# Involvement of Acyl Coenzyme A Oxidase Isozymes in Biotransformation of Methyl Ricinoleate into γ-Decalactone by *Yarrowia lipolytica*

YVES WACHÉ,[1]* CÉLINE LAROCHE,[1] KARIN BERGMARK,[1] CHARLOTTE MØLLER-ANDERSEN,[1]
MARIO AGUEDO,[1] MARIE-THÉRÈSE LE DALL,[2] HUIJIE WANG,[2]
JEAN-MARC NICAUD,[2] AND JEAN-MARC BELIN[1]

*Laboratoire de Biotechnologie, Ecole Nationale Supérieure de Biologie Appliquée à la Nutrition et à l'Alimentation,
Université de Bourgogne, 21000 Dijon,[1] and Laboratoire de Génétique des Micro-organismes,
INRA-CNRS, URA1925, 78850 Thiverval-Grignon,[2] France*

We reported previously on the function of acyl coenzyme A (acyl-CoA) oxidase isozymes in the yeast *Yarrowia lipolytica* by investigating strains disrupted in one or several acyl-CoA oxidase-encoding genes (*POX1* through *POX5*) (H. Wang et al., J. Bacteriol. 181:5140–5148, 1999). Here, these mutants were studied for lactone production. Monodisrupted strains produced similar levels of lactone as the wild-type strain (50 mg/liter) except for Δ*pox3*, which produced 220 mg of γ-decalactone per liter after 24 h. The Δ*pox2* Δ*pox3* double-disrupted strain, although slightly affected in growth, produced about 150 mg of lactone per liter, indicating that Aox2p was not essential for the biotransformation. The Δ*pox2* Δ*pox3* Δ*pox5* triple-disrupted strain produced and consumed lactone very slowly. On the contrary, the Δ*pox2* Δ*pox3* Δ*pox4* Δ*pox5* multidisrupted strain did not grow or biotransform methyl ricinoleate into γ-decalactone, demonstrating that Aox4p is essential for the biotransformation.

The biotransformation of fatty acids into γ-decalactone, a compound with a peach-like aroma, has led to numerous patents. Most processes are based on the use of yeast strains to transform ricinoleic acid or its derivatives into lactones, as reviewed by Endrizzi et al. (4). The development of such processes initially involves optimizing physical and chemical conditions for the biotransformation or screening to find new organisms that produce γ-decalactone. This stage of development is continuing, but finding ways to increase lactone production is increasingly demanding improvements in our knowledge of the relevant cellular mechanisms. Endrizzi et al. (2) showed that the β-oxidation pathway is involved in the biotransformation, and studies of intermediates have intensified (9, 10, 14, 20). Féron et al. (6, 7) have focused on the toxicity of lactone for the yeast that produces it, and Endrizzi-Joran (5) has focused on the reconsumption of lactone. Pagot (15) has been looking for critical steps and has found two potentially rate-limiting steps: the entry of fatty acids into peroxisomes, which is at least partially carnitine dependent (16), and the first and limiting (13, 18) reaction of peroxisomal β-oxidation (17), involving acyl coenzyme A (acyl-CoA) thioester oxidation by an acyl-CoA oxidase (Aox) to yield *trans*-2-enoyl-CoA derivatives. All these studies have increased our understanding of the mechanisms involved but have not substantially increased lactone production.

In *Yarrowia lipolytica*, acyl-CoA oxidases are encoded by five genes (*POX1* through *POX5*). An investigation of the function of the Aox isozymes demonstrated a chain length specificity for Aox2p (long chain) and Aox3p (short chain). Aox2p and Aox3p together account for 70 to 80% of global Aox activity

(22). Pagot et al. (17) disrupted *POX1* (encoding Aox1p) and observed higher levels of β-oxidation and higher Aox activities but lower levels of lactone production, thereby demonstrating that Aox plays a fundamental role in lactone production. So far, no technological applications have resulted from the research of yeast acyl-CoA oxidases apart from that of Picataggio et al. (19), who blocked β-oxidation by sequential disruption of Aox genes in *Candida tropicalis* to redirect fatty acids to the ω-oxidation pathway, leading to the production of long-chain dicarboxylic acids.

We decided to focus on this step with the goal of significantly increasing lactone production. We used the set of disrupted strains for the *POX* genes described previously (22), with the aim of determining their role in lactone production. We found that Aox composition affects lactone production, either positively or negatively. Particularly, mutants disrupted for the *POX3* gene encoding the short-chain-specific enzyme (Aox3p) had increased lactone productions.

The *Y. lipolytica* strains used in this study are derived from the wild-type *Y. lipolytica* W29 (ATCC 20460). Their constructions have been described elsewhere (21, 22). All strains were cultured for 48 h on malt extract agar (Difco) at 27°C and used to inoculate a 500-ml baffled Erlenmeyer flask containing 200 ml of glucose medium (15 g of glucose/liter, 2.5 g of ammonium chloride/liter, 0.1 g of yeast extract/liter, 2.1 g of monopotassium phosphate/liter, 4.51 g of disodium phosphate/liter, 0.2 g of magnesium sulfate/liter, 0.1 g of sodium chloride/liter, 9.14 mg of iron (II) sulfate heptahydrate/liter, 0.5 mg of zinc chloride/liter, 1.56 mg of copper sulfate/liter) to an optical density at 600 nm ($OD_{600}$) of 0.25 ($6 \times 10^6$ cells/ml). Flasks were shaken at 140 rpm for 18 h until the cultures reached the late logarithmic growth phase. Cells were harvested (10,000 × *g*, 5 min), washed twice with phosphate buffer (50 mM, pH 7.4), and resuspended in MD medium (6.7 g of yeast nitrogen base/liter, 5 g of ammonium chloride/liter, 1 g of methyl decanoate/liter) ($OD_{600}$ = 0.25) for growth experiments or in MR me-

* Corresponding author. Mailing address: Laboratoire de Biotechnologie, Ecole Nationale Supérieure de Biologie Appliquée à la Nutrition et à l'Alimentation, Université de Bourgogne, 1, esplanade Erasme, 21000 Dijon, France. Phone: 33 3 80 39 66 80. Fax: 33 3 80 39 66 41. E-mail: ywache@u-bourgogne.fr.
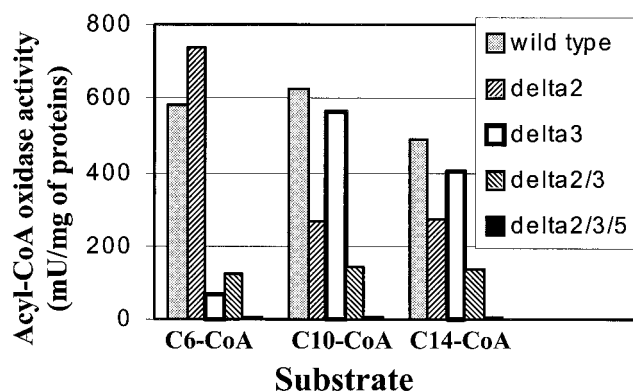
FIG. 1. Acyl-CoA oxidase activity profiles of mutant strains as a function of substrate chain length. Activity was measured independently with $C_6$-CoA, $C_{10}$-CoA, and $C_{14}$-CoA substrates and was standardized for protein concentration (21). The strains presented here are the wild type, $\Delta pox2$, $\Delta pox3$, $\Delta pox2$ $\Delta pox3$, and $\Delta pox2$ $\Delta pox3$ $\Delta pox5$.

TABLE 1. Growth in MR medium and γ-decalactone production kinetics for various mutant strains and the wild type[a]

| Strain | $\mu_{max}$ (h$^{-1}$) | $\lambda$ (h) | $(dP/dt)_{max}$ (mg/liter/h) | $q_{pmax}$ (mg/10$^6$ cells/h) |
|---|---|---|---|---|
| Wild type | 0.15 ± 0.01 | 2.2 ± 0.4 | 6.1 | 0.34 |
| $\Delta pox2$ | 0.18 ± 0.01 | 2.7 ± 0.2 | 13.5 | 0.66 |
| $\Delta pox3$ | 0.15 ± 0.02 | 1.3 ± 0.5 | 31.1 | 1.30 |
| $\Delta pox5$ | 0.15 ± 0.01 | 2.9 ± 0.5 | 12.0 | 0.62 |
| $\Delta pox2$ $\Delta pox3$ | 0.07 ± 0.01 | 3.7 ± 0.4 | 9.4 | 0.48 |
| $\Delta pox3$ $\Delta pox5$ | 0.16 ± 0.01 | 1.4 ± 0.5 | 22.0 | 1.22 |
| $\Delta pox2$ $\Delta pox3$ $\Delta pox5$ | ND | ND | 7.1 | 0.67 |
| $\Delta pox2$ $\Delta pox3$ $\Delta pox4$ $\Delta pox5$ | 0 | ∞ | 0 | 0 |

[a] Maximal growth rate ($\mu_{max}$) and lag phase ($\lambda$) were calculated with the Gompertz parameters. Maximal rate of production [$(dP/dt)_{max}$] and maximal rate of specific production ($q_{pmax}$) were determined from the production curves shown in Fig. 2. ND, not determined.

dium (MD medium with 0.2 g of Tween 80/liter and with 1 g of methyl decanoate/liter replaced with 5 g of methyl ricinoleate/liter [Dubois, Boulogne, France]) (OD$_{600}$ = 0.25) for growth and lactone production kinetic experiments.

Cells grown in MR or MD medium were counted in a Malassez cell. Slide Write+ (Advanced Graphics Software Inc., Carlsbad, Calif.) was used to fit curves and to calculate the parameters corresponding to the Gompertz model as modified by Zwietering et al. (23): $X_{max}$, the maximal amount of biomass; $\mu_{max}$, the maximal growth rate (h$^{-1}$); and $\lambda$, the lag phase (h).

For γ-decalactone extraction and analysis, 1.5 ml was removed and centrifuged. The supernatant (both aqueous and oil phases) was mixed, and its pH was lowered to 2 with HCl (6 N). The internal standard (γ-undecalactone) was added, and the mixture was extracted with 1.5 ml of diethyl ether in 4-ml glass vials by shaking for 1.5 min. The ether phase was then analyzed in an HP6890 gas chromatograph with an HP-INNOWax capillary column (30.0 m by 320 μm by 0.25 μm) using N$_2$ as a carrier gas at a linear flow rate of 4.3 ml/min. The split injector (split ratio, 7.1:1) temperature was set to 250°C, and that of the flame ionization detector to 300°C. The oven temperature was programmed to increase from 60 to 145°C at a rate of 5°C/min and then at a rate of 2°C/min to 180°C.

Contributions of each Aox isozyme to the global acyl-CoA oxidase activity can be deduced from Fig. 1, which represents the effect of gene disruption on the activity of the remaining enzymes in the cell extracts depending on substrate chain length (21, 22). As shown in Table 1, a POX monodisruption had no significant effect on the growth characteristics of the mutant strains. Consistent with Aox2 and Aox3 playing the major roles, the growth of the double-disrupted strain, $\Delta pox2$ $\Delta pox3$, was altered, with a lower $\mu_{max}$ (0.07 h$^{-1}$ compared to 0.15 h$^{-1}$ for the wild type) and a longer $\lambda$ (3.7 h, compared to 2.2 h). However, there were no significant differences in $X_{max}$ (results not shown) except for $\Delta pox2$ $\Delta pox3$ $\Delta pox4$ $\Delta pox5$ cells, which did not grow at all due to the lack of activity of the enzyme encoded by POX1 in those conditions.

To determine whether particular Aox isozymes were involved in $C_{10}$ consumption, we studied the growth of various mutants on the $C_{10}$ substrate methyl decanoate instead of the toxic γ-decalactone (Table 2). $\mu_{max}$ was significantly higher for $\Delta pox2$ and lower for $\Delta pox3$ than for the wild type. At least one of the two enzymes (Aox2p and Aox3p) must be present if the

yeast is to grow in MD medium, as the double-disrupted strain, $\Delta pox2$ $\Delta pox3$, cannot grow on this substrate.

Figure 2 shows lactone production kinetics of various strains for the first 24 h of culture in MR medium. We can distinguish the mutants with $\Delta pox3$ ($\Delta pox3$ and $\Delta pox3$ $\Delta pox5$) and $\Delta pox2$ $\Delta pox3$ disruptions (Fig. 2B) from those with another disruption ($\Delta pox2$, $\Delta pox5$) or no disruption (wild type) (Fig. 2A). The strains with POX2 and POX3 produced γ-decalactone in the first 12 h, and thereafter, the reconsumption rate was at least equal to the production rate. For strains containing the POX3 deletion, lactone was produced during the first 24 h of culture to higher levels. The rate of productivity for $\Delta pox3$ and $\Delta pox3$ $\Delta pox5$ in the first 12 h was significantly higher (more than 12.5 mg/liter/h) than that of all the other strains (between 3 and 7 mg/liter/h). However, strains with $\Delta pox2$ $\Delta pox3$ disruptions produced lactone more slowly, with maximal production occurring, as for the maximal biomass production, between 12 and 24 h.

Table 1 shows the maximal rate of lactone production [$(dP/dt)_{max}$] and maximal rate of specific production [$q_{pmax} = (dP/dt X)_{max}$]. $\Delta pox2$ $\Delta pox3$ $\Delta pox4$ $\Delta pox5$ cells did not grow or produce any lactone. The rates of production (6.1 mg/liter/h) and specific production were the lowest in wild-type cells. The strain with $\Delta pox2$ $\Delta pox3$ simultaneously disrupted had a slightly higher rate of lactone production (9.4 mg/liter/h). Higher rates (twice that of the wild type) were obtained with $\Delta pox2$ and $\Delta pox5$ strains, and the highest rate was obtained with the $\Delta pox3$-disrupted strains, which had rates of production four to five times that of the wild type (22.0 mg/liter/h for $\Delta pox3$ $\Delta pox5$ and 31.1 mg/liter/h for $\Delta pox3$). The $q_{pmax}$ for $\Delta pox3$ strains was also three to four times higher than that of the wild type: 1.3 mg/10$^6$ cells/h for $\Delta pox3$ and 1.22 mg/10$^6$ cells/h for $\Delta pox3$ $\Delta pox5$. For the double-disrupted strain $\Delta pox2$ $\Delta pox3$, $q_{pmax}$ was only slightly higher than that of the wild type: 0.48 mg/10$^6$ cells/h instead of 0.34 mg/10$^6$ cells/h, but the period of $(dP/dt X)_{max}$ was much longer.

TABLE 2. Growth kinetics of various mutant strains and the wild type in methyl decanoate

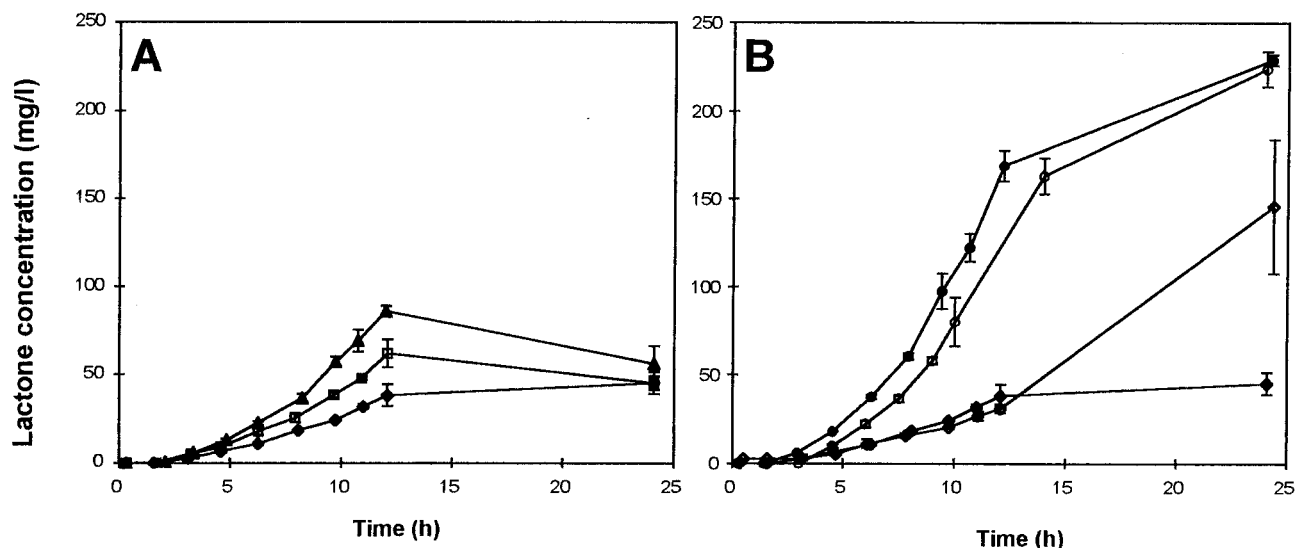| Strain | $X_{max}$ (cells/ml$^{-1}$) | $\mu_{max}$ (h$^{-1}$) |
|---|---|---|
| Wild type | 9.0 × 10$^7$ | 0.16 |
| $\Delta pox2$ | 10.8 × 10$^7$ | 0.23 |
| $\Delta pox3$ | 4.0 × 10$^7$ | 0.10 |
| $\Delta pox2$ $\Delta pox3$ | 6.0 × 10$^6$ (=X$_0$) | 0 |

FIG. 2. Lactone production by the various strains. (A) Monodisrupted strains other than $\Delta pox3$: $\Delta pox2$ (▲); $\Delta pox5$ (□), and wild type (◆). (B) $\Delta pox3$ strains: $\Delta pox3$ (●); $\Delta pox2$ $\Delta pox3$ (◇); $\Delta pox3$ $\Delta pox5$ (○), and wild type (◆). Lactone concentration was determined in the culture medium after extraction.

Aox2 and Aox3 combined provide the main Aox activity (70 to 80% of the total) (Fig. 1), but the other three Aox proteins are involved in β-oxidation. For some gene disruptions, cells compensate for the lack of the corresponding Aox, most probably through regulation of the other *POX* gene(s), resulting in global Aox activity higher than that of the wild type (22). One of the genes causing an enhanced Aox activity (*POX1*) was not functional or not expressed in our conditions, but its disruption affected not only the level of Aox activity but also the lactone productivity of the mutant strain (17). The double disruption of *POX2* and *POX3* clearly shows that at least one other gene product is involved in the production pathway. Aox5p may be functional, as the triple-disrupted strain ($\Delta pox2$ $\Delta pox3$ $\Delta pox5$) had no detectable activity but grew and biotransformed methyl ricinoleate into γ-decalactone. Another gene product, Aox4p, is functional in *Yarrowia* cells because the $\Delta pox2$ $\Delta pox3$ $\Delta pox4$ $\Delta pox5$ mutant did not grow or produce lactone, contrary to $\Delta pox2$ $\Delta pox3$ $\Delta pox5$. Methyl ricinoleate biotransformation in yeast utilizes the peroxisomal β-oxidation pathway (2). The search for intermediates established that ricinoleyl-CoA is shortened by two carbons four times, resulting in the accumu-lation of γ-decalactone (9, 14, 20). The precursor of this lactone is 4-hydroxydecanoyl-CoA, or 4-hydroxydecanoic acid (6), which can be oxidized in one cycle, leading to 2-hydroxyoctanoic acid (14). Alternatively, it may be oxidized in half a cycle, yielding 3,4-dihydroxydecanoic acid and 3-hydroxy-γ-decalactone (9). 4-Hydroxydecanoic acid and its CoA form give rise easily to the more stable lactone form, accounting for the accumulation of γ-decalactone (9, 14). However, a decrease in lactone concentration has often been observed because some yeasts are able to reconsume this metabolic product (3, 8, 14). One mechanism which could be implicated in the reconsumption is ω-oxidation followed by the β-oxidation of the carbon chain extremity (5), but other possibilities include delactonization by a lactonase producing a hydroxy acid which is fed into the β-oxidation pathway (1, 11, 12).

The significant increase in the lactone production of $\Delta pox3$ strains most likely results from two effects, as schematically presented in Fig. 3. Reconsumption is particularly evident because for *POX3*-possessing strains, lactone concentration in the culture medium drastically decreases after 12 h. Presumably, disrupting the short-chain-specific Aox gene slows down
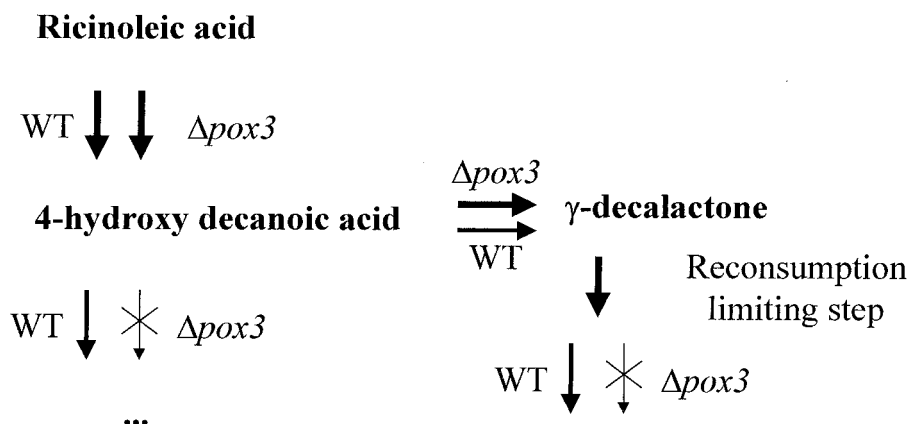


FIG. 3. Schematic representation of the role of Aox3p in the pathway of production of γ-decalactone.

β-oxidation cycles at the $C_{10}$ level as the growth of $\Delta pox3$ strains is greatly affected on $C_{10}$ substrate. This will increase the size of the 4-hydroxydecanoic acid pool and therefore slightly increase γ-decalactone production, and it will also stop any β-oxidation following the possible ω-oxidation or delactonization and, hence, will lower the rate of decalactone disappearance. The initial step of lactone reconsumption may involve an ω-oxidation reaction or opening of the lactone ring, and the Aox step only affects the fluxes. We added γ-decalactone to the medium in the presence of the wild type, $\Delta pox2$, $\Delta pox3$, and $\Delta pox2$ $\Delta pox3$ and monitored its disappearance. We found no significant differences between the strains in 4 h of reaction (results not shown).

This study, showing a fivefold increase in the production rate, demonstrates that mastering the control of Aox, particularly lowering the impact of short-chain-specific Aox, is a promising way to increase lactone production. However, it will still be necessary to overcome lactone toxicity and to inhibit reconsumption.

## REFERENCES

1. **Barton, P., and M. I. Page.** 1993. The esterase catalysed resolution of lactones and spirodilactone. J. Chem. Soc. Perkin. Trans. I **2:**2317–2318.
2. **Endrizzi, A., A. C. Awadé, and J.-M. Belin.** 1993. Presumptive involvement of methyl ricinoleate beta-oxidation in the production of gamma-decalactone by the yeast *Pichia guilliermondii*. FEMS Microbiol. Lett. **114:**153–160.
3. **Endrizzi, A., and J.-M. Belin.** 1995. Bioconversion of methyl ricinoleate to 4-hydroxy-decanoic acid and to gamma-decalactone by yeasts of the genus *Candida*. J. Basic Microbiol. **35:**285–292.
4. **Endrizzi, A., Y. Pagot, A. Le Clainche, J.-M. Nicaud, and J.-M. Belin.** 1996. Production of lactones and peroxisomal beta-oxidation in yeasts. Crit. Rev. Biotechnol. **16:**301–329.
5. **Endrizzi-Joran, A.** 1994. Thèse de Doctorat. Université de Bourgogne, Dijon, France.
6. **Féron, G., L. Dufossé, G. Mauvais, P. Bonnarme, and H. E. Spinnler.** 1997. Fatty acid accumulation in the yeast *Sporidiobolus salmonicolor* during batch production of gamma-decalactone. FEMS Microbiol. Lett. **149:**17–24.
7. **Féron, G., L. Dufossé, E. Pierard, P. Bonnarme, J. L. Le Queré, and H. E. Spinnler.** 1996. Production, identification, and toxicity of gamma-decalactone and 4-hydroxydecanoic acid from *Sporidiobolus* spp. Appl. Environ. Microbiol. **62:**2826–2831.
8. **Fuganti, C., P. Grasselli, and M. Barbeni.** 1991. Biogeneration of aromas: gamma and delta lactones from C6 to C12. NATO ASI Ser. Ser. A **207:**1–17.
9. **Gatfield, I. L., M. Güntert, H. Sommer, and P. Werkhoff.** 1993. Some aspects of the microbiological production of flavor-active lactones with particular reference to gamma-decalactone. Chem. Mikrobiol. Technol. Lebensm. **15:**165–170.
10. **Haffner, T., and R. Tressl.** 1996. Biosynthesis of (R)-gamma-decanolactone in the yeast *Sporobolomyces odorus*. J. Agric. Food Chem. **44:**1218–1223.
11. **Khalameyzer, V., I. Fischer, U. T. Bornscheuer, and J. Altenbuchner.** 1999. Screening, nucleotide sequence, and biochemical characterization of an esterase from *Pseudomonas fluorescens* with high activity towards lactones. Appl. Environ. Microbiol. **65:**477–482.
12. **Kim, C. H., T. Endo, and H. Yonehara.** 1988. Studies on mikamycin B lactonase V. Metabolic control in mikamycin B fermentation. J. Antibiot. **41:**73–80.
13. **Lazarow, P. B., and C. De Duve.** 1976. A fatty acyl-CoA oxidizing system in rat liver peroxisomes; enhancement by clofibrate, a hypolipidemic drug. Proc. Natl. Acad. Sci. USA **73:**2043–2046.
14. **Okui, S., M. Uchiyama, and M. Mizugaki.** 1963. Metabolism of hydroxy fatty acids. 2. Intermediates of the oxidative breakdown of ricinoleic acid by genus *Candida*. J. Biochem. **54:**536–540.
15. **Pagot, Y.** 1997. Thèse de Doctorat. Université de Bourgogne, Dijon, France.
16. **Pagot, Y., and J.-M. Belin.** 1996. Involvement of carnitine acyltransferases in peroxisomal fatty acid metabolism by the yeast *Pichia guilliermondii*. Appl. Environ. Microbiol. **62:**3864–3867.
17. **Pagot, Y., A. Le Clainche, J.-M. Nicaud, Y. Waché, and J.-M. Belin.** 1998. Peroxisomal beta-oxidation activities and gamma-decalactone production by the yeast *Yarrowia lipolytica*. Appl. Microbiol. Biotechnol. **49:**295–300.
18. **Pedersen, J. I., G. Eggersten, U. Hellman, U. Anderson, and I. Björkhem.** 1997. Molecular cloning and expression of cDNA encoding 3alpha, 7alpha, 12alpha-trihydroxy-5beta-cholestanoyl-CoA oxidase from rabbit liver. J. Biol. Chem. **272:**18481–18489.
19. **Picataggio, S., T. Rohrer, K. Deanda, D. Lanning, R. Reynolds, J. Mielenz, and L. D. Eirich.** 1992. Metabolic engineering of *Candida tropicalis* for the production of long chain dicarboxylic acids. Bio/Technology **10:**894–898.
20. **Spinnler, H. E., C. Ginies, J. A. Khan, and E. N. Vulfson.** 1996. Analysis of metabolic pathways by the growth of cells in the presence of organic solvents. Proc. Natl. Acad. Sci. USA **93:**3373–3376.
21. **Wang, H., A. Le Clainche, M.-T. Le Dall, Y. Waché, Y. Pagot, J. M. Belin, C. Gaillardin, and J. M. Nicaud.** 1998. Cloning and characterization of the peroxisomal acyl CoA oxidase *ACO3* gene from the alkane-utilizing yeast *Yarrowia lipolytica*. Yeast **14:**1373–1386.
22. **Wang, H. J., M.-T. Le Dall, Y. Waché, C. Laroche, J.-M. Belin, C. Gaillardin, and J.-M. Nicaud.** 1999. Evaluation of acyl Coenzyme A oxidase (Aox) isozyme function in the *n*-alkane-assimilating yeast *Yarrowia lipolytica*. J. Bacteriol. **181:**5140–5148.
23. **Zwietering, M. H., I. Jongenburger, F. M. Rombouts, and K. Van't Riet.** 1990. Modelling of the bacterial growth curve. Appl. Environ. Microbiol. **56:**1875–1881.