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Cloning, Sequencing, and Characterization of Five Genes Coding for Acyl-CoA Oxidase Isozymes in the Yeast *Yarrowia lipolytica*

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ABSTRACT

The Acyl-CoA oxidase (AOX) isozymes catalyze the first steps of peroxisomal β-oxidation, which is important for the degradation of fatty acids. Using conserved blocks in previously identified yeast *POX* genes encoding AOXs, the authors have shown that five *POX* genes are present in the yeast *Yarrowia lipolytica*. These genes show approx 63% identity among themselves, and 42% identity with the *POX* genes from other yeasts. Mono-disrupted *Y. lipolytica* strains were constructed using a variation of the sticky-end polymerase chain reaction method. AOX activity in the mono-disrupted strains revealed that a long-chain oxidase is encoded by the *POX2* gene and a short-chain oxidase by the *POX3* gene.

Index Entries: Yeast; *Yarrowia lipolytica*; Acyl-CoA oxidase; lactone.

INTRODUCTION

The yeast *Yarrowia lipolytica* is able to utilize hydrophobic substrates like alkanes, triglycerides, and fatty acids (FAs) as carbon source for growth *(1)*. Degradation of alkanes involved three enzymatic steps in the endoplasmic reticulum to produce a FA *(2)*. Similarly, degradation of triglycerides is performed by secreted lipases that release the corresponding FAs, which are then metabolized. β oxidation is a very important peroxisomal cycle involved in the degradation of these FAs *(3)*. It also plays a crucial role in the production of lactone, for example, γ-decalactone (a peach flavor), from oil or derivatives (castor oil, ricinoleic acid, or methyl ricinoleate) *(4)*.

Mutants in *Y. lipolytica* affected in alkane utilization have been isolated *(2,5,6)*. Similarly,

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mutants affected for growth on FA were selected in the laboratory of Rachubinski *(7)*. In addition, Nga et al. *(8)* attempted to isolate mutants affected in lipase production. Although these approaches were successful for the isolation of mutants affected in different steps in the degradation of hydrophobic substrates, or in the characterization of genes involved in peroxisomes biogenesis *(9–11)*, they were not successful for the isolation of genes encoding lipases and acyl CoA oxidases (AOXs), probably because of multiple family members for these genes. Therefore, a reverse genetic approach was used to isolate the genes encoding these enzymes.

Here is described the characterization of a multicomponent family encoding AOXs from the yeast *Y. lipolytica*, the construction of mono-disrupted strains, and an analysis of global AOX activity in these modified strains.

MATERIALS AND METHODS

Strains

Y. lipolytica strains used in this study are Po1d (*MatA, ura3-302, leu2-270, xpr2-322*) *(1)* and derivatives MTLY25 *pox1*KO (*pox1::URA3*), MTLY12 *pox2*KO (*pox2::URA3*), MTLY13 *pox3*KO (*pox3::URA3*), MTLY14 *pox4*KO (*pox4::URA3*), and MTLY15 *pox5*KO (*pox5::URA3*).

Media

The media YPD and yeast nitrogen base (YNB) were prepared as described previously *(1)*. Minimum fatty acid media YNBO is composed of: YNB (0.17%), ammonium chloride (0.4%), uracil (0.01%), leucine (0.032%), as required, and methyl oleate at a 1% final concentration. A FA stock solution was prepared as follows: A mixture of methyl oleate (10%) and Tween 80 (1%) was prepared and sonicated 3× for 1 min on ice. For solid medium, 2% agarose was added.

Sequence Determination and Analysis

Double-stranded templates were purified on a qiawell8 column (Qiagen). Sequence analysis was performed on an automated sequencer (ABI model 373A, Perkin Elmer), using synthetic primers and the dye terminator procedure. The complete nucleotide sequence was compiled using the Staden package of programs *(12)*. DNA and protein sequences were analyzed using custom-made Staden programs and software from the University of Wisconsin Genetics Computer Group (version 8) *(13)*.

Construction of Disruption Cassettes (PT and PUT)

Amplifications of the promoter (P) and terminator (T) regions, and the production of a promoter–terminator (PT) fragment containing a central *Sce*I site, were performed according to the sticky-end polymerase chain reaction (PCR) method (SEP method) *(14)*. The resulting PT fragment (disrupt 2 cassette) was treated with T4 DNA polymerase, or by adding Pyrococcus furiosus DNA polymerase in the PCR buffer, to render the ends blunt, was cloned into the *Eco*RV site of pBluescript II KS$^+$ (Stratagene) to give the disrupt 2 cassette (PT cassette). Those clones containing the disrupt 2 cassette were digested with *Sce*I, and the URA3 *Sce*I cassette, which contains one *Sce*I restriction site on either side, was inserted, yielding the disrupt 1 cassettes (PUT cassette).

Yeast Transformation and Verification of Disruption by PCR

Y. lipolytica cells were transformed using the lithium acetate method, as described in Barth and Gaillardin *(1)*, and correct disruption of *POX* genes was verified by PCR, as described by Güssow and Clarkson *(15)*.

AOX Activity Assays

AOX activity was measured as described previously *(16)*. Long-chain AOX activity was measured, using hexanoyl-CoA (C6), decanoyl-CoA (C10), and myristoyl-CoA (C14) as substrates. Results are means of at least three separate experiments.

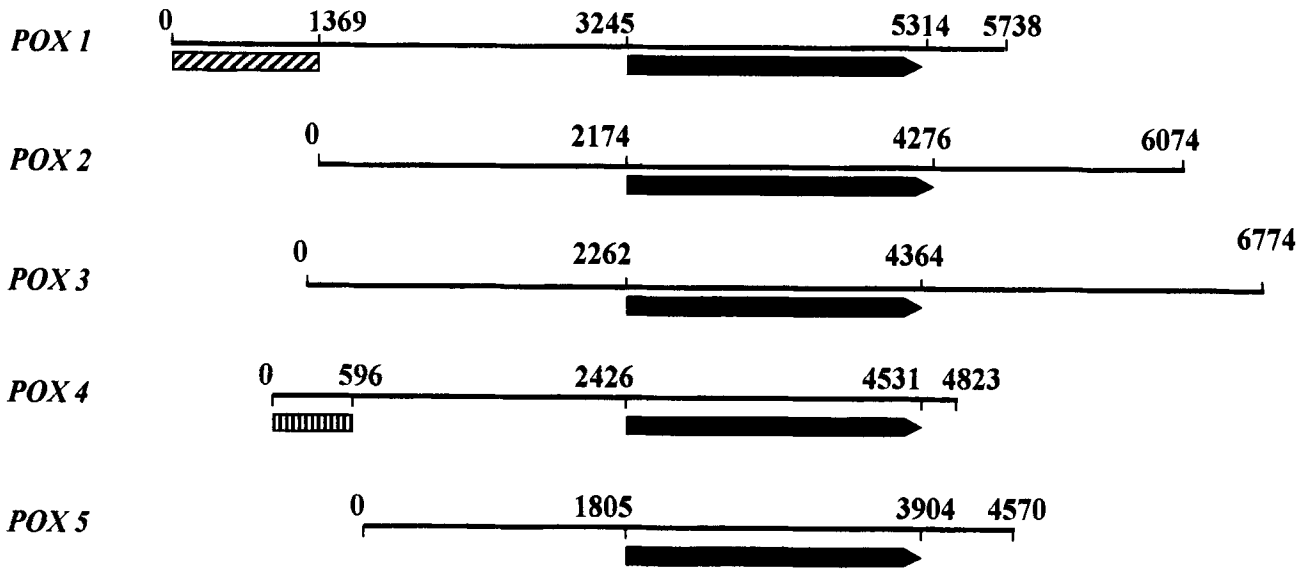

Fig. 1. Schematic representation of the genomic regions containing *Y. lipolytica POX* genes. Black arrow corresponds to the *POX* ORFs. Hatched box in *POX1* sequence indicate part of the ORF homologous to yLPH17 (S62012). Hatched box in *POX4* sequence represents the location of the ORF homologous to *S. cerevisiae* DNA-directed RNA polymerase I and III (P28000).

RESULTS

Cloning and Sequencing of Genes for Y. lipolytica *AOXs*

Comparison of the *POX1* gene from *Saccharomyces cerevisiae* (M27515), the *AOX1* and *POX1* genes from *Candida maltosa* (X06721, D21228), and the *PXP4*, *PXP5*, and *PXP2* genes from *Candida tropicalis* (M12160, M12161, P18259) revealed conserved nucleotide blocks that were used to amplify fragments of the genes encoding *Y. lipolytica* AOXs. The amplified fragments were used for the isolation of plasmids containing the corresponding genes by colony hybridization of recombinants from the Xuan library *(17)*, or by divergent PCR on DNA from C. Neuvéglise gene library *(18)*.

As shown in Fig. 1, the sequences were determined over 4.3–6.7 kbp. For *POX1*, the complete 5738 bp sequence revealed two open-reading frames (ORF), a 1369-bp region coding for the NH2 terminus of a protein, with 42.9% identity to the ORFAN yLPH17 identified during the systematic sequencing of *S. cerevisiae* genome (major intrinsic protein sequence S62012). The second ORF, of 2067 bp, corresponding to the yl*POX1* gene, codes for a protein composed of 689 amino acids (aa) (77,232 Daltons). The second 6074-bp sequence revealed a single ORF corresponding to the yl*POX2* gene, which codes for a 700-aa protein (78,641 Daltons). The yl*POX3* gene is contained within a 6774-bp sequence, and encodes a 700-aa protein (77,960 Daltons). In the 4823-bp sequence containing the yl*POX4* gene, which codes for a protein of 701 aa (79,241 Daltons), a second ORF, showing 47% identity (64% similarity) to RPC9 (Swissprot accession no. P28000) encoding *S. cerevisiae* DNA-directed RNA polymerase I and III, and encoding a 16-kDa polypeptide, was observed. Finally, the yl*POX5* gene contained within a 4570-bp fragment and encodes a protein of 699 aa (78,300 Daltons). Sequences will appear in the (EMBL) database under the accession no. AJOO1299–AJOO1303.

The ATG environments of the *POX* genes are as follows; CCGACAATGA (*POX1*),

GACGCCATGA (*POX2*), CACACAATGA (*POX3*), ACAACAATGA (*POX4*), and CCAAACATGA (*POX5*). All except *POX2* have an A at the −3, −1 and +3 positions, a feature that was shown to be important for high gene expression in *Y. lipolytica*. Using β-galactosidase as a reporter gene, the authors have shown that these genes are not expressed on glucose, and are induced on fatty acid media. In oleic media, *POX2*, *POX3*, and *POX5* present similar strength: *POX4* a lower one and *POX1* is a low expressed gene (data not shown).

No sequence similar to the peroxisome box (URE) *19–21)*, or to the oleic acid-responsive element (OAR) observed in *C. tropicalis (22)* and *S. cerevisiae (23)*, could be observed in *POX* promoter regions. However, a putative oleate response element (ORE), whose sequence is CGG (N15-17)CCG *(24)* is observed in *POX1*, *POX2*, and *POX3* promoters.

A comparison of the promoter region, using the MACAW program of the NCBI, showed that t/aCCCCACAc and the GTAC(N2)Gt/aAC motifs were observed in all yl*POX* genes, although their functions and roles in AOX expression remain to be determined.

Comparison of AOX Proteins

Comparison of the deduced aa sequences of the five AOX genes of *Y. lipolytica* with those of *S. cerevisiae*, *C. maltosa*, and *C. tropicalis* is presented in Fig. 2. Alignment and comparison of deduced aa sequences showed that 22% of the aa are conserved in all AOXs (Fig. 2, consensus). Two highly conserved blocks are located at aa 240 and 490, whose sequences are Block 1, ATKWWIGGAAH; and block2, QXCGGHGYSXYNGF(X5)DWXVQCTWEGD N. *Y. lipolytica* AOXs are only about 45% identical (50% similar) to AOXs from other yeasts, but they are 55–70% identical amongst themselves (65–76% similar), as shown in Table 1. AOXs from *C. tropicalis* and *C. maltosa* are less conserved, showing 51–63% identity between them (65–76% similar) for *C. tropicalis*, and 50% identity between cm*POX1* and cm*POX4* (65–76% similar) for *C. maltosa*. The highest identity was observed between ct*AOX2* and cm*AOX1* (84% similar) and ct*AOX4* and cm*AOX4* (83% similar), indicating that cm*AOX4* may correspond to a short-chain AOX. Such higher identity was not observed with any of the *Y. lipolytica* proteins, giving no evidence for their potential specific activity.

Members of the *Y. lipolytica* AOX family are often 55–70% identical to one another, but this identity was not evenly dispersed along the proteins. As shown in Fig. 2, regions with lower identity are at the NH2 terminus, from AA_{370} to AA_{400}, AA_{480} to AA_{550} and at the COOH terminus from AA_{660}. These regions may therefore be involved in chain-length specificity.

As in other yeast AOXs, *Y. lipolytica* AOXs lack any conserved variant of PTS1 or PTS2 motifs, in contrast to rat liver AOX, which is targeted to peroxisomes by a PTS1 motif *(31)*. The most striking feature among them is that the COOH termini of the proteins are rich in acidic aa (aspartic acid D and glutamine E). Acidic COOH termini were not observed in the AOX proteins of the other yeasts.

Construction of Mono-disrupted Strains

In order to determine the roles and functions of the five *POX* genes found in the yeast *Y. lipolytica*, the authors first decided to construct mono-disrupted strains. The approach was to construct disruption cassettes by the SEP method, developed in this laboratory for gene disruption in *S. cerevisiae*, as described in Material and Methods (SEP Method) *(14)*. Gene disruption was achieved by gene replacement *(32)*, using the disrupt 1 cassette containing P and T fragments, separated by an *URA3* gene. On average, about 800 bp of the P and of the T regions were amplified. The cassette 1 was amplified by PCR, and the amplified 2.2-kbp fragments were used to transform Po1d strain. Transformants were selected for complementation of the uracile auxotrophy. Typically, 10^2–10^3 transformants were obtained per μg PCR fragment, and 50% contained a disrupted allele, as shown by PCR and confirmed by Southern blot (data not

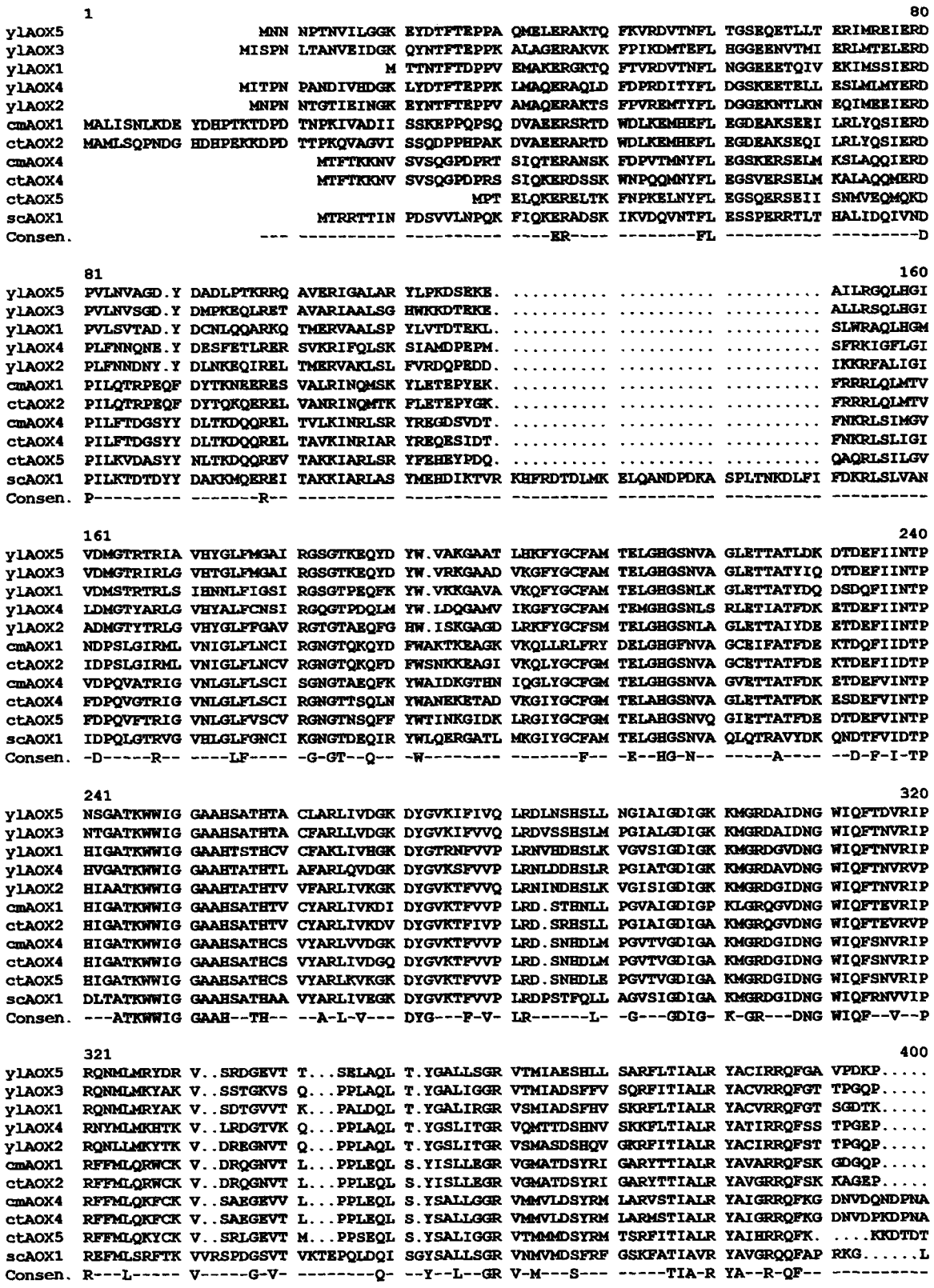

Fig. 2. Comparison of aa sequences of yeast AOX. Alignment of *Y. lipolytica* (*POX1–POX5*, this work), *S. cerevisiae POX1 (25)*, *C. maltosa maltosa POX2* and *POX4 (26,27)*, and *C. tropicalis POX2, POX4*, and *POX5 (28–30)*. Identical aa in all four species are indicated in the consensus sequence. Gaps indicated by dots were introduced for optimal alignment. EMBL accession no. AJ001299–AJ001303.

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          401                                                                                                  480
ylAOX5    .ETKLIDYPY HQRRLLPLLA YTYAMKMGAD EAQQQYNSSF GALLKLNPVK DAEK..FAVA TADLKALFAS SAGMKAFTTW
ylAOX3    .ETKIIDYPY HQRRLLPLLA FTYAMKMAAD QSQIQYDQTT DLLQTIDP.K DKGA..LGKA IVDLKELFAS SAGLKAFTTW
ylAOX1    .ETKIIDYPY HQRRLLPLLA YCYAMKMGAD EAQKTWIETT DRILALNPND PAQKNDLEKA VTDTKELFAA SAGMKAFTTW
ylAOX4    .ETRLIDYLY HQRRLLPLMA YSYAMKLAGD HVRELFFAS. ..QEKAESLK EDDKAGVESY VQDIKELFSV SAGLKAATTW
ylAOX2    .ETKIIDYPY HQRRLLPLLA YVYALKMTAD EVGALFSRT. ..MLKMDDLK PDDKAGLNEV VSDVKELFSV SAGLKAFSTW
cmAOX1    .ETKLIDYTL HQRRLLPYLA LTYLAALGTD KLERQHDQLL KNLDKA..LA TNNKLLLKNT IQSTKSMFVD SGSLKSTLTW
ctAOX2    .ETKLIDYTL HQRRLLPYLA LTYAAAVGTD RLERQHEELL ANLDIA..LA KKDKLLLKNT ITGTKSMFVD SGSLKSTLTW
cmAOX4    LETQLIDYPL HQKRLFPYLA AAYVVSTGAL KVEHTIQSTL ATLDAA..VE NNDTTAIFKS IDDMKSLFID SGSLKATTTW
ctAOX4    LETQLIDYPL HQKRLFPYLA AAYVISAGAL KVEDTIHNTL AELDAA..VE KNDTKAIFKS IDDMKSLFVD SGSLKSTATW
ctAOX5    IETKLIDYPL HQKRLFPFLA AAYLFSQGAL YLEQTMNATN DKLDEA..VS AGEKEAIDAA IVESKKLFVA SGCLKSTCTW
scAOX1    SETQLIDYPL HQYRVLPQLC VPYLVSPVAF KLMDNYYSTL DELYNAS..S SAYKAALVTV SKKLKNLFID SATLKATNTW
Consen.   -ET--IDY-- HQ-R--P--- --Y------- ---------- ---------- ---------- ----K--F-- S---K---TW

          481                                                                                                  560
ylAOX5    AAAKIIDECR QACGGHGYSG YNGFGQAYAD WVVQCTWEGD NNVLCLSMGR SLIQSCIAMR KKKGHVGKSV EYLQRRDELQ
ylAOX3    TCANIIDQCR QACGGHGYSG YNGFGQAYAD WVVQCTWEGD NNVLCLSMGR GLIQSCLGHR KGK.PLGSSV GYLANKG.LE
ylAOX1    GCAKIIDECR QACGGHGYSG YNGFGQGYAD WVVQCTWEGD NNVLCLSMGR GLVQSALQIL AGK.HVGASI QYVGDKSKIS
ylAOX4    ACADIIDKAR QACGGHGYSA YNGFGQAFQD WVVQCTWEGD NTVLTLSAGR ALIQSALVYR KE.GKLGNAT KYLSRSKELA
ylAOX2    ACADVIDKTR QACGGHGYSG YNGFGQAYAD WVVQCTWEGD NNILTLSAGR ALIQSAVALR KG.EPVGNAV SYLKRYKDLA
cmAOX1    LASDLINEAR QSCGGHGYSA YNGFGKTYGD WAVQCTWEGD NNVLGMSAGK TIIKTVQQVL NGKQLKDSTL EFLNDAPAL.
ctAOX2    LAADLINETR QACGGHGYSS YNGFGKTYDD WVVQCTWEGD NNVLAMSAGK TIIKTVQQVL NGKELKDSTL EFLNAAPEL.
cmAOX4    LAAEAIDQCR QACGGHGYSS YNGFAKAFND WVVQCTWEGD NNVLSLSVGK PIIKQIIGIE DNGKTVRGST AFLNQVKDFT
ctAOX4    LGAEAIDQCR QACGGHGYSS YNGFGKAYND WVVQCTWEGD NNVLAMSVGK PIVKQVISIE DAGKTVRGST AFLNQLKDYT
ctAOX5    LTAEAIDEAR QACGGHGYSS YNGFGKAYSD WVVQCTWEGD NNILAMNVAK PMVRDLLKEP E......... ..........
scAOX1    LIATLIDELR QTCGGHGYSQ YNGFGKGYDD WVVQCTWEGD NNVLSLTSAK SILKKFID.S ATKGRFDNTL DVDSFSYLKP
Consen.   -----I---R Q-CGGHGYS- YNGF-----D W-VQCTWEGD N--L------ ---------- ---------- ----------

          561                                                                                                  640
ylAOX5    N..ARVDNKP LTDPAVLITA WEKVACEAIN RATDSFIKLT QEGLSPDQAF EELSQQRFEC ARIHTRKHLI TSFYARI.SK
ylAOX3    Q..ATLSGRD LKDPKVLIEA WEKVANGAIQ RATDKFVELT KGGLSPDQAF EELSQQRFQC AKIHTRKHLV TAFYERINAS
ylAOX1    QNGQGTPREQ LLSPEFLVEA FRTASRNNIL RTTDKYQELV KT.LNPDQAF EELSQQRFQC ARIHTRQHLI SSFYARI.AT
ylAOX4    N..AKRNGRS LEDPKLLVEA WEAVSAGAIN AATDAYEELS KQGVSVDECF EQVSQERFQA ARIHTRRALI EAFYSRIAT.
ylAOX2    N..AKLNGRS LTDPKVLVEA WEVAAGNIIN RATDQYEKLI GEGLNADQAF EVLSQQRFQA AKVHTRRHLI AAFFSRIDTE
cmAOX1    .SSAKKAVIR IKSHVDDTDR VLKAIAGLIS KYAKDL.IPV S.....YQSW DSIGPQRVVL SKFRCHYYLL ETFNERLNDR
ctAOX2    .SKAKKAVIR IRDHVDDVDR VLKAIAGLIS KFSKDL.IPI S.....YQSW DSIGAQRVIL SKLRCHYYLL ETFNERLNDK
cmAOX4    GSNASKVVLN NTSDLNDINK VIKSIEVAII RLAHEAAISV R.....KESL DFAGAELVQI SKLKAHHYLL TEFVKRVGE.
ctAOX4    GSNSSKVVLN TVADLDDIKT VIKAIEVAII RLSQEAASIV K.....KESF DYVGAELVQL SKLKAHHYLL TEYIRRIDT.
ctAOX5    ...QKGLVLS SVADLDDPAK LVKAFDHALS GLARDIGAVA E.....DKGF DITGPSLVLV SKLNAHRFLI DGFFKRITP.
scAOX1    QYIGSVVSGE IKSGLKELGD YTEIWSITLI KLLAHIGTLV EKS....RSI DSVSKLLVLV SKFHALRCML KTYYDKLNSR
Consen.   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

          641                                                                                                  720
ylAOX5    ....AKARVK PHLTVLANLF AVWSI.EEDS GLFLREGCFE PAEMDEIT.A LVDELCCEAR EQVIGFTDAF NLSDFFINAP
ylAOX3    ....AKADVK PYLINLANLF TLWSI.EEDS GLFLREGFLQ PKDIDQVT.E LVNHYCKEVR DQVAGYTDAF GLSDWFINAP
ylAOX1    ....AKDDIK PHLLKLANLF ALWSI.EEDT GIFLRENILT PGDIDLIN.S LVDELCVAVR DQVIGLTDAF GLSDFFINAP
ylAOX4    ....ADEKVK PHLIPLANLF ALWSI.EEDS ALFLAEGYFE PEDIIEVT.S LVNKYCGIVR KNVIGYTDAF NLSDYFINAA
ylAOX2    ....AGEAIK QPLLNLALLF ALWSI.EEDS GLFLREGFLE PKDIDTVT.E LVNKYCTTVR EEVIGYTDAF NLSDYFINAP
cmAOX1    IK..AKSPAR PHLENIIKLY YVTNVLGPFI DEFLRFGVIS PSVAKYITTE YPQKLCAAIR PYVIGLTDSF QQPDNFINSL
ctAOX2    IK..AKSPAR PHLENIIKLY YVTNILGPFI DEFLRFGVIS PQVAKYITYE YPQKLCANIR PYVIGLTDSF QQPDNFINSL
cmAOX4    .F..EHKELV PFLNTIGRLY SATVVLDKFA GVFLTFNVAS PQAITDLAST QIPKLCAEVR PNVVAYTDSF QQSDMVINSA
ctAOX4    .F..DQKDLV PYLITLGKLY AATIVLDRFA GVFLTFNVAS TEAITALASV QIPKLCAEVR PNVVAYTDSF QQSDMIVNSA
ctAOX5    .E..WSEVLR P....LGFLY .ADWILTNFG ATFLQYGIIT PDVSRKISSE HFPALCAKVR PNVVGLTDGF NLTDMMTNAA
scAOX1    DSHISDEITK ESMWNVYKLF SLYFI.DKHS GEFQQFKIFT PDQISKVVQP QLLALLPIVR KDCIGLTDSF ELPDAMLNSP
Consen.   ---------- --------L- ---------- --F------- ---------- ---------R ------TD-F ---D---N--

          721                                                                  784
ylAOX5    IGRFDGDAYK HYMDEVKAAN N..PRNTHAP YYETKLRPFL FRPDEDEEIC DLDE
ylAOX3    IGNYDGDVYK HYFAKVNQQN P..AQNPRPP YYESTLRPFL FREDEDDDIC ELDEE
ylAOX1    IGSYDGNVYE KYFAKVNQQN P..ATNPRPP YYESTLKPFL FREEEDDEIC DLDE
ylAOX4    IGRYDGDVYK NYFEKVKQQY P..PEGGKPH YYEDVMKPFL HRERIPDVPM EPEDIQ
ylAOX2    IGCYDGDAYR HYFQKVNEQN P..ARDPRPP YYASTLKPFL FREEEDDDIC ELDEE
cmAOX1    IGRYDGNVYT NYLTNVTNVN D..PTNYKAP YSEALEAMLN RASLERERRF EKSKAVAAKL SQ
ctAOX2    IGKYDGNIYT NYLESVKDVN D..PSNYKAP YSEALEAMLN RSALENRERS ERGKAAADIL SK
cmAOX4    IGKYDGDVYE NYFDLVKQLN P..PKNTKAP YTAALEGMLN RPSLEARERY EKSDETAAIL SK
ctAOX4    IGRYDGDIYE NYFDLVKLQN P..PSKTKAP YSDALEAMLN RPTLDERERF EKSDETAAIL SK
ctAOX5    IGRYDGNVYE HYFETVKALN P..PENTKAP YSKALEDMLN RPDLEVRERG EKSEEAAEIL SS
scAOX1    IGYFDGDIYH NYFNEVCRNN PVEADGAGKP SYHALLSSML GRGFEFDQKL GGAANAEILS KINK
Consen.   IG--DG--Y- -Y---V---- ---------- ---------- ---------- -----
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Fig. 2. *(Continued)*

Table 1
Comparison of AOXs

	Percent identity										
	yl*POX1*	yl*POX2*	yl*POX3*	yl*POX4*	yl*POX5*	sc*POX1*	ct*POX2*	ct*POX4*	ct*POX5*	cm*POX2*	cm*POX4*
yl*POX1*		63.3	67.1	55.1	64.1	39.3	41.8	41.3	45.2	40.3	42.3
yl*POX2*	69.9		67.0	63.7	61.4	40.6	42.9	42.64	46.5	40.0	41.0
yl*POX3*	73.9	74.1		60.0	69.4	40.4	43.4	43.3	45.9	41.1	44.3
yl*POX4*	62.8	71.4	67.3		56.7	36.4	41.6	41.5	42.5	39.4	42.4
yl*POX5*	70.2	69.9	76.2	64.9		39.9	42.7	43.3	45.5	40.3	44.1
sc*POX1*	47.3	49.7	48.2	45.6	47.6		40.6	48.2	48.4	38.9	47.9
ct*POX2*	52.7	51.7	53.0	50.6	50.8	49.6		53.8	51.3	84.7	52.1
ct*POX4*	51.4	51.7	53.4	50.9	51.6	55.8	62.4		63.9	51.6	83.2
ct*POX5*	54.8	55.8	54.9	53.7	54.3	56.3	60.2	71.0		49.6	62.5
cm*POX2*	50.3	49.2	50.7	48.3	48.3	46.5	84.7	59.0	57.9		50.7
cm*POX4*	51.5	51.8	52.7	51.1	52.8	55.8	61.0	88.4	69.8	58.6	

Degrees of aa sequence identity and similarity between AOXs from different yeasts. Degree of sequence identity and similarity (%) was calculated using gap program in GCG software. yl*AOX1* through yl*AOX5* are from *Y. lipolytica*; scAOX1 is from *S. cerevisiae*; ct*AOX2*, ct*AOX4*, and ct*AOX5* are from *C. tropicalis*; and cm*AOX1* and cm*AOX4* are from *C. maltosa*.

Table 2
AOX Activity in WT Strain and Mono-disrupted Strains

Strain	Substrate		
	C6-CoA	C10-CoA	C14-CoA
Pold (WT)	0.45 +/−0.10	0.49 +/−0.17	0.45 +/−0.06
MTLY25 (*pox1*KO)	1.31 +/−0.19	1.76 +/−0.10	0.83 +/−0.06
MTLY12 (*pox2*KO)	0.69 +/−0.13	0.41 +/−0.10	0.27 +/−0.11
MTLY13 (*pox3*KO)	0.03 +/−0.02	0.56 +/−0.08	0.40 +/−0.13
MTLY14 (*pox4*KO)	1.04 +/−0.11	1.15 +/−0.07	0.72 +/−0.06
MTLY15 (*pox5*KO)	1.61 +/−0.03	1.93 +/−0.16	1.45 +/−0.08

AOX was measured using hexanoyl-CoA (C6), decanoyl-CoA (C10), or Myristyl-CoA (C14) as substrates. Strains are Pold (WT), MTLY25 (*pox1*KO), MTLY12 (*pox2*KO), MTLY13 (*pox3*KO), MTLY14 (*pox4*KO), and MTLY15 (*pox5*KO). Activity is expressed in U/mg protein. Total protein was measured by the Bradford method *(33)*.

shown). The mono-disrupted strains were MTLY25 *pox1*KO (*pox1::URA3*), MTLY12 *pox2*KO (*pox2::URA3*), MTLY13 *pox3*KO (*pox3::URA3*), MTLY14 *pox4*KO (*pox4::URA3*), and MTLY15 *pox5*KO (*pox5::URA3*).

AOX Activity in Mono-disrupted Strains

AOX activity was measured in the above deleted strains. Strains were grown on YNB, and transferred into YNBO media for induction. Table 2 shows the AOX activity in deleted strains 5 h after transfer into induction medium. The authors have compared AOX isozyme activity in the wild-type (WT) strain, Pold, and in the mono-disrupted strains, using C6-CoA, C10-CoA and C14-CoA as substrates. The AOX isozyme activities of each strain differs, depending on the length of the substrate carbon chain. As shown in Table 2, the AOX activity of strains deleted for *pox1*, *pox4*, and *pox5* is higher for the three substrates than in the WT strain. Similar results were obtained by Picataggio et al. *(34)*, who observed that a strain deleted for *POX4* has higher AOX activity than does the WT strain. Strains Δ*pox2* and Δ*pox3* had AOX activities similar to those of the WT strain, except for C14 substrate in Δ*pox2* strain and the C6 substrate in Δ*pox3* strain, which suggests that *POX2* codes for an AOX that is more active toward long-chain FAs (C14), and that *POX3* codes for an AOX that is more active toward short-chain FAs (C6). These results were confirmed when the activity of the AOX3 protein expressed in *Escherichia coli* was tested (not shown).

It seems that the enzymatic substrates for AOX2p and AOX3p are different and complementary for the growth on long-FA-containing medium. In contrast, the strains deleted for *pox*1, *pox*4, *pox*5 showed higher AOX activity than the WT strain on any FA substrate. It seems that deletion of these genes caused an increase in the activity of other AOXs (possibly AOX2p, AOX3p). Whether these effects are transcriptional or posttranscriptional is currently being investigated.

DISCUSSION

A multicomponent family encoding AOXs was identified in the yeast *Y. lipolytica*. The construction of mono-disrupted strains allowed the authors to demonstrate that AOX2p corresponds to a long-chain AOX, and that AOX3p is a short-chain AOX. Similar results have been observed by Picataggio et al. *(34)* in *C. tropicalis*, where AOX4 has high specific activities for short-chain substrates, and AOX5 is a long-chain AOX. The role of AXO1,

AOX4, and AOX5 remains to be determined, but the authors already demonstrated an effect of yl*POX1* gene disruption on γ-decalactone production and consumption *(35)*.

Construction of strains presenting double and multiple gene disruption will be necessary to go further in the understanding of the role of the Acyl-CoA oxidases in the yeast *Y. lipolytica*. This will open the opportunity to further modify *Y. lipolytica* strain for the improvement of the γ-lactone production, or for dicarboxylic acid production, as shown by Picataggio et al. *(36)*.

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REFERENCES

1. Barth, G. and Gaillardin, C. (1996) The dimorphic fungus *Yarrowia lipolytica*, in *Nonconventional Yeasts in Biotechnology* (Wolf, K. ed.), Springer-Verlag, Berlin, pp. 313–388.
2. Mauersberger, S. (1991) Mutants of alkane oxidation in the yeasts *Yarrowia lipolytica* and *Candida maltosa*, in Alkane metabolism and oversynthesis of metabolites by microorganisms (Finogenova, T. V., Sharyshev, A. A., eds.), Centre for Biological Research, USSR Academy of Sciences, Pushchino, Russia, pp. 59–78.
3. Tanaka, A., Osumi, M., and Fukui, S. (1982). Peroxisomes of alkane-grown yeast: fundamental and practical aspects. *Ann. NY Acad. Sci.* **386,** 183–198.
4. Endrizzi A., Pagot Y., Le Clainche A., Nicaud J-M., and Belin J-M. (1996) Production of lactones and peroxisomal beta-oxidation in yeasts. *Crit. Rev. Biotechnol.* **16,** 301–329.
5. Bassel J. and Mortimer R. (1982) Genetic and biochemical studies of n-alkane non-utilizing mutants of *Saccharomycopsis lipolytica*. *Curr. Genet.* **5,** 77–88.
6. Bassel J. and Mortimer R. (1985) Identification of mutations preventing n-hexadecane uptake among 26 n-alkane non-utilizing mutants of *Yarrowia (Saccharomycopsis) lipolytica*. *Curr. Genet.* **9,** 579–586.
7. Nuttley, W. M., Brade, A. M., Gaillardin, C., Eitzen, G. A., Glover, J. R., Aitchison, J. D., and Rachubinski, R. A. (1993) Rapid identification and characterization of peroxisomal assembly mutants in *Yarrowia lipolytica*. *Yeast* **9,** 507–517.
8. Nga, B., Gaillardin, C., Fournier, P., and Heslot, H. (1989) Genetic analysis of lipase low-producing mutants of *Yarrowia lipolytica*. *J. Gen. Microbiol.* **135,** 2439–2443.
9. Nuttley, W. M., Brade, A. M., Eitzen, G. A., Veenhuis, M., Aitchison, J. D., Szilard, R. K., Glover, J. R., and Rachubinski, R. A. (1994) *PAY4*, a gene required for peroxisome assembly in the yeast *Yarrowia lipolytica*, encodes a novel member of a family of putative ATPases. *J. Biol. Chem.* **269,** 556–566.
10. Eitzen, G. A., Aitchison, J. D., Szilard, R. K., Veenhuis, M., Nuttley, W. M., and Rachubinski, R. A. (1995) The *Yarrowia lipolytica* gene PAY2 encodes a 42–kDa peroxisomal integral membrane protein essential for matrix protein import and peroxisome enlargement but not for peroxisome membrane proliferation. *J. Biol. Chem.* **270,** 1429–1436.
11. Eitzen, G. A., Titorenko, V. I., Smith, J. J., Veenhuis, M., Szilard, R. K., and Rachubinski, R. A. (1996) The *Yarrowia lipolytica* gene *PAY5* encodes a peroxisomal integral membrane protein homologous to the mammalian peroxisome factor PAF-1. *J. Biol. Chem.* **271,** 20,300–20,306.
12. Dear, S. and Staden, R. (1991) A sequence assembly and editing program for efficient management of large sequence projects. *Nucl. Acids Res.* **19,** 3907–3911.
13. Devereux, J., Haeberli, P., and Smithies, O. (1984) A comprehensive set of sequence analysis programs for a VAX. *Nucleic Acids Res.* **12,** 387–395.
14. Maftahi, M., Nicaud, J.-M., and Gaillardin, C. (1996) Sticky-end polymerase chain reaction method for systematic gene disruption in *Saccharomyces cerevisiae*. *Yeast* **12,** 859–868.
15. Güssow, D. and Clackson, T. (1989) Direct clone characterization from plaques and colonies by the polymerase chain reaction. *Nucl. Acids Res.* **17,** 4000.
16. Pagot, Y., Endrizzi, A., Nicaud, J.-M., and Belin, J.-M. (1997) Utilisation of an auxotrophic strain of the yeast *Yarrowia lipolytica* to improve γ-decalactone production yields. *Lett. Appl. Microbiol.* **25,** 113–116.

17. Xuan, J. W., Fournier, P., and Gaillardin, C. (1988) Cloning of the *LYS5* gene encoding saccharopine dehydrogenase from the yeast *Yarrowia lipolytica* by target integration. *Curr. Genet.* **14,** 15–21.
18. Neuvéglise, C., Nicaud, J-M., Ross-Macdonald, P., and Gaillardin, C. (1998) A shuttle mutagenesis system for tagging genes in the yeast *Yarrowia lipolytica*. *Gene* **213,** 37–46.
19. Einerhand, A. W., Van der Leij, I., Kos, W. T., Distel, B., and Tabak, H. F. (1992) Transcriptional regulation of genes encoding proteins involved in biogenesis of peroxisomes in *Saccharomyces cerevisiae*. *Cell Biochem. Function* **10,** 185–191.
20. Einerhand, A. W., Kos, W. T., Distel, B., and Tabak, H. F. (1993) Characterization of a transcriptional control element involved in proliferation of peroxisomes in yeast in response to oleate. *Eur. J. Biochem.* **214,** 323–331.
21. Filipits, M., Simon, M. M., Rapatz, W., Hamilton, B., and Ruis, H. (1993) A *Saccharomyces cerevisiae* upstream activating sequence mediates induction of peroxisome proliferation by fatty acids. *Gene* **132,** 49–55.
22. Sloots, J. A., Aitchison, J. D., and Rachubinski, R. A. (1991) Glucose responsive and oleic acid responsive elements in the gene encoding the peroxisomal trifunctional enzyme of *Candida tropicalis*. *Gene* **105,** 129–134.
23. Simon, M., Binder, M., Adam, G., Hartg, A., and Ruis, H. (1992) Control of peroxisome proliferation in *Saccharomyces cerevisiae* by *ADR1*, *SNF1* (*CAT1*, *CCR1*) and *SNF4* (*CAT3*). *Yeast* **8,** 303–309.
24. Wang, T., Luo, Y., and Small, G. M. (1994) The *POX1* gene encoding peroxisomal acyl-CoA oxidase in *Saccharomyces cerevisiae* is under the control of multiple regulatory elements. *J. Biol. Chem.* **269,** 24,480–24,485.
25. Dmochowska, A., Dignard, D., Maleszka, R., and Thomas, D. Y. (1990) Structure and transcriptional control of the *Saccharomyces cerevisiae POX1* gene encoding acyl-CoA oxidase. *Gene* **88,** 247–252.
26. Masuda, Y., Park, S. M., Ohta, A., and Takagi, M. (1995) Cloning and characterization of the *POX2* gene in *Candida maltosa*. *Gene* **167,** 157–161.
27. Hill, D. E., Boulay, R., and Rogers, D. (1988) Complete nucleotide sequence of the peroxisomal acyl-CoA oxidase from the alkane-utilizing yeast *Candida maltosa*. *Nucl. Acids. Res.* **16,** 365–366.
28. Okasaki, K., Takechi, T., Kambara, N., Fukui, S., Kubota, I., and Kamiryo, T. (1986) Two acyl-CoA oxidases in peroxisomes of the yeast *Candida tropicalis*: primary structures deduced from genomic DNA sequence. *Proc. Natl. Acad. Sci. USA* **83,** 1232–1236.
29. Okasaki, K., Tan, H., Fukui, S., Kubota, I., and Kamiryo, T. (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.
30. Murray, W. W. and Rachubinski, R. A. (1987) The primary structure of a peroxisomal fatty acyl-CoA oxidase from the yeast *Candida tropicalis* pK233. *Gene* **51,** 119–128.
31. Miyazawa, S., Osumi, T., Hashimoto, T., Ohno, K., Miura, S., and Fujiki, Y. (1989) Peroxisome targeting signal of rat liver acyl-CoA oxidase resides at the carboxy terminus. *Mol. Cell. Biol.* **9,** 83–91.
32. Rothstein, R. J. (1983) One-step gene disruption in yeast. *Methods Enzymol.* **101,** 202–211.
33. Bradford, M. (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **216,** 607–613.
34. Picataggio, S., Deanda, K., and Mielenz, J. (1991) Determination of *Candida tropicalis* acyl-coenzyme A oxidase isozyme function by sequential gene disruption. *Mol. Cell. Biol.* **11,** 4333–4339.
35. Pagot, Y., Le Clainche, A., Nicaud, J.-M., Wache, Y., and Belin, J.-M., (1998) Peroxisomal β-oxidation activities and γ-decalactone production by the yeast *Yarrowia lipolytica*. *Appl. Microbiol. Biotechnol.* **49,** 295–300.
36. Picataggio, S., Rohrer, T., Deanda, K., Lanning, D., Reynolds, R., Mielenz, J., and Eirich, L. D. (1992) Metabolic engineering of *Candida tropicalis* for the production of long-chain dicarboxylic acids. *Bio/Technology* **10,** 894–898.