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Yarrowia lipolytica, a yeast model for the genetic studies of hydroxy fatty acids biotransformation into lactones

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Abstract

The Acyl-CoA oxidase isozymes (ACO) play an important role in the degradation of fatty acids and in the production of lactones by bioconversion. Using conserved blocks in yeast ACO genes, we have shown that five genes were present in the yeast *Yarrowia lipolytica*. We have previously isolated three complete genes (*ACO1*, *ACO2* and *ACO3*) and part of the *ACO5* gene. Using divergent PCR, we isolated clones coding for the end of *ACO5* and for the *ACO4* genes. These genes show about 70% identity between them and 60% with the ACO of other yeast. Mono-disrupted strains were constructed using a variation of the SEP method. ACO activity in the disrupted strains revealed that a long-chain oxidase is encoded by *ACO2* and a short-chain oxidase by *ACO3*.

Keywords: Yeast; *Yarrowia lipolytica*; Acyl-CoA oxidase; Lactone

1. Introduction

Lactones are very interesting molecules for the food industry because of their highly aromatic fruity aroma [1]. One of these lactones, γ-decalactone, which presents a peach flavour, is obtained by bioconversion of ricinoleic acid by yeast. Productions of 1–10 g l^{-1} were obtained from oil or derivatives (castor oil, ricinoleic acid or methyl ricinoleate) using yeasts like *Saccharomyces cerevisiae*, *Pichia etchelsii*, *Sporidiobolus ruinenii* and *Yarrowia lipolytica* [1]. Peroxisomal β-oxidation was shown to play an important role in this bioconversion [2], where Acyl-CoA oxidase (ACO) represent a key step [3]. We have focused our attention on the rate limiting step catalysed by this enzyme.

Since *Y. lipolytica* utilises hydrophobic substrates such as alkanes, oil, and fatty acids, we searched to understand how this yeast degrades these products as an overall process. Recent work in our laboratory is related to three questions. First, why is there a multiplicity of ACO encoding genes? Second, what are the true functions of the individual ACO? Third, how can one exploit variation of ACO gene copies for strain improvement?

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Here, we describe the cloning of the five ACO genes encoding ACOs in the yeast *Y. lipolytica* and the construction of mono-disrupted strains.

2. Materials and methods

2.1. Strains and media

The *Y. lipolytica* strains used in this study are Po1d (*MatA, ura3-302, leu2-270, xpr2-322*) [4] and derivatives ALCY22 (*aco1::LEU2*), MTLY16 (*aco2::URA3*), MTLY17 (*aco3::URA3*), MTLY18 (*aco4::URA3*), MTLY19 (*aco5::URA3*) and MTLY24 (*Δaco5*).

2.2. Media

The media YPD and YNB were prepared as described previously [4]. Fatty acids media are MM media supplemented with 1% of the corresponding fatty acids. Fatty acid minimum media (MM) are composed of: YNB (0.17%), ammonium chloride (0.4%), uracile (0.01%), leucine (0.032%) when required and the corresponding fatty acid at a 1% final concentration. A fatty acid stock solution was prepared as follows: a mixture of fatty acids (10%) and Tween 80 (1%) was prepared and sonicated three times for 1 min in ice. For solid medium, 2% agarose was added. For the selection of Ura^- colonies after transformation [4], 5-Fluororotic acid medium (5FOA medium) was used, which contains uracile (15 μg/l) and 5-FOA (1.25 g/l).

2.3. 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) using synthetic primers and the dye terminator procedure. The complete nucleotide sequence was compiled using the Staden package of programs [5]. DNA and protein sequences were analyzed using custom-made programs Staden and GCG.

2.4. Divergent PCR for ACO4 and ACO5 genes sequencing

The amplification was done for 25 cycles with 250 ng of denatured plasmid template DNA from C. Neuveglise library, appropriate primer pairs (100 pmol each) and 1 unit of both Taq (Appligene) and Pfu (Stratagene) DNA polymerases. Amplification was performed on a Perkin Elmer Gene Amp 9600. The process was started by denaturation during 5 min at 95°C and amplification was performed as follows: 45 s at 94°C, 40 s at 60°C and 4 min at 72°C. The last cycle was followed by 4 min at 74°C.

2.5. Construction of the URA3 cassette

The *URA3* gene was amplified by PCR using plasmid pINA156 [4] as template together with 1 μM of the long primer pair ura3s1/ura3s2 containing the I-*Sce*I site using Pfu DNA polymerase (Stratagene). The 1247-bp fragment was cloned into pBluescript II KS^- vector, giving rise to the plasmid pINA-URA3-I-*Sce*I.

2.6. Construction of disruption cassettes (PT and PUT)

The amplifications were performed according to the SEP method [6]. Typical PCR with Taq was utilised for generating disruption cassettes. There are three steps: PCR1 amplify separately promoter (P) and terminator (T) regions with primers d1/d2, d3/d4; d2/d3 contain the I-*Sec*I endonuclease recognition sequence [5′-TAAGGGATAA|CAGGGTAAT-3′ (3′-ATTCCC|TATTGCCCATTA-5′)]. PCR2 synthesizes the PT fragment; anneals and amplifies PCR1P, PCR1T to produce PCR2 fragment containing the rare restriction site I-*Sce*I in the middle. The resulting PT fragment (disrupt 2 cassette) was treated with T4 DNA polymerase or adding Pfu into

PCR reaction buffer to render the ends blunt and were cloned into the *Eco*RV site of pBluescript II KS$^-$ (Stratagene) giving rise to disrupt 2 cassettes (see Fig. 2A3). Those clones containing disrupt 2 cassettes were digested with I-*Sce*I and the *URA3 Sce*I cassette which contains one *I-Sec*I restriction site on both sides were inserted resulting in disrupt 1 cassettes (Fig. 2A4).

2.7. Verification of yeast transformants by PCR

The resulting disrupt 1 or disrupt 2 cassettes were integrated in the ACO genes as represented schematically in Fig. 2B (Refs. [6,7] respectively). Correct disruption was verified by PCR as described previously [6]. Primer pairs acover1/acover2 were used for PCR analysis to amplify the disrupted ORF (acover1 and acover2 are indicated in Fig. 2).

2.8. Acyl-CoA oxidase activity assays

The ACO activity were measured as described previously [3]. Long chain fatty ACO activity was measured using Hexanoyl-CoA (C6), Decanoyl-CoA (C10), Lauroyl-CoA (C12), Myristoyl-CoA (C14) and Palmitoyl-CoA (C16) as substrates. Results are means of at least three separate experiments.

3. Results and discussion

Comparison of the yeast ACO encoded by the *PXP4*, *PXP5* and *PXP2* genes from *C.*

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ACO1                MTTNTF TDPPVEMAKE RGKTQFTVRD VTNFLNGGEE ETQIVEKIMS SIERDPVLSV TADYDCNLQQ ARKQTMERVA ALSPYLVTDT 100
ACO2   MNPNNTGTI EINGKEYNTF TEPPVAMAQE RAKTSFPVRE MTYFLDGGEK NTLKNEQIME EIERDPLFNN DNYYDLNKEQ IRELTMERVA KLSLFVRDQP
ACO3   MISPNLTANV EIDGKQYNTF TEPPKALAGE RAKVKFPIKD MTEFLHGGEE NVTMIERLMT ELERDPVLNV SGDYDMPKEQ LRETAVARIA ALSGHWKKDT
ACO4   MITPNPANDI VHDGKLYDTF TEPPKLMAQE RAQLDFDPRD ITYFLDGSKE ETELLESLML MYERDPLFNN QNEYDESFET LRERSVKRIF QLSKSIAMDP
ACO5     MNNNPTNV ILGGKEYDTF TEPPAQMELE RAKTQFKVRD VTNFLTGSEQ ETLLTERIMR EIERDPVLNV AGDYDADLPT KRRQAVERIG ALARYLPKDS
                       ** * **     * *              ** *        * *    ****        **       *    *    *

ACO1   EKLSLWRAQL HGMVDMSTRT RLSIHNNLFI GSIRGSGTPE QFKYWVKKGA VAVKQFYGCF AMTELGHGSN LKGLETTATY DQDSDQFIIN TPHIGATKWW 200
ACO2   EDDIKKRFAL IGIADMGTYT RLGVHYGLFF GAVRGTGTAE QFGHWISKGA GDLRKFYGCF SMTELGHGSN LAGLETTAIY DEETDEFIIN TPHIAATKWW
ACO3   EKEALLRSQL HGIVDMGTRI RLGVHTGLFM GAIRGSGTKE QYDYWVRKGA ADVKGFYGCF AMTELGHGSN VAGLETTATY IQDTDEFIIN TPNTGATKWW
ACO4   EPMSFRKIGF LGILDMGTYA RLGVHYALFC NSIRGQGTPD QLMYWLDQGA MVIKGFYGCF AMTEMGHGSN LSRLETIATF DKETDEFIIN TPHVGATKWW
ACO5   EKEAILRGQL HGIVDMGTRT RIAVHYGLFM GAIRGSGTKE QYDYWVAKGA ATLHKFYGCF AMTELGHGSN VAGLETTATL DKDTDEFIIN TPNSGATKWW
       *            ** *   *   *  **     ** **   *   *    **       *****  *** *****   **** *       * **** **   *****

ACO1   IGGAAHTSTH CVCFAKLIVH GKDYGTRNFV VPLRNVHDHS LKVGVSIGDI GKKMGRDGVD NGWIQFTNVR IPRQNMLMRY AKVSDTGVVT KPALDQLTYG 300
ACO2   IGGAAHTATH TVVFARLIVK GKDYGVKTFV VQLRNINDHS LKVGISIGDI GKKMGRDGID NGWIQFTNVR IPRQNLLMKY TKVDREGNVT QPPLAQLTYG
ACO3   IGGAAHSATH TACFARLLVD GKDYGVKIFV VQLRDVSSHS LMPGIALGDI GKKMGRDAID NGWIQFTNVR IPRQNMLMKY AKVSSTGKVS QPPLAQLTYG
ACO4   IGGAAHTATH TLAFARLQVD GKDYGVKSFV VPLRNLDDHS LRPGIATGDI GKKMGRDAVD NGWIQFTNVR VPRNYMLMKH TKVLRDGTVK QPPLAQLTYG
ACO5   IGGAAHSATH TACLARLIVD GKDYGVKIFI VQLRDLNSHS LLNGIAIGDI GKKMGRDAID NGWIQFTDVR IPRQNMLMRY DRVSRDGEVT TSELAQLTYG
       ****** **     * *   *****      * **    ** *  *   *** *******  * ******* **   **   **     *     *     * *****

ACO1   ALIRGRVSMI ADSFHVSKRF LTIALRYACV RRQFGTSGDT KETKIIDYPY HQRRLLPLLA YCYAMKMGAD EAQKTWIETT DRILALNPND PAQKNDLEKA 400
ACO2   SLITGRVSMA SDSHQVGKRF ITIALRYACI RRQFSTTPGQ PETKIIDYPY HQRRLLPLLA YVYALKMTAD EVGALFSRT. ..MLKMDDLK PDDKAGLNEV
ACO3   ALIGGRVTMI ADSFFVSQRF ITIALRYACV RRQFGTTPGQ PETKIIDYPY HQRRLLPLLA FTYAMKMAAD QSQIQYDQTT DLLQTIDP.K DKGA..LGKA
ACO4   SLITGRVQMT TDSHNVSKKF LTIALRYATI RRQFSSTPGE PETRLIDYLY HQRRLLPLMA YSYAMKLAGD HVRELFFAS. ..QEKAESLK EDDKAGVESY
ACO5   ALLSGRVTMI AESHLLSARF LTIALRYACI RRQFGAVPDK PETKLIDYPY HQRRLLPLLA YTYAMKMGAD EAQQQYNSSF GALLKLNPVK DAEK..FAVA
         *  *** *    *      *  *******   ****         **  *** * ******** *   ** *  **

ACO1   VTDTKELFAA SAGMKAFTTW GCAKIIDECR QACGGHGYSG YNGFGQGYAD WVVQCTWEGD NNVLCLSMGR GLVQSALQIL AGK.HVGASI QYVGDKSKIS 500
ACO2   VSDVKELFSV SAGLKAFSTW ACADVIDKTR QACGGHGYSG YNGFGQAYAD WVVQCTWEGD NNILTLSAGR ALIQSAVALR KG.EPVGNAV SYLKRYKDLA
ACO3   IVDLKELFAS SAGLKAFTTW TCANIIDQCR QACGGHGYSG YNGFGQAYAD WVVQCTWEGD NNVLCLSMGR GLIQSCLGHR KGK.PLGSSV GYLANKG.LE
ACO4   VQDIKELFSV SAGLKAATTW ACADIIDKAR QACGGHGYSA YNGFGQAFQD WVVQCTWEGD NTVLTLSAGR ALIQSALVYR KE.GKLGNAT KYLSRSKELA
ACO5   TADLKALFAS SAGMKAFTTW AAAKIIDECR QACGGHGYSG YNGFGQAYAD WVVQCTWEGD NNVLCLSMGR SLIQSCIAMR KKKGHVGKSV EYLQRRDELQ
         * * **   *** **  **   *  **  * *********  ******   * ********** *  * ** **  * **

ACO1   QNGQGTPREQ LLSPEFLVEA FRTASRNNIL RTTDKYQELV KT.LNPDQAF EELSQQRFQC ARIHTRQHLI SSFYARI.AT AKDDIKPHLL KLANLFALWS 600
ACO2   N..AKLNGRS LTDPKVLVEA WEVAAGNIIN RATDQYEKLI GEGLNADQAF EVLSQQRFQA AKVHTRRHLI AAFFSRIDTE AGEAIKQPLL NLALLFALWS
ACO3   Q..ATLSGRD LKDPKVLIEA WEKVANGAIQ RATDKFVELT KGGLSPDQAF EELSQQRFQC AKIHTRKHLV TAFYERINAS AKADVKPYLI NLANLFTLWS
ACO4   N..AKRNGRS LEDPKLLVEA WEAVSAGAIN AATDAYEELS KQGVSVDECF EQVSQERFQA ARIHTRRALI EAFYSRIAT. ADEKVKPHLI PLANLFALWS
ACO5   N..ARVDNKP LTDPAVLITA WEKVACEAIN RATDSFIKLT QEGLSPDQAF EELSQQRFEC ARIHTRKHLI TSFYARI.SK AKARVKPHLT VLANLFAVWS
                 * *  *  *          *    **    *       * *  *  ** **   *  ***  *    *  **    *    *  *   ** **  **    706

ACO1   IEEDTGIFLR ENILTPGDID LINSLVDELC VAVRDQVIGL TDAFGLSDFF INAPIGSYDG NVYEKYFAKV NQQNPATNPR PPYYESTLKP FLFREEEDDE ICDLDE
ACO2   IEEDSGLFLR EGFLEPKDID TVTELVNKYC TTVREEVIGY TDAFNLSDYF INAPIGCYDG DAYRHYFQKV NEQNPARDPR PPYYASTLKP FLFREEEDDD ICELDEE
ACO3   IEEDSGLFLR EGFLQPKDID QVTELVNHYC KEVRDQVAGY TDAFGLSDWF INAPIGNYDG DVYKHYFAKV NQQNPAQNPR PPYYESTLRP FLFREDEDDD ICELDEE
ACO4   IEEDSALFLA EGYFEPEDII EVTSLVNKYC GIVRKNVIGY TDAFNLSDYF INAAIGRYDG DVYKNYFEKV KQQYPPEGGK PHYYEDVMKP FLHRERIPDV PMEPEDIQ
ACO5   IEEDSGLFLR EGCFEPAEMD EITALVDELC CEAREQVIGF TDAFNLSDFF INAPIGRFDG DAYKHYMDEV KAANNPRNTH APYYETKLRP FLFRPDEDEE ICDLDE
       ****   **  *    *   *     **   *    *  * *  **** *** * *** **  **       *   *                **     * ** *
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Fig. 1. Comparison of deduced amino acid sequences of the five ACO genes of the yeast *Y. lipolytica* (*ACO1* to *ACO5*). Symbol (*) denotes amino acid identity and boxes I, II and III show regions of extended identity with other yeast ACO genes that was used for the design of oligonucleotides (grey box). Points indicate gaps introduced to optimise alignment. EMBL accession No. AJ001299 to AJ001303.

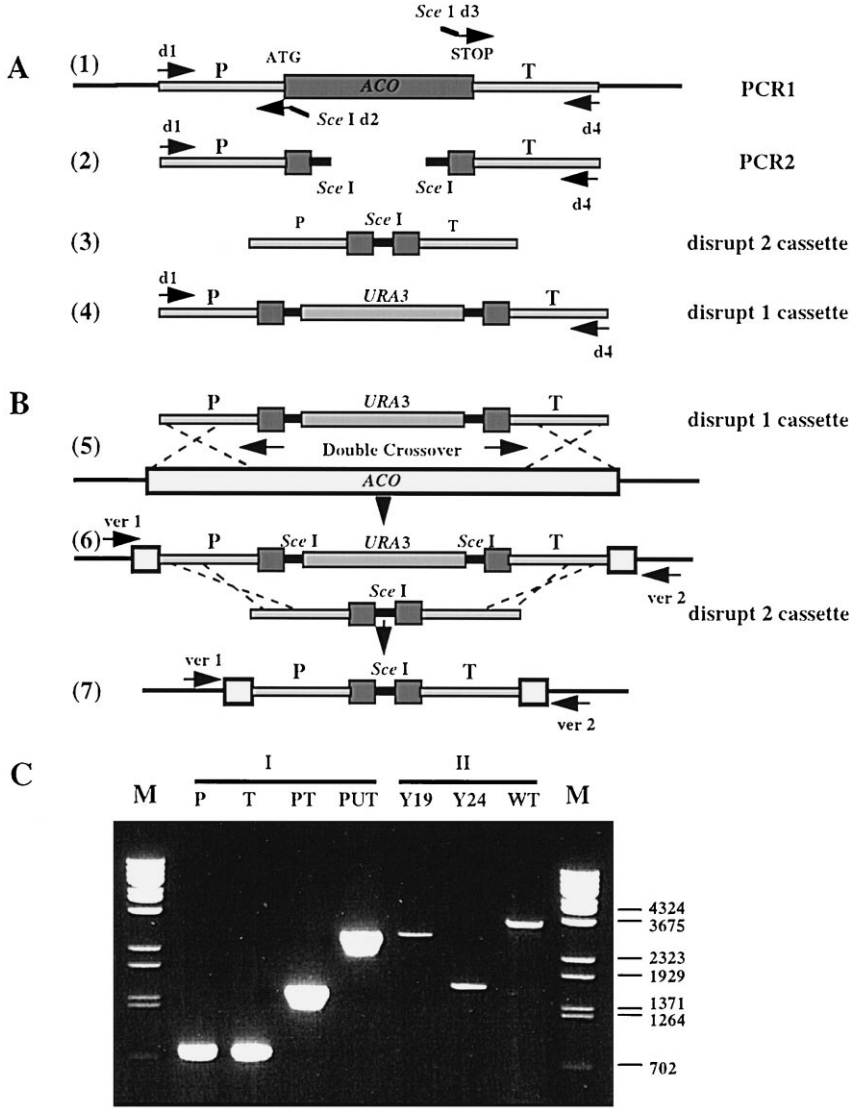

Fig. 2. Schematic view of PCR-based gene disruption. (A) Preparation of disrupt 1 and 2 cassettes; (1) The promoter and the terminator regions of the ACO genes were amplified using the specific oligonucleotides pairs d1/d2 (P) and d3/d4 (T), respectively; oligonucleotides d2 and d3 each contains one strand of a 20-bp additional sequence, which can be cleaved by the rare cutting restriction enzyme I-*Sce*I. (2) A second PCR is performed using oligonucleotides pairs d1/d4. (3) The resulting PCR product corresponding to the disrupt 2 cassette is cloned into Bluescript vector KS^+. (4) The disrupt 1 cassette was obtained by insertion of an I-*Sce*I *URA3* cassette. (B) PCR-based gene disruption: (5) First, the disrupt 1 cassette was used for generating a *aco*::*URA3* disruption (6) by selection of URA + clones. (7) An *Δaco* strain is then obtained by transformation with disrupt 2 cassette and selection of URA- strain on 5-FOA plates. (C) Disruption *ACO5*. (I) Production of promoter (P), terminator (T) and disrupt 2 cassette (PT) using SEP method. Also shown the PCR product resulting from amplification of disrupt 1 cassette (PUT). (II) PCR analysis of deleted strains for *ACO5* (MTLY19, MTLY24) compared with the wild type strain (WT). Molecular weight marker (M) is λ*Bst*EII.

tropicalis (M12160, M12161, P18259), the *AOX1* and *POX1* genes from *C. maltosa* (X06721, D21228) and the *POX1* gene from *S. cerevisiae* (M27515) revealed four highly conserved blocks which sequence are; block 1: HIGATKWWIGGAAHSATH; block 2: DNGWIQF; block 3: RQXCGGHGYSXYNGF; and block 4: DWVVQCTWEGDNN. Oligonu-

cleotides corresponding to part of these blocks (underlined aa), designed using *Y. lipolytica* codon usage, were used for PCR amplification on the six pools of the Xuan *Y. lipolytica* genomic library [4]. This allowed us to show that five ACO encoding genes were present in this yeast [7]; three of them, *ACO1*, *ACO2* and *ACO3* have been cloned and sequenced, while most of the *ACO5* sequence has been determined, the sequence coding for the amino acids at the COOH end was not present in the cloned insert. In addition, for the *ACO4* gene, only an 830-bp fragment was amplified and sequenced. Since that *ACO4* gene was not cloned by hybridisation during the isolation of the other ACOs, we decided to isolate the gene by divergent PCR using C. Neuveglise gene library. This library was constructed by insertion of 2-kb genomic DNA of *Y. lipolytica* into 2-kb *Escherichia coli* vector carrying kanamycine resistance gene as selective marker. Divergent primer pair located within the initial 830-bp known sequence, were used for amplification. The amplified 4-kb fragments were purified on agarose gel and T4 DNA polymerase polishing prior ligation. After transformation into *E. coli*, Kan^r resistant colonies were isolated. Four clones were used for sequence determination by primer walking. A second PCR reaction was performed using a new primer pair located at the 5′ end of the determined sequence. The complete 4823-bp sequence revealed a 2109-bp ORF corresponding to *ACO4* gene. It encodes a 701 amino acid protein (79,241 Da) presenting 70% identity and 75% similarity to the other *Y. lipolytica* ACO genes (see Fig. 1). A similar approach was used to determine the sequence of the 3′ end of the *ACO5*. The final 4570-bp contain the *ACO5* gene which encodes a 699 amino acid protein. Comparison of the deduced amino acid sequences of the five ACO oxidase genes of *Y. lipolytica* is shown in Fig. 1 (EMBL accession No AJOO1299 through AJOO1303). Alignment and comparison of deduced amino acid between ylACO genes showed 60 to 70% identities (75 to 80% similarities), while only about 45% identities and 60% similarities were found with the yeast ACO.

3.1. Construction of mono-disrupted strains

In order to determine the roles and functions of the five ACO genes found in the yeast *Y. lipolytica*, we first decided to construct mono-disrupted strains. Indeed, our question was: why are five genes present in this yeast while one ACO gene is found in *S. cerevisiae*, two in *C. maltosa* and three in *C. tropicalis*? Our approach was to combine the gene replacement method with the recently SEP method developed in our laboratory for gene disruption in *S. cerevisiae* [6]. PCR gene disruption was achieved by gene replacement as outlined in Fig. 2 using two disruption cassettes: disrupt 1 cassette contained promoter (P) and terminator (T) fragment separated by an *URA3* gene and disrupt 2 cassette contained promoter (P) linked to the terminator (T). The disruption cassettes were constructed as described in Fig. 3A and M&M. For the construction of disrupt 2 cassette, two PCR are needed; the amplified promoter (P) and terminator (T) fragments (Fig. 2A2) are used for the second PCR and were linked with a 18-bp fragment which was recognised by endonuclease I-*Sce*I (Boehringer Mannheim). The resulting PCR fragment (Fig. 2A3) is then cloned in a vector. The disrupt 1 cassette is obtained by insertion of the URA3-I-*Sce*I cassette (Fig. 2A4). This meganuclease restriction site was introduced since it could allow us to determine the chromosomal location of the gene as described by Casaregola et al. [8].

The disrupt cassettes were used as shown in Fig. 2B. In the first step, the ACO genes were disrupted using disrupt 1 cassette and selection of URA + transformants (Fig. 2B5). We obtained 10^2–10^3 transformants per microgram of DNA. After transformation with disrupt 2 cassette, we verified transformants with primer ver which are located a few basepairs outside of primers d1 and/or d4. (Fig. 2B). In the first

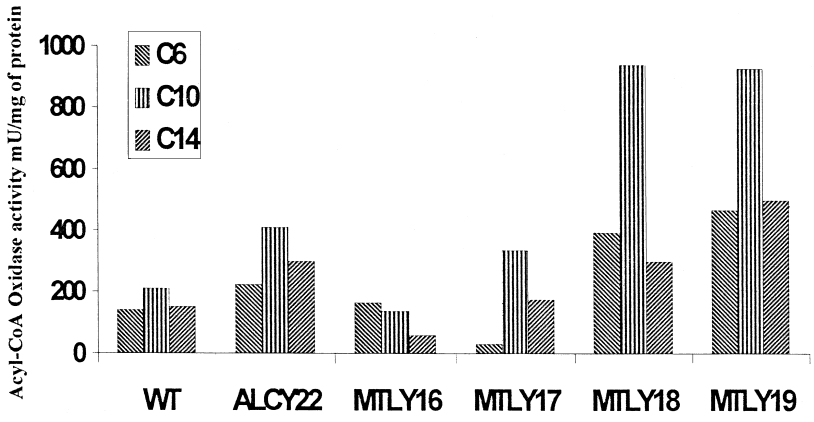

Fig. 3. ACO activity in wild type strain (WT) and mono-disrupted strains. Acyl-CoA was measured using Hexanoyl-CoA (C6), Decanoyl-CoA (C10) or Myristyl-CoA (C14) as substrates. Strains are Po1d (WT), ALCY22 (del ACO1), MTLY16 (del ACO2), MTLY17 (del ACO3), MTLY18 (del ACO4) and MTLY19 (del ACO5). Activity are expressed in mU/mg of protein.

transformation step, we obtained a disruption efficiency of about 50%. DNA from eight transformants (for each disrupt 1 cassettes) were prepared and used for PCR verification as described in M&M. As an example, for ACO5 disruption (Fig. 2C), four clones present the expected 2844-bp fragment while the four other clones present the 3500 bp-WT fragment. This results in a *aco5*::*URA3* deleted strain (MTLY19). Details of the PCR fragments and disrupt cassettes are shown in Fig. 3CI. The mono-disrupted strain could be transformed using disrupt 2 cassettes as outlined in Fig. 2B6 giving rise to an *Δaco5* strain which could be selected on 5FOA plates. As shown in Fig. 2CII, transformant MTLY24 presents the expected 1617 bp PCR fragment indicating that it contains the *Δaco5* allele. Similarly, we obtained strains MTLY16 (*aco2*::*URA3*), MTLY17 (*aco3*::*URA3*), and MTLY18 (*aco4*::*URA3*).

3.2. Acyl-CoA oxidase activity in mono-disrupted strains

The above deleted strains together with the *ACO1* deleted strain ALCY22 (*aco1*::*LEU2*) [7] were used for ACO activity measurement. Strains were grown on YNB and transferred into methyloleate media for induction. Fig. 3 shows the activity of ACO of deleted aco_s strains 6 h after transfer. We have compared ACO isozyme activity in the wild type strain and in the mono-disrupted ones using C6-CoA, C10-CoA and C14-CoA as substrate. The ACO isozyme activities of each strain differs depending on the substrate carbon chain length. As shown in Fig. 3, the activity of ACO of strains deleted for *ACO1*, *ACO4* and *ACO5* is higher than WT. Similar results were obtained by Picataggio et al. [9] who observed that a strain deleted for *POX4* presents higher ACO activity than the wild type. For strains *Δaco2* and *Δaco3* similar activities with the wild type are found except for C14 substrate in *Δaco2* strain and for C6 in *Δaco3* strain. This suggests that *ACO2* codes for an ACO which is more active toward long chain fatty acids (C14) while *ACO3* codes for an ACO which is more active toward short chain fatty acids (C6). The latter results were confirmed when we tested activity of the ACO3 protein expressed in *E. coli*. In conclusion, it seems that aco2p and aco3p enzymatic substrates are different and complement for the growth on long fatty acid containing medium. In contrast, the strains deleted for aco1, aco4, aco5 showed higher activity of ACO on every fatty acid as substrate than the WT strain. It seems that their deletions caused an increase of the activity of other ACO (aco2p, aco3p).

Whether these effects are transcriptional or post-transcriptional is currently investigated.

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