functionally inactivate *POX4* activity.

Regeneration of the *URA3* selectable marker. Since the initial round of transformations resulted in a Ura$^+$ phenotype, methods to restore the uracil requirement to transformed strains were developed to permit reutilization of the *URA3A* selectable marker for selective disruption of the remaining functional *POX4* and *POX5* genes. The methods used to repeatedly regenerate the selectable marker in a single strain are based either on selection for spontaneous mutations which inactivate the selectable marker or on directed deletion of a portion of the *URA3* coding sequence. Both approaches make use of the ability to readily select isolates demonstrating a Ura$^-$ phenotype by their resistance to 5-FOA, an analog of a uracil pathway intermediate which is toxic to cells that can synthesize their own uracil (1). For example, *C. tropicalis* SU-2 (Ura$^-$) demonstrated 71.6, 50.3, and 14.8% survival in the presence of 5-FOA at 500, 750, and 1,000 μg/ml, respectively, whereas under comparable conditions, strain H51 (Ura$^+$) demonstrated survival of less than 3.6×10^{-6}. Selection in the presence of 5-FOA at 750 μg/ml was found to be suitable for the selection of Ura$^-$ isolates.

To generate a chromosomal deletion within the *URA3A* selectable marker, *C. tropicalis* H51 (*pox5*::*URA3A*/*POX5*) was transformed with *Eco*RI-*Pst*I-digested pCU3Δ*Kpn*I, previously characterized as containing a 50-bp deletion spanning the *Kpn*I site of the *URA3A* gene. Ura$^-$ cells were isolated following nystatin enrichment and selection for 5-FOA resistance. Characterization of 25 Ura$^-$ isolates by DNA blot hybridization of *Eco*RI- and *Kpn*I-digested genomic DNA to *POX5* and *URA3* probes, respectively, showed 13 of the isolates to contain the expected deletion within the *URA3* selectable marker gene at the disrupted *POX5* locus (not shown). All demonstrated Ura$^+$ reversion frequencies of less than 10^{-8}. One of these strains was isolated and designated H51Δ*Kpn*I (*pox5*::Δ*ura3A*/*POX5*).

Several 5-FOA-resistant isolates that were recovered from strain H51 in the absence of transforming DNA were found to be identical to H51 in their *POX5 Eco*RI hybridization pattern but were phenotypically Ura$^-$. We reasoned that these strains might contain spontaneous point mutations within the *URA3A* selectable marker and could thus be retransformed with the *POX5-URA3A* disruption cassette to inactivate the remaining functional *POX5* gene. Three strains with low Ura$^+$ reversion frequencies were separately transformed to Ura$^+$ with *Eco*RI-digested pKD1-URA3A. Characterization of 28 Ura$^+$ transformants by DNA blot hybridization of *Eco*RI-digested genomic DNA to a *POX5* probe identified 9 transformants which demonstrated the sole presence of a 6.1-kb *Eco*RI fragment with twice the hybridization intensity of strain H51. These transformants represent a disruption of both copies of the *POX5* gene, one of which was isolated and designated strain H53 (*pox5*::*ura3A*/*pox5*::*URA3A*) (Fig. 2). This approach, therefore, provides a convenient means for multiple genetic modifications in a single strain of *C. tropicalis* by reutilizing the same *URA3A* selection marker. The absence of any additional wild-type *POX5* restriction fragments in this strain confirmed that *C. tropicalis* contains no more than two *POX5* genes and reflects its diploid nature.

Development of mutants containing multiple *POX4* and *POX5* gene disruptions. As a first step to disrupt the remaining functional *POX4* or *POX5* genes in strains previously transformed to uracil prototrophy with either disruption cassette, Ura$^-$ derivatives of these strains were obtained by selection for spontaneous mutations within the *URA3A* selection marker. The genotypes of the strains developed are listed in Table 1, and a DNA blot hybridization demonstrating the gene disruptions in each of these strains is shown in Fig. 2. Strain H534 (*pox4A*::*URA3A*/*POX4B pox5*::*ura3A*/*pox5*::*ura3A*) was isolated following transformation of a Ura$^-$ derivative of H53 (*pox5*::*ura3A*/*pox5*::*ura3A*) with the *POX4* disruption cassette from pKD3-URA3A. Strain H45 (*pox4A*::*ura3A*/*POX4B pox5*::*URA3A*/*POX5*) was isolated following transformation of a Ura$^-$ derivative of H41 (*pox4A*::*ura3A*/*POX4B*) with the *POX5* disruption cassette from pKD1-URA3A. Strain H43 (*pox4A*::*URA3A*/*pox4B*::*ura3A*) was isolated following transformation of a uracil-requiring derivative of H41B (*POX4A*/*pox4B*::*ura3A*) to Ura$^+$ with the *POX4A* disruption cassette from pKD3-URA3A. The absence of any additional wild-type *POX4* restriction fragments in this strain confirmed that *C. tropicalis* is diploid at the *POX4* locus as well. Strain H534B (*POX4A*/*pox4B*::*URA3A pox5*::*ura3A*/*pox5*::*ura3A*) was isolated following transformation of a uracil-requiring derivative of H53 (*pox5*::*ura3A*/pox5::*ura3A*) with a truncated *POX4A* disruption cassette in order to target the *POX4B* gene. Strain H435 (*pox4A*::*ura3A*/*pox4B*::*ura3A pox5*::*URA3A*/*POX5*) was constructed by transformation of a uracil-requiring derivative of H43 (*pox4A*::*ura3A*/*pox4B*::*ura3A*) with the *POX5* disruption cassette from pKD1-URA3A. Strain H5343 (*pox4A*::*ura3A*/*pox4B*::*URA3A pox5*::*ura3A*/*pox5*::*ura3A*), in which all *POX4* and *POX5* genes were disrupted, was isolated following transformation of a uracil-requiring derivative of H534 (*pox4A*::*ura3A*/*POX4B pox5*::*ura3A*/*pox5*::*ura3A*) to Ura$^+$ with the truncated *POX4A* disruption cassette from pKD3-URA3A.

Unlike all previous mutants in the lineage, strain H5343 cannot utilize alkanes or fatty acids as sole carbon sources for growth, indicating that these sequential gene disruptions have resulted in a complete functional block of the β-oxidation pathway.

Characterization of acyl-CoA oxidase isozyme activity. The availability of this collection of *POX4* and *POX5* mutants permitted an evaluation of the regulation and function of the individual acyl-CoA oxidase isozymes. Therefore, the acyl-CoA oxidase activity of each strain was measured independently on three assay substrates of different chain length following growth on glucose or induction with alkane or fatty acid substrates (Table 2).

No acyl-CoA oxidase activity was detected in strain H5343 grown on glucose or induced with either dodecane or methyl laurate on any of the acyl-CoA substrates tested. Additional experiments confirmed the lack of activity on any acyl-CoA substrates with chain lengths between 4 and 18 carbons. This confirmed that all functional acyl-CoA oxidase genes had been inactivated, resulting in the complete absence of any functional acyl-CoA oxidase in this strain. It is this lack of enzymatic activity which results in a functional

TABLE 2. Acyl-CoA oxidase activities of *pox4* and *pox5* mutants of *C. tropicalis*

Strain	Acyl-CoA oxidase activity (U/mg) with:								
	Growth on glucose and assay substrate:			Dodecane induction and assay substrate:			Methyl-laurate induction and assay substrate:		
	C_6-CoA	C_{10}-CoA	C_{12}-CoA	C_6-CoA	C_{10}-CoA	C_{12}-CoA	C_6-CoA	C_{10}-CoA	C_{12}-CoA
SU-2	0.23	0.12	0.15	0.24	0.41	0.47	0.38	0.47	0.60
H51	0.25	0.35	0.28	0.34	0.23	0.31	1.62	2.16	1.08
H53	0.26	0.39	0.26	6.87	2.99	2.41	3.43	4.71	2.14
H534	0.08	0.10	0.08	1.73	0.82	0.77	0.84	1.52	0.42
H534B	0.03	0.01	0.02	2.50	1.21	1.39	0.63	0.66	0.59
H45	0.03	0.05	0.03	0.24	0.16	0.19	0.92	1.11	0.55
H41	0.04	0.04	0.04	0.61	0.50	0.39	0.92	1.28	0.92
H41B	0.08	0.03	0.07	0.89	1.34	1.39	0.78	0.92	0.86
H43	ND[a]	ND	0.01	0.03	0.96	1.56	ND	0.05	0.06
H435	ND	ND	ND	0.01	0.19	0.29	ND	0.03	0.05
H5343	ND	ND	ND	ND	ND	ND	ND	ND	ND

[a] ND, no activity detected.

block of the β-oxidation pathway and consequently prevents the strain from utilizing alkanes or fatty acids as carbon sources for growth.

In the absence of any other functional acyl-CoA oxidase activity, a comparison of strain H53 with strain H43 thus allowed direct measurement of PXP4 and PXP5 isozyme activities, respectively. Extracts from strain H53 demonstrated wild-type levels of acyl-CoA oxidase following growth on glucose, while those from strain H43 showed little or no activity. When grown on glucose, only mutants containing PXP4 demonstrated functional acyl-CoA oxidase activity, suggesting that this is the sole constitutively expressed acyl-CoA oxidase isozyme in the wild type. Also, the level of activity was reduced below wild-type levels only in mutants containing *POX4* gene disruptions. The absence of activity in strain H43 grown on glucose indicates that *POX5* is not expressed under these conditions.

Upon induction with dodecane or methyl laurate, several mutants containing partial β-oxidation blockage demonstrated greater than wild-type activities, apparently to compensate for the loss of one gene product by overexpression of any remaining functional *POX4* or *POX5* genes. *POX4* was strongly induced by either dodecane or methyl laurate and to a greater extent than *POX5*, which was induced primarily by dodecane. Since the enzymatic activity in methyl laurate-induced strain H43 was 10-fold less than wild-type levels, PXP4 appears to be the principal isozyme induced by these substrates. This conclusion is consistent with the observation that PXP4 is the most abundant peroxisomal polypeptide in oleate-grown cells (8).

The elevated activity in these mutants suggested that the two isozymes might have different functions in the β-oxidation pathway. The specific activities in strain H43 were higher when assayed on substrates with 12 carbons than on those with either 10 or 6 carbons. In contrast, strain H53 demonstrated higher specific activities on the shorter-chain substrates. These results suggested that PXP4 and PXP5 differ in chain length specificity. To further characterize these differences, the specific activities of the extracts from dodecane-induced strains H43, H53, and SU-2 were determined on C_4-CoA through C_{18}-CoA substrates at concentrations ranging from 6.25 to 100μM (Fig. 3). The specific activities in strain H43 were the highest on C_{12}-CoA and increased with substrate concentrations between 6.25 and 100 μM. Little or no activity was detected on substrates shorter than 8 carbons or longer than 16 carbons. Thus, PXP5 appears to have a narrow-spectrum long-chain activity. In contrast, PXP4 in strain H53 exhibited a broader substrate range than did PXP5, demonstrating activity on all substrates from C_4-CoA through C_{18}-CoA. The highest specific activity was observed on substrates with six carbons when tested at substrate concentrations of 25 μM or higher. At concentrations lower than 25 μM, the highest activity was observed on substrates with 12 carbons. In addition, the activity on substrates longer than C_{10}-CoA decreased with substrate concentrations higher than 12.5 μM, suggesting inhibition of this isozyme at the higher substrate concentrations. Increasing the peroxidase and flavin adenine dinucleotide concentrations in these assays did not increase the specific activities. Thus, the specificity of PXP4, at least in vitro, appeared to be modulated by the substrate concentration: at high concentrations, the isozyme demonstrated maximal activity on short-chain substrates, while at low substrate concentrations, the chain length specificity of PXP4 shifted to the longer substrates. A comparison of the activities in strains H534 and H534B showed that the isozymes encoded by the two *POX4* alleles demonstrate similar chain length specificity at substrate concentrations ranging from 12.5 to 100 μM (results not shown). The wild-type acyl-CoA oxidase activity detected in strain SU-2 demonstrated a broader substrate spectrum than either of the individual isozymes. While the maximum specific activity was observed on C_{12}-CoA, as reported by Shimizu et al. (12) for the purified enzyme from *C. tropicalis*, substrate inhibition on the longer chain lengths was also observed (Fig. 3). These results suggested that the two isozymes differ both in chain length specificity and in kinetic properties, since only the PXP4 isozyme was sensitive to substrate inhibition on the longer-chain substrates.

DISCUSSION

These results represent a direct demonstration of gene disruption for the site-specific modification of the *C. tropicalis* genome and have revealed the diploid nature of this yeast by showing that it contains two copies of each *POX4* and *POX5* gene. The high frequency at which the desired mutants were recovered illustrates the specificity of the modifications to *C. tropicalis* genome. For example, all of the 11 transformants analyzed following transformation with

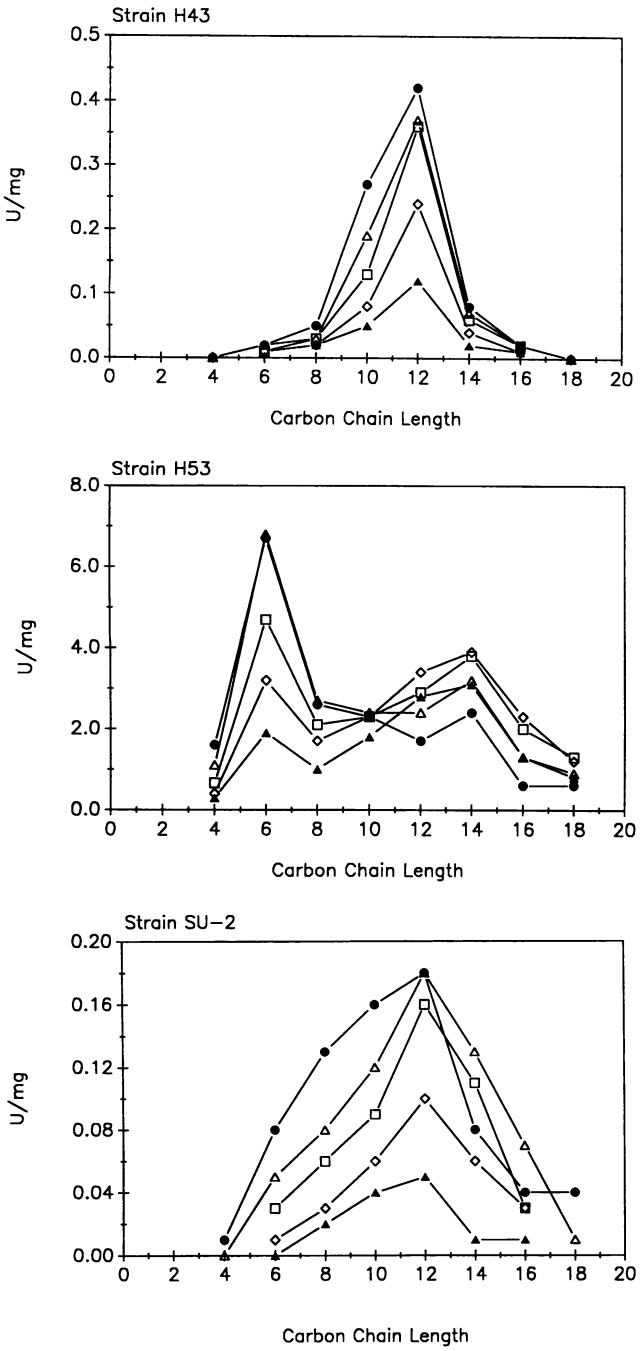

FIG. 3. Acyl-CoA oxidase isozyme activity profile as a function of substrate chain length. Strains were cultured for 30 h in YEPD and then induced for 40 h in yeast nitrogen base containing yeast extract (3 g/liter) and dodecane (15 g/liter). Extracts were prepared by repeated passage of washed cell suspensions through a French pressure cell (1,260 lb/in^2), and cell debris was removed by centrifugation (13,000 × *g*). Activity was measured independently on C_4-CoA through C_{18}-CoA substrates (12) and normalized to the protein concentration. The *POX5*-encoded acyl-CoA oxidase was measured in strain H43. The POX4-encoded isozyme was measured in strain H53. Wild-type activity was measured in strain SU-2. Symbols: ▲, 6.25 μM; ◇, 12.5 μM; □, 25 μM; △, 50 μM; ●, 100 μM.

the *POX5* disruption cassette contained the expected *POX5* gene disruption at the chromosomal *POX5* locus. None of the Ura$^+$ transformants were recovered as the result of integration at the chromosomal *ura3A* locus. Therefore, in *C. tropicalis*, the ends of linear transforming DNA are both highly recombinogenic and dictate the site of integration into the genome. Furthermore, the utility of the *URA3A* transformation system was illustrated by its ability to complement the SU-2 uracil requirement even when the subcloned *URA3A* gene fragment was integrated at a site other than the chromosomal *ura3A* locus. This approach permits site-specific genetic modifications to *C. tropicalis* for the first time.

The *POX4* gene disruptions also revealed the allelic heterogeneity of the two *POX4* homologs. The increased size of the disrupted *POX4A* gene fragment in H41 allowed easy identification of both homologs. By a comparison of *C. tropicalis* SU-2 and H41 digestion patterns, restriction site polymorphism was observed for endonucleases *Hpa*I, *Hin*dIII, *Sac*I, *Bgl*II, *Bam*HI, *Xho*I, and *Sal*I, indicating significant divergence between the two homologs. When restriction fragments derived from the 5′ and 3′ regions of the cloned *POX4A* gene were used to probe restriction digests of H41 DNA, the downstream sequences detected both *POX4* homologs but the upstream sequence detected only the disrupted *POX4A* gene. This finding suggested that the divergence lies immediately upstream of *POX4B* but certainly does not rule out downstream divergence beyond the regions tested. Clearly, this divergence between *POX4* homologs had initially prevented disruption of *POX4B* with the *POX4A* disruption cassette. It seems likely that the *POX4* DNA sequence differences previously reported (7, 8) reflect the allelic heterogeneity of these two homologs.

Methods to restore the uracil requirement to cells previously transformed with the *C. tropicalis URA3A* gene to Ura$^+$ have also been developed to allow sequential gene disruptions within a single strain. The mutagenesis of the *URA3A* gene has been accomplished either by directed deletion of a portion of the gene or by selection for spontaneous mutations that inactivate the gene, using the toxic analog 5-FOA. The resulting Ura$^-$ strains can serve as the host strain for another round of targeted mutagenesis by reutilizing the *URA3A* selectable marker for gene disruption by selection for the Ura$^+$ phenotype. The directed deletion approach is particularly useful since it provides a nonreverting *ura3* mutation and eliminates any Ura$^+$ background, thus facilitating the identification of subsequent Ura$^+$ transformants. Both approaches have been used to develop *C. tropicalis* strains with multiple *POX4* and *POX5* gene disruptions. Therefore, the selectable marker transformation system can be regenerated to permit multiple genetic modifications in a single strain of *C. tropicalis* without the need for UV-induced mitotic recombination as in *Candida albicans* (6). Furthermore, the mitotic stability of the multiple gene disruptions contained in strain H5343 has been demonstrated by their retention following successive transfer for over 90 generations.

A comparison of mutants in which both *POX4* or both *POX5* genes were disrupted has shown that the isozymes encoded by these two genes are differentially regulated and display unique substrate profiles and kinetic properties. While both genes were induced on alkane and fatty acid substrates, only *POX4* was constitutively expressed on glucose. The *POX4*-encoded isozyme functions over a broader substrate range than does the isozyme encoded by *POX5*. Several mutants containing partial β-oxidation blockage demonstrated specific activities as much as 20-fold

greater than the wild-type levels, apparently to compensate for the loss of one gene product by overexpression of any remaining functional *POX4* or *POX5* genes. Despite the ability of these mutants to grow on alkane or fatty acid substrates and the elevated levels of acyl-CoA oxidase, we have also observed that these strains demonstrate increased synthesis and excretion of dicarboxylic acids, suggesting that a significant portion of alkane or fatty acid substrate is redirected to the ω-oxidation pathway (unpublished data). Taken together, these observations suggest that the two isozymes are neither functionally identical nor physiologically capable, independently, of maintaining normal substrate flow through the β-oxidation pathway. The data presented here support this hypothesis. In general, the PXP4 isozyme demonstrated higher specific activities on shorter-chain substrates and the PXP5 isozyme demonstrated higher specific activities on longer-chain substrates. The chain length specificity observed here for PXP5 appears to be very similar to that reported by Shimizu et al. (12) for the purified enzyme from *C. tropicalis*, in agreement with Okasaki et al. (8), who suggested that they had, in fact, purified the isozyme encoded by *POX5*.

The absence of detectable acyl-CoA oxidase activity in strain H5343, in which all *POX4* and *POX5* genes had been disrupted, confirmed that all functional acyl-CoA oxidase genes had been inactivated. This strain cannot utilize alkanes or fatty acids for growth, indicating that the β-oxidation pathway has been functionally blocked. Apparently, the *POX2* gene previously reported to be a third gene in the peroxisomal acyl-CoA oxidase multigene family (9) either represents a nonfunctional gene duplication of *POX4* or encodes a structural component devoid of enzymatic activity.

The primary structure of acyl-CoA oxidase has been of particular interest since internal topogenic signals postulated to direct its localization to the peroxisome have been identified. However, the ability to study the localization of these enzymes in *C. tropicalis* has been hampered by the lack of a gene transfer system and the availability of appropriate mutants. Consequently, it has not been possible to study peroxisomal localization in vivo. The selectively blocked strains described here will be useful for further study of the localization and import of peroxisomal acyl-CoA oxidase in *C. tropicalis* by allowing an in vivo analysis of deletion mutants and hybrid proteins designed to study the internal targeting signals.

REFERENCES

1. **Boeke, J. D., F. LaCroute, and G. R. Fink.** 1984. A positive selection for mutants lacking orotidine-5′-phosphate decarboxylase activity in yeast: 5-fluoro-orotic acid resistance. Mol. Gen. Genet. **197**:345–346.
2. **Haas, L. O. C., J. M. Cregg, and M. A. G. Gleeson.** 1990. Development of an integrative DNA transformation system for the yeast *Candida tropicalis*. J. Bacteriol. **172**:4571–4577.
3. **Hanahan, D., and M. Messelson.** 1983. Plasmid screening at high colony density. Methods Enzymol. **100**:333–342.
4. **Kamiryo, T., and K. Okazaki.** 1984. High-level expression and molecular cloning of genes encoding *Candida tropicalis* peroxisomal proteins. Mol. Cell. Biol. **4**:2136–2141.
5. **Kamiryo, T., Y. Sakasegawa, and H. Tan.** 1989. Expression and transport of *Candida tropicalis* peroxisomal acyl-coenzyme A oxidase in the yeast *Candida maltosa*. Agric. Biol. Chem. **53**:179–186.
6. **Kelley, R., S. M. Miller, M. B. Kurtz, and D. R. Kirsch.** 1987. Directed mutagenesis in *Candida albicans*: one-step gene disruption to isolate *ura3* mutants. Mol. Cell. Biol. **7**:199–207.
7. **Murray, W. W., and R. A. Rachubinski.** 1987. The primary structure of a peroxisomal fatty acyl-CoA oxidase from the yeast *Candida tropicalis* pK233. Gene **51**:119–128.
8. **Okazaki, K., T. Takechi, N. Kambara, S. Fukui, I. Kubota, and T. Kamiryo.** 1986. Two acyl-coenzyme A oxidases in peroxisomes of the yeast *Candida tropicalis*: primary structures deduced from genomic DNA sequence. Proc. Natl. Acad. Sci. USA **83**:1232–1236.
9. **Okazaki, K., H. Tan, S. Fukui, I. Kubota, and T. Kamiryo.** 1987. Peroxisomal acyl-coenzyme A oxidase multigene family of the yeast *Candida tropicalis*; nucleotide sequence of a third gene and its protein product. Gene **58**:37–44.
10. **Osumi, M., F. Fukuzumi, Y. Treanishi, A. Tanaka, and S. Fukui.** 1975. Development of microbodies in *Candida tropicalis* during incubation in *n*-alkane medium. Arch. Microbiol. **103**:1–11.
11. **Santos, M. J., T. Imanaka, H. Shio, and P. B. Lazarow.** 1988. Peroxisomal integral membrane proteins in control and Zellweger fibroblasts. J. Biol. Chem. **263**:10502–10509.
12. **Shimizu, S., K. Yasui, Y. Tani, and H. Yamada.** 1979. Acyl-CoA oxidase from *Candida tropicalis*. Biochem. Biophys. Res. Commun. **91**:108–113.
13. **Small, G. M., L. J. Szabo, and P. B. Lazaro.** 1988. Acyl-CoA oxidase contains two targeting sequences each of which can mediate protein import into peroxisomes. EMBO J. **7**:1167–1173.
14. **Ueda, M., K. Yamanoi, T. Morikawa, H. Okada, and A. Tanaka.** 1985. Peroxisomal localization of enzymes related to fatty acid β-oxidation in an n-alkane-grown yeast, *Candida tropicalis*. Agric. Biol. Chem. **49**:1821–1828.