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Acyl-CoA oxidase, a key step for lactone production by *Yarrowia lipolytica*

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Abstract

γ-Decalactone is a peachy aroma compound resulting from the peroxisomal β-oxidation of ricinoleic acid by yeasts. During this oxidation, the action of acyl-CoA oxidase is fundamental. In *Yarrowia lipolytica*, it was shown, using conserved blocks, that five acyl-CoA-oxidase genes (*ACO1* to *ACO5*) were present. In order to investigate the role of each ACO isozymes, mono-disrupted strains were constructed (*Δaco1* to *Δaco5*). The acyl-CoA activity was measured for each strain showing that a long-chain oxidase was encoded by *ACO2* and a short one by *ACO3*. Lactone production, for its part, was increased for *Δaco3* and, to a lesser extend, for *Δaco5* whereas lactone consumption was higher for *Δaco4*.

Keywords: Acyl-CoA oxidase; Lactone; Peroxisomal β-oxidation; *Yarrowia lipolytica*

1. Introduction

Peroxisomal β-oxidation (Fig. 1a) is an important reaction in yeasts since it plays a role in the biotransformation of hydroxyfatty acids into lactones. These molecules are odorous compounds of great significance to the food industry thanks to their fruity and more or less buttered odour. Particularly, the biotransformation of ricinoleic acid into γ-decalactone (Fig. 1b) has been studied as an example of fatty acid degradation presenting both a physiological and an industrial interest [1]. Indeed, γ-decalactone is a molecule with a fruity peachy-like odour which, if produced by yeasts, can obtain a natural label. β-oxidation is a fatty acid cyclic oxidation system which consists of four main reactions catalyzed by three enzymes among which acyl-CoA oxidase controls a key step [2].

The implication of acyl-CoA oxidases isozymes with different regulation, substrate profile or kinetic properties has been shown in some yeasts. In *Yarrowia lipolytica*, which is able to biotransform methyl ricinoleate into γ-decalactone, the presence of different isozymes is an interesting topic to study in order to control the lactone production. The role of each

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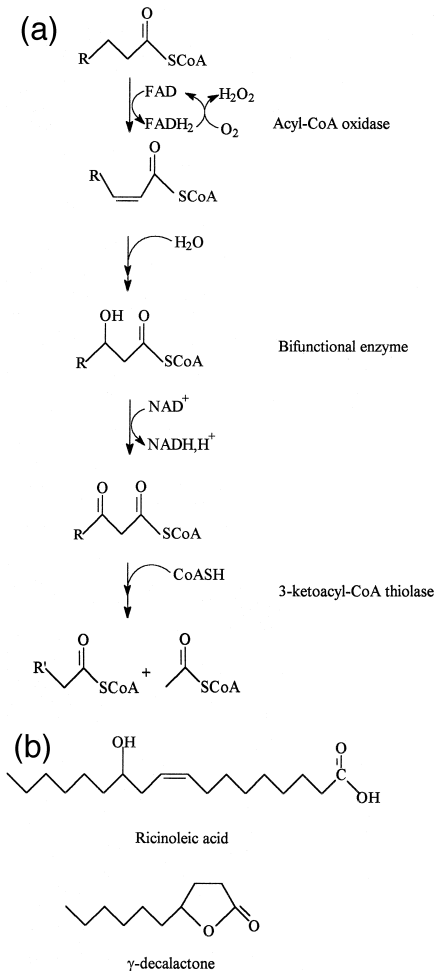

Fig. 1. (a) Reactions and enzymes implicated in peroxisomal β-oxidation. (b) Ricinoleic acid and γ-decalactone.

isozyme can be investigated with the help of mono-disrupted strains.

2. Materials and methods

2.1. Media and strains

Strains: The *Y. lipolytica* strains used in this study are *PO1d* (*MatA, ura3-302, leu2-270, xpr2-322* [3]) and derivatives: *MTLY26* (*aco1::URA3*); *MTLY16* (*aco2::URA3*); *MTLY17* (*aco3::URA3*); *MTLY18* (*aco4::URA3*) and *MTLY19* (*aco5::URA3*).

Media: Culture, bioconversion and lactone consumption media were prepared as described previously [4]. Induction medium was composed of YNB (Yeast Nitrogen Base) (0.17%), ammonium chloride (0.5%), methyl palmitate (0.1%) and Tween 80 (0.01%). For each medium used for *PO1d*, leucine (0.005%) and uracil (0.005%) were added.

2.2. Growth conditions

200 ml of growth medium was inoculated with 6–7.10^6 cells/ml and incubated in a 500 ml erlenmeyer flask at 27°C under agitation (140 rpm). In log-phase, cells were harvested by centrifugation (6000 *g*, 5 min) and transferred to the induction medium at an inoculation OD_{600} of 0.8. For the acyl-CoA activity assay, 10 ml were taken at $T = 0$, 5, 10, 25 and 50 h. For the lactone experiments, cells were transferred after 24 h to the bioconversion medium or to the lactone consumption medium.

2.3. Acyl-CoA activity assays

The activity assay was carried out as described elsewhere [2] without protein precipitation and using myristoyl-CoA (C14), decanoyl-CoA (C10) and hexanoyl-CoA (C6) as substrates.

2.4. Extraction and analysis of volatile compounds

γ-Decalactone was extracted and analysed as described previously [2].

3. Results and discussion

3.1. Presence of Acyl-CoA oxidase isozymes [5]

The comparison of yeast Acyl-CoA oxidases encoded by the *PXP4*, *PXP5* and *PXP2* genes from *Candida tropicalis* (*M12160*, *M12161*,

P18259), the *AOX1* and *POX1* genes from *Candida maltosa* (*X06721*, *D21228*) and the *POX1* gene from *Saccharomyces cerevisiae* (*M27515*) revealed four highly conserved blocks which sequences are: block 1, HIGATKWWIG-GAAHSATH; block 2, DNGWIQF; block 3, RQXCGGHGYSXYNGF and block 4, DWVVQCTWEGDNN. Oligonucleotides corresponding to part of these blocks (underlined), designed using *Y. lipolytica* codon usage, were used for PCR amplification on the six pools of the Xuan *Y. lipolytica* genomic library [6]. This allowed us to show that five Acyl-CoA oxidase encoding genes were present in this yeast.

3.2. Construction of mono-disrupted strains [5]

Disruption cassettes were constructed by PCR using the SEP method [7] as outlined by Nicaud et al. [5]. The complete ORF was replaced by the *Y. lipolytica URA3* gene. The disruption cassettes were introduced into *PO1d* by transformation and selection of transformants for Ura + phenotype. Correct gene replacement was verified by both PCR and Southern blots.

3.3. Acyl-CoA oxidase activity of mono-disrupted strains

3.3.1. Activity levels and spectral characteristics

ACO activities were assayed on three substrates differing in their carbon chain length (C6, C10 and C14) (Fig. 2). The activity levels discriminated the mono-disrupted strains into two groups. One group (*Δaco1*, *Δaco2* and *Δaco3*) showed activities in the same order of magnitude as *PO1d* while the other (*Δaco4* and *Δaco5*) presented four to five-fold higher activities. The observation of spectral characteristics gave also two groups: one (*PO1d*, *Δaco1*, *Δaco4* and *Δaco5*) with an activity much higher for C10 than for C6 and C14 substrates and a second where this spectrum was perturbed. Actually, *Δaco2* showed an activity close to *PO1d*-activity for C6 but this activity was lowered for C10 and collapsed to a near zero-activity for C14 whereas *Δaco3* had a C14 activity similar to PO1d, a slightly increased one for C10 and no activity at all for C6.

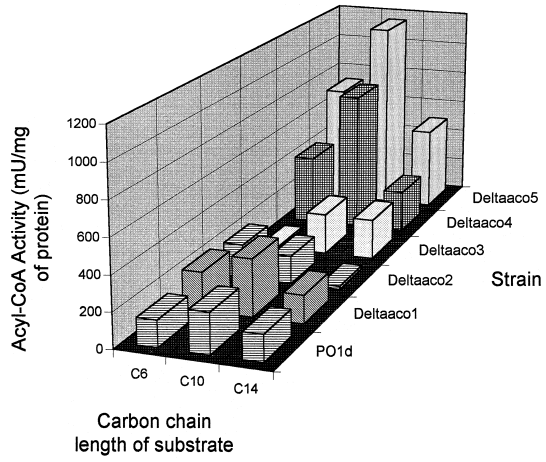

Fig. 2. Acyl-CoA activities of the reference strain (*PO1d*) and of the mono-disrupted strains assayed on hexanoyl-CoA (C6), decanoyl–CoA (C10) and myristoyl–CoA (C14). Strains are *PO1d*, *MTLY26* (*Δaco1*), *MTLY16* (*Δaco2*), *MTLY17* (*Δaco3*), *MTLY18* (*Δaco4*) and *MTLY19* (*Δaco5*).

3.3.2. Induction kinetics

The induction of acyl-CoA oxidase activity was very high right from 5 h and declined very slowly after two days for *Δaco1*, *Δaco2* and *Δaco3* (Fig. 3). On the other hand, the activities of *Δaco4* and *Δaco5* increased also quite

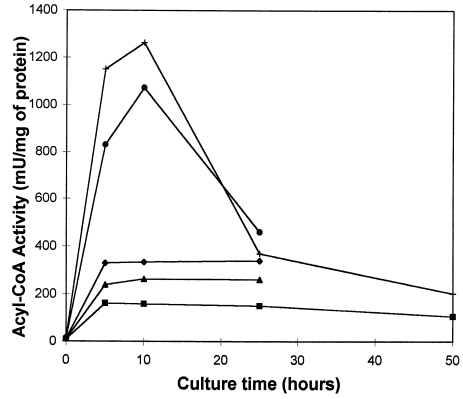

Fig. 3. Kinetic of acyl-CoA induction. Strains: *MTLY26-Δaco1* (lozenge), *MTLY16-Δaco2* (square), *MTLY17-Δaco3* (triangle), *MTLY18-Δaco4* (circle) and *MTLY19-Δaco5* (cross).

quickly but collapsed dramatically before 24 h to values more similar to those of *PO1d*, though still high.

From the spectral characteristics it was possible to conclude that *ACO2* codes for an acyl-CoA oxidase more active toward long chain fatty acids whereas the enzyme encoded by *ACO3* is more active for short chain fatty acids. The enzymes encoded by *ACO4* and *ACO5* have a quite different effect since the activities of *Δaco4* and *Δaco5* are much higher. Similar results have been observed by Picataggio et al. [8] in a strain of *C. tropicalis* deleted for *POX4*. The role of such enzymes could deal with the regulation of the other *ACO*'s but this requires further investigations. For its part, the effect of the enzyme encoded by *ACO1* is not so obvious. As a matter of fact, the activity of *Δaco1* is not so very different, although slightly higher, from *PO1d*.

3.4. Lactone production and consumption

γ-Decalactone production was rather different from one mono-disrupted strain to another (Fig. 4). Indeed, *Δaco3* produced up to 195 mg/l, *Δaco5*, 156 mg/l and *Δaco2* and *Δaco4* only 111 mg/l in 10 h.

The production, as measured in this experiment, resulted from an equilibrium between the lactone actually produced and the one consumed. Therefore, we coupled the experiment with a measurement of lactone consumption. *Δaco4* consumed 41 mg/h of lactone whereas *Δaco2*, *Δaco3* and *Δaco5* consumed only about 30 mg/h.

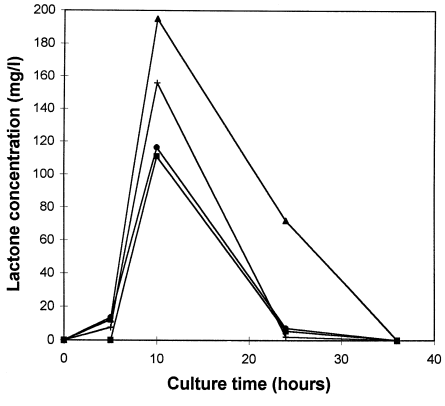

Fig. 4. Kinetic of lactone production. Strains: *MTLY16-Δaco2* (circle), *MTLY17-Δaco3* (triangle), *MTLY18-Δaco4* (square) and *MTLY19-Δaco5* (cross).

The *Δaco3* important production may be related to the poor Acyl-CoA oxidase activity on short carbon chain substrates for this strain. Without C6 activity, the β-oxidation cycle gives eventually C6, C8 or C10. The β-oxidation could be stopped there to prevent excess of products even if C10-consumption is still working.

About *Δaco5*, we notice that production is important in the first hours but is then stopped. This can be related to the oxidase activity which is very important during the first day but declines afterwards.

Another explanation to this decrease may be that, in vivo, a too high acyl-CoA activity can alter the metabolic equilibria. In fact, the reaction catalyzed by acyl-CoA oxidase leads to the reduction of FAD which, in being reoxidized, forms a H_2O_2 molecule, a substrate for catalase [9]. A higher acyl-CoA activity is likely to lead to an accumulation of $FADH_2$ or H_2O_2.

Two carnitine acyl-transferase activities are also important, one to bring the acyl-CoA into the peroxisome and the other to bring out the acetyl-CoA eventually produced by β-oxidation. This activity has been identified as a potential rate limiting step in the biotransformation of ricinoleic acid into lactone [10]. In the case of a very high acyl-CoA activity, the shortage of carnitine acyl-transferase would become more limiting.

4. Conclusion

The implication of *ACO* genes disruption on the γ-decalactone production and consumption could lead to a promising way of improving the lactone production by appropriate disruptions.

To go further in the understanding of the role of acyl-CoA oxidase isozymes, a study by double disruption should be necessary.

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