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Peroxisomal β-oxidation and Production of γ-decalactone by the Yeast *Rhodotorula aurantiaca*

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Abstract

γ-Decalactone is a fruity aroma compound resulting from the peroxisomal β-oxidation of ricinoleic acid by yeasts. During the β-oxidation of fatty acids, the acyl-CoA oxidase and thioesterase play an important role. In *R. aurantiaca*, we demonstrated the presence of partial gene sequences homologous to acyl-CoA oxidase and thioesterase involved in the pathways synthesis of γ-decalactone. This preliminary work is expected to characterize the relationship between the γ-decalactone production and the transcription of these partial gene sequences of *R. aurantiaca* probably involved in the oxidation of ricinoleic acid.

Keywords: γ-Decalactone, *R. aurantiaca*, acyl-CoA oxydase and thioesterase

Introduction

Lactones are very interesting molecules for the food industry because of their highly aromatic fruity aroma. Among these lactones, γ-decalactone, which presents a peach flavor, is obtained by biotransformation of ricinoleic acid by yeasts. The biosynthesis of γ-decalactone includes two procedures: the biotransformation of ricinoleic acid to 4-hydroxydecanoic acid by selected yeasts and then the acidification and heat treatment of the culture broth.

β-Oxidation is the metabolic pathway responsible for the production of γ-decalactone by yeasts (Endrizzi *et al.*, 1993; Pagot and Belin, 1996; Waché *et al.,* 2001; Waché *et al.*, 2002). It consists of four successive reactions (oxidation, hydratation, oxidase and cleavage) catalyzed by three enzymes among which acyl-CoA oxidase controls a key-step yielding an acyl-CoA which has two carbons less and an acetyl-CoA. This sequence is repeated several times until the complete breakdown of the compound.

The acyl-CoA oxidase, which catalyses the initial step of β-oxidation of fatty acids in peroxisomes, has been identified in several organisms including yeasts as *Saccharomyces cerevisiae, Candida maltosa, Candida tropicalis* and *Yarrowia lipolytica*. The biotransformation of ricinoleic acid into γ-decalactone by *Candida* is presented in Fig. 1.

We have previously studied the effects of temperature, initial pH and castor oil concentration on the production of γ-decalactone by the psychrophilic yeast *Rhodotorula aurantiaca* A19 (Alchihab *et al.*, 2009). However, the mechanism involved in the production of γ-decalactone from castor oil by *R. aurantiaca* was not discussed before.

The objective of this study was to bring new insights to explain and to understand the ability of *R. aurantiaca* to produce γ-decalactone and its precursor 4-hydroxydecanoic acid.

Materials and methods

Microorganisms, growth media and culture conditions

The psychrophilic strain *R. aurantiaca* A19 was previously isolated near the Antarctic station Dumont d'Urville and deposited at the MUCL under registration N° 40267. This strain was unable to grow at 20°C (Sabri *et al.*, 2000). Medium 863 is a complex medium containing: 6 g/l peptone casein, 3 g/l yeast extract and 20 g/l glucose. Medium 868 was prepared by adding 17 g/l agar to the medium 863.

The seed culture used was 3% (v/v) of the working volume and was prepared by inoculating 863 broth (100 mL) with a loopful of yeast cells from isolated colonies of a fresh culture (5 days) on 868 agar at 12°C. This preculture was incubated under agitation (150 rpm) for 4 days then transferred in 3 l of medium of biotansformation containing 20 g/l of castor oil, 6 g/l peptone casein, 3 g/l yeast extract, this medium was incubated for 4 days in the same conditions.

Genomic DNA isolation

The genomic DNA of *R. aurantiaca* was extracted from growing cultures as described below. Growing cells (1.5 ml) were collected by centrifugation at 13,000 × g for 5 min. Cells were washed with an EDTA buffer 50 mM

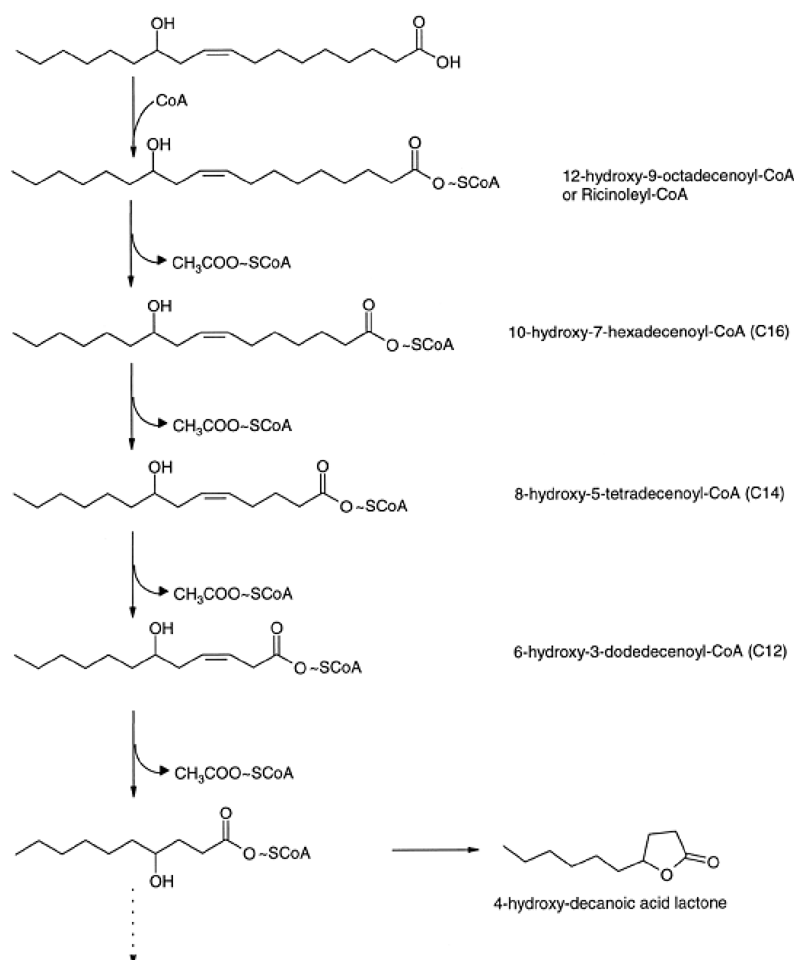

Fig. 1. β-oxidation of ricinoleic acid and 4-hydroxy decanoic lactone. Original pathway as deduced from the work of Okui *et al.* (1963) in the yeast Candida

pH 8 and digested by lyticase 8 mg/ml for 1 h at 37°C. DNA was purified using the kit genomic DNA purification Wizard (Promega, Madison, USA). DNA was resuspended in milli-Q water and DNA concentration was estimated by measuring the absorbance at 260 nm.

PCR amplification of the Genomic DNA

PCR was used to amplify the acyl-CoA oxidase and thioesterase genes of *R. aurantiaca*. Primers used for PCR and DNA sequencing are presented in Tab. 1. Amplification of DNA was performed in a Mastercycler personal

Tab. 1. Primers used for PCR and sequencing of acyl-CoA oxidase

Name	Sequence	Degenerate	Target	Yeast	Reference
aco107	CATATTGGTGCTACTAAGTGGTGG	No	*pox3*	*Y. lipolytica*	Wang *et al.* (1998)
aco108	CCCAGGTACACTGGACAACCC	No	*pox3*	*Y. lipolytica*	Wang *et al.* (1998)
aco3ver1	GTATCAGGTGTGGTTGTGGG	No	*pox3*	*Y. lipolytica*	Wang *et al.* (1998)
aco3ver2	CGAGAGTATTTGTGAGACACGC	No	*pox3*	*Y. lipolytica*	Wang *et al.* (1998)
aco3d1	GGTATCAAAGCCGAGGTTGG	No	*pox3*	*Y. lipolytica*	Wang *et al.* (1998)
aco3d2	CATTACCCTGTTAGCCCTAGGTTG	No	*pox3*	*Y. lipolytica*	Wang *et al.* (1998)
aco3d3	CTAGGGATAACAGGGTAATGCGAG	No	*pox3*	*Y. lipolytica*	Wang *et al.* (1998)
aco3d4	CTCTTGCCCTACTATTTCGTCG	No	*pox3*	*Y. lipolytica*	Wang *et al.* (1998)
aco-F	GCCTTTGTTGTCCCTATCCGT	No	*pox3*	*Y. lipolytica*	Wang *et al.* (1998)
aco-R	CGATATCCCCAACAGTGATGC	No	*pox3*	*Y. lipolytica*	Wang *et al.* (1998)
Pox3prime	AAYTGDATCCANCCRTTRTC	Yes	*pox1*	*P. pastoris*	Koller *et al.* (1999)
Pox5prime	GCNACNAARTGGTGGATHGG	Yes	*pox1*	*P. pastoris*	Koller *et al.* (1999)

thermal cycler. PCR reaction was monitored in an eppendorf tube (0.2 mL) containing: 10 × Taq Buffer, 2 mM dNTP-Mix, 25 Mm $MgCl_2$, 50 ng/μL of DNA solution, Taq DNA Polymerase 5 U/μl and supplemented by sterile milli-Q water to a final volume of 50 μl. PCR conditions included a hot start at 95°C for 5 min, 25 cycles consisting of denaturation at 95°C for 30 seconds, hybridation at 46°C for 30 seconds, polymerisation at 72°C for 2 min and a final extension cycle at 72°C for 10 min. PCR products were resolved by electrophoresis in 1% agarose gel at 100 volt/min and visualized by ethidium bromide (1 μl/10 ml) staining. The molecular weight of the reactional products was estimated in reference to a molecular weigth marker (100 bp DNA ladder plus, GeneRuler, Fermentas Life Sciences, Germany). The PCR reaction product was purified using the Kit PCR preps Wizard (Promega, Madison, USA) and quantified on agarose gel.

Sequence determination and analysis

The purified PCR products were sequenced using the same primer and BigDye Terminator cycle v3.0 kit as specified by the supplier. After each reaction, the fluorochromes in excess were eliminated by precipitation of ethanol. The products of sequencing reaction were charged on an ABI PRISM™ DNA sequencer (Perkin-Elmer) belonging to the Progenius company (Gembloux, Belgium). The sequences obtained were then assembled in silico (Vector NTI) using overlapping zones between the various sequences to form the contiguous sequence.

The required nucleotide sequences and deduced amino acid sequences were aligned and compared with acyl-CoA oxidase and thioesterase gene sequences of others isolates available from GenBank database using Blast (NCBI) program (The Basic Local Alignment search tool). The multiple sequences alignment was generated by Vector NTI program.

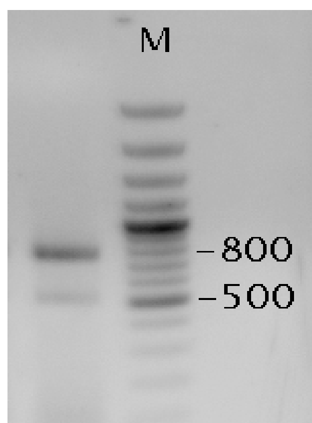

Fig. 2. PCR amplification of *R. aurantiaca* tamplate DNA with primers specific designed for acyl-CoA oxidase in *Yarrowia lipolytica*

Nucleotide sequence accession number

The nucleotide sequences of acyl-CoA oxidase and thioesterase genes, reported here, have been deposited in the GeneBank database under the respectively accession nos GU994135 and GU994134.

Results and disscusion

Peroxisomal β-oxidation enzymes were shown to be responsible for the production of γ-decalactone in other yeast strains (Endrizzi *et al*., 1993; Pagot and Belin., 1996; Waché *et al*., 2001; Waché *et al*., 2002). These enzymes are acyl-CoA oxidase, a bifunctional hydratase-oxidase and a thiolase. Recent study showed that the peroxisomal acyl-CoA thioesterase likely to be involved in fatty acids oxidation (unpublished results). The acyl-CoA oxidase was described as a key step enzyme in the degradation of fatty acids in peroxisome and in the production of lactones through biotransformation by yeast.

Moreover, in order to bring new insights to understand the high γ-decalactone production by *R. aurantiaca,* we focused on the β-oxidative system of this yeast and on the key step enzymes acyl-CoA oxidase and thioesterase. To highlight the presence of acyl-CoA oxidase and thioesterase genes in *R. aurantiaca*, oligonucleotide primers used for the amplification of acyl-CoA oxidase gene in *Yarrowia lipolytica* (aco107 and aco108) were used (Wang *et al*., 1998). Under optimized PCR conditions, the primer pairs resulted in amplification of DNA fragments of 500 and 800 pb from *R. aurantiaca* genome (Fig. 2). The two fragments were sequenced and the sequences obtained were submitted to GeneBank database (CWBI).

The first sequence obtained was submitted to the GeneBank database, we found that this sequence presented 49% of homology with a gene encoding for the acyl-CoA oxidase of *Y. lipolytica* (POX1). The deduced amino acid sequence from this fragment were aligned with the POX1

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ctPOX4   CGG HGYSSYNGFG KAYNDWVVQCTWE
ctPOX5   CGG HGYSSYNGFG KAYSDWVVQCTWE
ylPOX1   CGG HGYSGYNGFG QGYADWVVQCTWE
ylPOX2   CGG HGYSGYNGFG QAYADWVVQCTWE
ylPOX3   CGG HGYSGYNGFG QAYADWVVQCTWE
ylPOX4   CGG HGYSAYNGFG QAFQDWVVQCTWE
ylPOX5   CGG HGYSGYNGFG QAYADWVVQCTWE
cmPOX4   CGG HGYSSYNGFA KAFNDWVVQCTWE
scPOX1   CGG HGYSQYNGFG KGYDDWVVQCTWE
raPOX1   RDG GWRLSLIG.V YWRV.WVVQCTWG
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Fig. 3. Comparison of acyl-CoA oxidase partial protein sequences from some yeast species. Alignment of *R. aurantiaca* (rapox1, this work), *Y. lipolytica pox1* to *pox5* (AJ001299 to AJ001303) (Wang *et al*., 1999), *S. cerevisiae pox1* (M27515) (Dmochowska *et al*., 1990), *C. maltosa pox4* (X06721) (Hill *et al*, 1988), *C. tropicalis pox4* and *pox5* (M12160 and M12161 (Murray *et al*., 1987)

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                    1                                                     50
Ricinus_communis    .......... .......... .......... .......... ...MEKNSVS
R. aurantiaca       EWRLCVEGVS GFERSG.VGG .VDSGES.RE VW.HCGGGHD CGRMSCQSVS

                    51                                                   100
Ricinus communis    ......TTAS IAGEAQSSSE VTVAKSLPAE YVR......E IESFFIRKGS
R. aurantiaca       ..SALRMVTS CIDPYQIISL AETGGRLDGE VSD.TVSWVA VDVVDYLQAG

                    101                                                  150
Ricinus communis    SAHLPENHKS KDFYSHLFRH LLRANYVQRG RVSCLFSVLS AFANIFNGLH
R. aurantiaca       PSQ.VHPSSN KTDISYSFRH LLSSSWRPRS TFSPINPALT DIQIVA..IL

                    151                                                  200
Ricinus communis    GGVIGGIAER VAIACARTIV SEDKELFLGE LSMSYLSAAP ...LNEECVV
R. aurantiaca       CGVVWGLWEG FAIVCAGTFL GEIGNFYFFR YLVQGPSRQD .ERRT.TMRV

                    201                                       240
Ricinus_communis    DGSTVRSG.R NLTVVAMEFR IKKTGKLVYT ARATLYHMPI
R. aurantiaca       LAHVVREGGI IVVLVARSDS ATR.S.REEV RKRTDSHLQV
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Fig. 4. Comparison of deduced amino acid sequence from the presumptive *R. aurantiaca* acyl-CoA thioesterase gene with the *Ricinus communis* acyl-CoA thioesterase gene (XP_002511810)

protein from *Saccharomyces cerevisiae* (Dmochowska *et al*., 1990), POX4 and POX5 proteins from *Candida tropicalis* (Okazaki *et al*., 1986; Murray *et al*., 1987), POX4 protein from *C. maltosa* (Hill *et al*., 1988), and five acyl-CoA oxidase proteins (POX1 to POX5 proteins) from *Yarrowia lipolytica* (Wang *et al*., 1999), revealed one highly conserved block which sequence was WVVQCTW (Fig. 3). This allowed us to show that a putative acyl-CoA oxidase gene was present in *R. aurantiaca* and it was named raPOX1.

However, the presence of 4-hydroxydecanoic acid might be related to the presence of acyl-CoA thioesterase that hydrolyzes the acyl coenzyme A esters after they are released from the β-oxidation system. Feron *et al*., (1996) showed that the yeast *Sporidiobolus ruinenii* produced the 4-hydroxydecanoic acid and γ-decalactone. These authors proposed that the ability of the yeast to produce this acid comes from the presence of this enzyme.

In the current study, we confirmed the presumptive presence of a gene encoding for the acyl-CoA thioesterase. The primer pairs (aco107 and aco108) resulted a second fragment of the DNA isolated from *R. aurantiaca* of approximately 800 pb. This fragment was sequenced and the sequence revealed 714 pb. It encodes a 238 amino acid protein presenting 36% identity with the acyl-CoA thioesterase from *Ricinus communis*. The alignement of partial amino acid sequence of *R. aurantiaca* thioesterase is presented in Fig. 4. However, analysis of three internal peptide sequences obtained (MSCQSVS, SYSFRHLL and EGFAIVCAGT) exhibited, respectively, 57, 75 and 60% homology with three amino acids sequences located between residues 44-50, 115-122, 159–168 of the acyl-CoA thioesterase from *Ricinus communis.*

In previous studies in our laboratory, Sabri *et al*. (2001) purified and characterized the *R. aurantiaca* thioesterase. Substrate specificity was also determined using several acyl-CoA ester derivatives. *R. aurantiaca* thioesterase is active on thioesters with carbon chain lengths ranging from 8 to 18. No activity was detected with C2-CoA or C4-CoA and the preferred substrates were C14-CoA, C16-CoA and C18-CoA.

To study the role of acyl-CoA oxidase and thioesterase enzymes presents in *R. aurantiaca*, the yeast was cultivated in media containing γ-decalactone as sole source carbon. It was observed that the yeast is not able to grow and to metabolize γ-decalactone. Consequently, we suggest that these enzymes are long-chain-specific.

Conclusions

Further studies are required to obtain the complete sequence of the two genes. Construction of strains of *R. aurantiaca* deleted of the genes encoding for the acyl-CoA oxidase and thioesterase will be necessary to go further in the understanding of the role of these enzymes in the degradation of fatty acids and consequently in the production of γ-decalactone.

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