ELSEVIER

FEMS Microbiology Letters 250 (2005) 63–69

FEMS MICROBIOLOGY Letters

www.fems-microbiology.org

Metabolism of fatty acid in yeast: Characterisation of β-oxidation and ultrastructural changes in the genus *Sporidiobolus* sp. cultivated on ricinoleic acid methyl ester

Gilles Feron [a,*], Caroline Blin-Perrin [a], Isabelle Krasniewski [a], Geneviève Mauvais [a], Jeannine Lherminier [b]

[a] *Laboratoire de Microbiologie, UMR INRA-UB 1232, 17 rue Sully, BP 86510, 21065 Dijon Cedex, France*
[b] *Service Commun de Microscopie Electronique, INRA, BP 86510, 21065 Dijon Cedex, France*

Received 8 March 2005; received in revised form 22 June 2005; accepted 22 June 2005

First published online 11 July 2005

Edited by C. Remacle

Abstract

Cell structure modifications and β-oxidation induction were monitored in two strains of *Sporidiobolus*, *Sp. Ruinenii* and *Sp. pararoseus* after cultivation on ricinoleic acid methyl ester. Ultrastructural observations of the yeast before and after cultivation on fatty acid esters did not reveal major modifications in *Sp. ruinenii*. Unexpectedly, in *Sp. pararoseus* a proliferation of the mitochondrion was observed. After induction, *Sp. ruinenii* principally exhibited an increase in the activities of acyl-CoA oxidase (ACO), hydroxyacyl-CoA deshydrogenase (HAD), thiolase and catalase. In contrast, *Sp. pararoseus* lacked ACO and catalase activities, but an increase in acyl-CoA deshydrogenase (ACDH) and enoyl-CoA hydratase (ECH) activity was observed. These data suggest that in *Sp. ruinenii*, β-oxidation is preferentially localized in the microbody, whereas in *Sp. pararoseus* it might be localized in the mitochondria.

Keywords: Yeast β-oxidation; *Sporidiobolus* genus; Mitochondrion; Ricinoleic acid; γ-Decalactone

1. Introduction

Fatty acid β-oxidation is a well-recognised process which enables the degradation of a variety of fatty acids to acetate units. The overall process comprises activation of the fatty acid to its corresponding acyl-CoA thioester, which is subsequently completely oxidised with the release of acetyl-CoA. While β-oxidation is almost ubiquitous in nature, differences have been observed with respect to the subcellular compartmentalization, substrate specificity and structural organization of the β-oxidation complex. In general, mammalian β-oxidation is localized in both the mitochondrial and peroxisomal apparatus, while in lower eukaryotes it is considered to be located only in peroxisomes. However, in 1999 Baltazar et al. [1], working on the *Aspergillus niger* fungus, suspected the presence of β-oxidation in the mitochondrion. This hypothesis was confirmed very recently by Magio-Hall and Keller [2] in *Aspergillus nidulans*, when they presented unequivocal evidence for both peroxisomal and mitochondrial β-oxidation. *S. cerevisiae* and other yeasts generally lack the mitochondrial enzymes for β-oxidation, and the subcellular localisation of the oxidative complex remains unclear, although

* Corresponding author: Tel.: +33 3 80 69 32 78; fax: +33 3 80 69 32 27.
E-mail address: feron@arome.dijon.inra.fr (G. Feron).

0378-1097/$22.00
doi:10.1016/j.femsle.2005.06.045

it is considered to be localized in the peroxisome alone [3,4].

Previous work by our laboratory on a yeast from the *Sporidiobolus* genus highlighted differences in the metabolic flux through the oxidative enzymatic complex, thus questioning the possibility of mitochondrial compartmentalization in one species, i.e. *Sporidiobolus pararoseus* [5]. The present paper describes our further investigation of β-oxidation in the *Sporidiobolus* genus and throws new light on both enzyme induction and the ultrastructural modifications to the yeast when cultivated on fatty acids.

2. Materials and methods

2.1. Yeast strains and biochemicals

Sporidiobolus ruinenii (CBS 5001) and *Sporidiobolus pararoseus* (CBS 484) were cultivated in a medium and under conditions which have been described previously [6]. Briefly, cultures were performed in 500-ml flasks containing 100 ml of the following medium: glucose 15 g l^{-1}, bactotryptone 0.5 g l^{-1}, yeast extract 1 g l^{-1}, malt extract 1 g l^{-1}, casaminoacids 2 g l^{-1}, KH_2PO_4 2 g l^{-1}, $CaCl_2$-($2H_2O$) 0.13 g l^{-1}, $FeSO_4$($7H_2O$) 0.01 g l^{-1}, $MgSO_4$-($7H_2O$) 3 g l^{-1}, antifoam 0.1% (v/v). The pH was adjusted to 6.0 with 6N KOH. The medium was then sterilized at 121 °C for 20 min. Inoculation was performed using 2% (v/v) of a yeast suspension (6×10^8 cells ml^{-1}) previously cultivated in the same medium. The cultures were stirred at 250 rpm. Five per cent (v/v) of methyl ricinoleate (MeRi) was added to the medium after 24 h of culture in order to initiate the bioconversion process.

All substrates were obtained from Sigma (Les Ulis, France); however, when they were commercially unavailable, acyl-CoA and enoyl-CoA were synthesized according to the methods described by Kawagushi et al. [7] and Balthazar et al. [1], respectively. After synthesis, the different substrates were extracted using the method described by Broadway et al. [8] and purified by HPLC following the procedure developed by Norwood et al. [9].

2.2. Preparation of cell extracts

After 5 days of cultivation in 5% ricinoleic acid methyl ester (MeRi), cells from 100 ml of culture were harvested by 15 min centrifugation at 5000*g* and 4 °C. Cells were washed twice with distilled water and then three times with a 100 mM phosphate buffer, pH 7.5. After the final washing, the yeast cells were suspended in a 20 mM HEPES-KOH buffer pH 7.5 containing 1 mM PMSF (phenyl methyl sulfonyl fluoride) and 1 mM EDTA (ethylene diamine tetra acetic acid). Cells were disrupted by three passages through a French pressure cell (35 MPa) to produce a crude extract. Whole cells and cell debris were removed by centrifugation at 100,000*g* for 30 min at 4 °C. The supernatant fraction, designated 'cell-free extract', was either used immediately or frozen in small portions in liquid N_2 and then stored at −80 °C until required. The solubilization of enzymes from the mitochondrial matrix and microbodies was tested by measuring respectively the citrate synthase (as used by Sass et al. [10] to determine cross-contamination between cytosolic and mitochondrial fractions in yeast), fumarase and catalase activities in the different fractions obtained during cell fractionation (crude extract, 100,000*g* pellet, 100,000*g* supernatant). The results (data not shown) indicated that $95 \pm 2\%$ of the total activity were recovered in the cell-free extract with respect to the enzyme tested and the yeast studied. The soluble protein concentration of the cell-free extract was determined by the method of Bradford [11] with bovine serum albumin as a standard.

2.3. Enzyme assays

All measurements were performed at 30 °C, using a double-beam spectrophotometer. Acyl-CoA oxidase (ACO) activity was assayed according to the method described by Shimizu et al. [12] on Tris–HCl 120 mM pH 8.6 buffer, 1 mM EDTA, by monitoring the formation of quinoneimine at 500 nm. In a final volume of 1.04 ml, a sample contained 0.8 μmol of 4-amino-antipyrine, 11 μmol of phenol, 50 nmol of FAD (flavin adenine dinucleotide), 50 units of peroxidase and 72 nmol of acyl-CoA (acyl-Coenzymes A). The reaction started with the addition of the enzyme extract. Acyl-Coa deshydrogenase (ACDH) activity was measured according to the method developed by Baltazar et al. [1]. On HEPES (N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid)/KOH buffer 50 mM pH 8.0 in a total volume of 1 ml, the reduction of 100 μmol of DCPIP (2,6-dichloro-phenolindophenol) was monitored at 600 nm in the presence of 50 μmol phenazine metosulfate, 72 nmol acyl CoA and a certain quantity of crude extract (50–200 μg proteins). When determining ACDH activity, account was always taken of thioesterase activity which might interfere with the measurement method. The assay for thioesterase activity contained 120 μmol DTP (4,4′-dithiopyridine), 72 nmol acyl-CoA in 1 ml of HEPES/KOH buffer 50 mM pH 7.5 and cell free extracts (50–200 μg proteins). Activity was determined by monitoring the reduction of DTP at 324 nm [13] and the resulting value was subtracted from that obtained with ACDH. Enoyl-CoA hydratase (ECH) was assayed at 30 °C following the procedure described by Binstock [14], in a total volume of 1 ml phosphate buffer 0.1 M pH 7.5, 28.8 nmol of crotonoyl-CoA or 2-*trans*-decenoyl-CoA and enzyme extract (10–50 μg protein). 3-hydroxyacyl-CoA dehydrogenase (HAD) activity was

measured in terms of the increase in absorbance at 340 nm due to the oxidation of 3-hydroxydecanoyl-CoA into 3-keto-decanoyl-CoA at 30 °C in the presence of NAD (nicotinamide adenine dinucleotide). In Tris (tris-(hydroxymethyl)-aminomethane)–HCl buffer 120 mM pH 8.6, each assay contained NAD 1.02 μmol, Triton-X100 0.05%, EDTA 1 mM, (*DL*)-3-hydroxydecanoyl-CoA 76 nmol. The reaction was triggered by addition of the cell-free extract solution (50–200 μg proteins). Catalase was tested using the method described by Lück [15], which consisted in following the reduction of H_2O_2 at 240 nm. In a total volume of 1 ml and in a potassium phosphate buffer 60 mM at pH 7, the assay contained 1 μmol H_2O_2 and a variable quantity of enzymatic extract (10–60 μg proteins). Fumarase was tested according to the method described by Stitt [16]. In a total volume of 595 μl and in a potassium phosphate buffer 100 mM, pH 7.5, the assay contained 27 μmol L-malate and cell-free extract (30–60 μg proteins). The accumulation of fumarate could be measured by the increase in absorbance at 240 nm. Citrate synthase was assayed as described by Kim et al. [17] by following the reduction of acetyl-CoA at 412 nm. Reactions contained 100 mM Tris hydrochloride, pH 8, 0.25 mM 5,5′-dithiobis-(2-nitrobenzoate), 0.1 mM acetyl coenzyme A, 0.2 mM oxaloacetate and cell extract (10–30 μg proteins).

2.4. Microscopy

The yeast fraction was obtained as described above. Aliquots of cells were washed with 100 mM sodium phosphate buffer, pH 7.2, containing 5 mM $MgCl_2$, and then fixed in the same sodium phosphate buffer containing 3% glutaraldehyde (v/v) and 2% formaldehyde (w/v) for 24 h at 4 °C. Throughout the protocol, cells were pelleted by low-speed centrifugation (3 min, 3000 rpm) between each step. After fixation, the cells were washed three times with the sodium phosphate buffer for a period of 30 min. They were then treated for 15 min in 1% sodium metaperiodate as recommended by Wright [18], and post-fixed with 0.5% (w/v) osmium tetroxide for 1 h at 4 °C. The samples were then dehydrated through a graded ethanol series and propylene oxide and embedded in Epon (TAAB, UK) according to the standard procedure [19]. Thick sections (0.5 μm) were mounted on glass slides and stained with 0.1% (w/v) toluidine blue at pH 11, prior to examination under bright field microscopy with a DMRB microscope (Leica, Rueil-Malmaison, France) coupled with a Hamamatsu Orca 100 camera. Morphometric analysis of the cells was performed using the Visilog image analysis software (Noesis, Les Ulis, France). Ultra-thin sections (90 nm), obtained with an ultramicrotome (Reichert, Ultracut E) fitted with a diamond knife, were collected and counterstained with uranyl acetate and lead citrate before observation with a Hitachi 7500 transmission electron microscope (operating at 80 kV). The H7500 was equipped with an AMT camera driven by AMT software (AMT, Danvers, USA). Locally developed programs made it possible to measure the total area of the cell (without taking account of cell wall thickness) and the areas of mitochondria and microbody compartments within each cell.

3. Results and discussion

3.1. Ultrastructural observations of Sporidiobolus spp cells

The differences observed previously in the *Sporidiobolus* [5] genus led us to investigate ultrastructural modifications to the cells using light and electron microscopy in two species, *Sp. ruinenii* and *Sp. pararoseus*. Whether methyl ricinoleate was present or not in the culture medium, and whatever the species considered, the average size of cells (9 μm length and 5 μm width) was not modified (data not shown). This result differed from those obtained by Wolf et al. [20] in *S. roseus* with a smaller size of yeast when the cells were cultured in methane. However, a more detailed investigation using electron microscopy revealed ultrastructural modifications to the cells after culture on fatty acid methyl ester.

Sp. ruinenii cells cultured on glucose exhibit a cell wall of about 71 nm, surrounded by a capsule which is characteristic of the basidiomycete family. The cytosolic compartment is dense and granular, suggesting strong protein synthesis. Both small vacuoles and mitochondrial and microbody structures could be observed (Fig. 1A and B). Microbodies contained a homogeneous matrix surrounded by a single-unit membrane. The number of microbodies observed per cell section appeared to be stable at around 1.5 organelles per cell (Table 1). After 5 days of culture on 5% methyl ricinoleate, *Sp. ruinenii* cells exhibited a thicker cell wall (approximately 106 nm). The cytoplasmic compartment contained numerous vacuoles, which were often large and occupied the major part of the cytoplasm (Fig. 1C and D). The mitochondrial apparatus could be seen clearly, and in some cases was concentrated at the top of the cell (Fig. 1F). Contrary to what has generally been observed in *C. tropicalis* [21], *S. cerevisiae* [22] and *Y. lypolitica* [23], microbodies did not proliferate in our yeast after cultivation on fatty acid, their number remaining at around 1.2–1.5 and covering a similar surface area (Table 1). The same observation had been made previously with *S. salmonicolor* [24] and was in accordance with the enzyme activity measurements described below. Furthermore, microbodies exhibited the presence of crystalline cores (Fig. 1E), which may correspond to the accumulation of alcohol oxidase proteins [25].

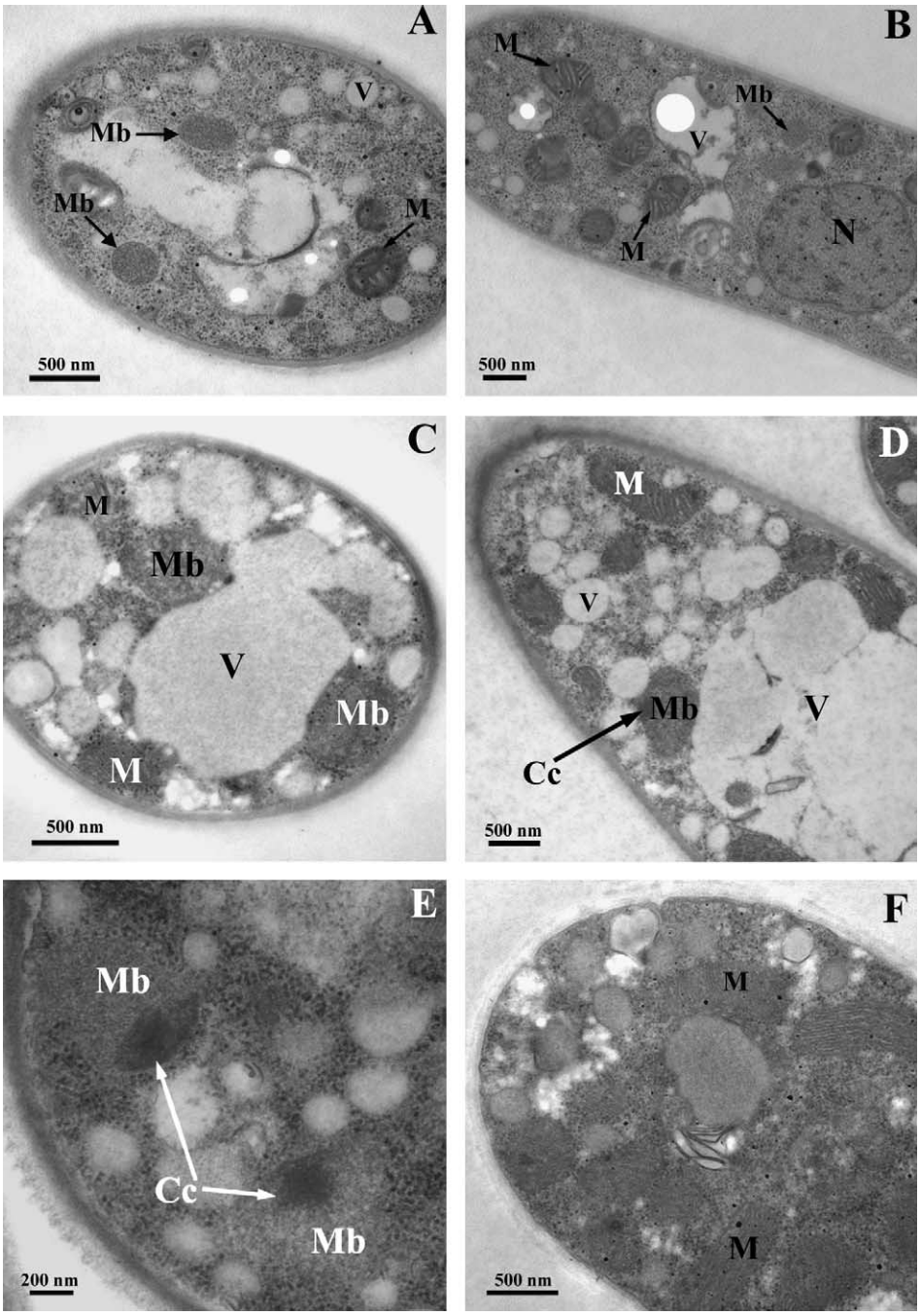

Fig. 1. Ultrathin sections of *Sp. ruinenii* grown without (A, B) and with (C, D, E, F) MeRi 5%. Micrographs were taken of 2% paraformaldehyde/3% glutaraldehyde-fixed, metaperiodate-treated, reduced osmium post-fixed and EPON embedded cells. *Abbreviations*: Cc: crystalline core, M: mitochondria, N: nucleus, Mb: microbody, V: vacuole.

By comparison with *Sp. ruinenii*, *Sp. pararoseus* cells were very difficult to infiltrate with resin, probably because of the different cell wall composition in the two species. Consequently, vacuoles or inclusions and their membranes were often poorly preserved, resulting in white areas in the micrographs (Fig. 2).

When cultivated on glucose, the cells exhibited a thick cell wall (about 95 nm) (Fig. 2A and B). The cytosolic compartment contained mitochondria (from 4 to 5 sections, as indicated in Table 1), less than one microbody and electron-dense inclusions (indicated by * in Fig. 2B) of unknown composition. However, with respect to the strong pink colour of the cells, it is possible to suggest that these inclusions may have corresponded to the accumulation of a pigment such as β-carotenoid. When cultured for five days with 5% methyl ricinoleate, cells developed a thicker wall (160 nm). Moreover, they exhibited a higher degree of vacuolisation (Fig. 2D)

Table 1
Average number and surface area covered (% of the total surface area of the cell) of visible mitochondrial sections and microbodies in *Sporidiobolus* sp. yeasts cultivated without (non induced) or with (induced) ricinoleic acid methyl ester

	Mitochondrion		Microbodies	
	Number	Occupied surface (%)	Number	Occupied surface (%)
Sp. ruinenii				
Non-induced	$5.3^{a} \pm 3.6$	$5.2^{a} \pm 2.6$	$1.5^{a} \pm 3.2$	$2.2^{a} \pm 1.9$
Induced	$3.6^{a} \pm 2.6$	$6.8^{a} \pm 2.8$	$1.2^{a} \pm 0.8$	$2.8^{a} \pm 2.5$
Sp. pararoseus				
Non-induced	$4.5^{a} \pm 2.6$	$6.4^{a} \pm 3.7$	$0.4^{a} \pm 0.7$	$0.5^{a} \pm 1.1$
Induced	$9^{b} \pm 3.3$	$13^{b} \pm 6.5$	$0.15^{a} \pm 0.45$	$0.12^{a} \pm 0.5$

Values are means ± SD from 15 to 20 cells analyzed.
[a,b] Means within columns and for one species with different letters are statistically different ($P < 0.05$).

which could correspond to an accumulation of lipid in some of the vacuoles, as has been observed elsewhere [24]. As shown in Table 1, microbodies were not induced in the presence of MeRi. The most surprising observation concerned development of the mitochondrial apparatus (Fig. 2C and E). Both the average surface area occupied in the cell, and the number of mitochondrial sections, doubled after cultivation on MeRi (Table 1), and in some cases this area could constitute up to 27% of the total surface of the cell. Moreover, many ribosome-like granules were observed on the surfaces of outer membranes (cytoplasmic side, indicated by an arrow on Fig. 2E). As has previously been suggested regarding other yeasts [26], these ribosomes, similar to those on the endoplasmic reticulum, probably synthesize proteins which may be transferred via a vector inside the mitochondrion. Similarly, in 1980, Wolf et al. [20] demonstrated the presence of numerous ribosomes surrounding the mitochondria of *S. roseus* and *R. glutinis* yeasts cultured in methane, but to our knowledge, such a proliferation of the mitochondrial apparatus has never before been described in a yeast.

3.2. Level of β-oxidation in Sporidiobolus spp cells

In *Sporidiobolus pararoseus*, the large mitochondrial apparatus induced in several fatty acid methyl esters utilizing yeasts may reflect a sub-cellular localization of some enzymes involved in the catabolism of fatty acids. For this reason, cell-free extracts of *Sp. ruinenii* and *Sp. salmonicolor* were tested and compared for enzymes of β-oxidation, with or without ricinoleic acid methyl ester in the culture medium. The results are shown in Table 2.

In *Sp. ruinenii*, the four activities of peroxisomial β-oxidation were detected in both extracts. Furthermore, ACDH activity ranged from 0.47 nkat mg^{-1} protein in the non-induced extract to about 1 nkat mg^{-1} protein in the induced extract. Levels of ACO and thiolase were enhanced 25-fold and 8-fold, respectively, after cultivation on fatty acid, although ECH and HAD remained at similar levels under both conditions of culture. This simultaneous presence of ACDH and ACO in the cells was surprising because it had never been observed before. This raises the question of the localization of these activities in the cell. It is well known that ACO is strictly localized in the peroxisome, but ACDH can be located in either the mitochondrial apparatus or in microbodies [27].

Similarly, catalase activity was enhanced 1.8-fold, from 180 ± 12 nkat mg^{-1} protein to 330 ± 17 nkat mg^{-1} protein. This low level of induction was unlike that commonly observed in *S. cerevisiae*, *Y. lipolytica*, *P. Pastoris* or *C. tropicalis* cultivated with fatty acids [22,28] but was in line with the ultrastructural observations. However, in *Candida* spp [29] and *S. cerevisiae* [22], this induction phenomenon has principally been observed during the exponential growth phase (with an average induction of 30–50 times in *Candida*) rather than the stationary phase where induction is noticeably lower (about 5 times in *Candida*), and that appeared to be mainly the case during our study. Moreover, more recent results have shown that the induction of catalase activity during transfer from a glucose-rich medium to an alcane-rich medium is moderate when compared to ACO and thiolase activities [30].

In *Sp. pararoseus*, the β-oxidation activity profile differed markedly from that observed in *Sp. ruinenii*. Levels of ACO remained extremely low in both non-induced and induced extracts. In contrast, all other activities were enhanced after cultivation on methyl ricinoleate; from approximately 3-fold for ECH, HAD and thiolase to 8-fold for ACDH. Neither catalase nor malate synthetase could be detected in either extract, thus confirming the very limited development of microbodies (peroxisomal and/or glyoxisomal apparatus). This profile of β-oxidation enzymes can be compared to the results obtained with *Neurospora crassa* by Kionka and Kunau [31]. However, concomitantly to induction (from 10-fold to 20-fold), these authors demonstrated a preferential increase of the glyoxisomal apparatus (about 25-fold) rather than the mitochondrion.

The presence and strong induction of ACDH, the very low levels of catalase and acyl-CoA oxidase during the first step of β-oxidation, the channelling phenomenon

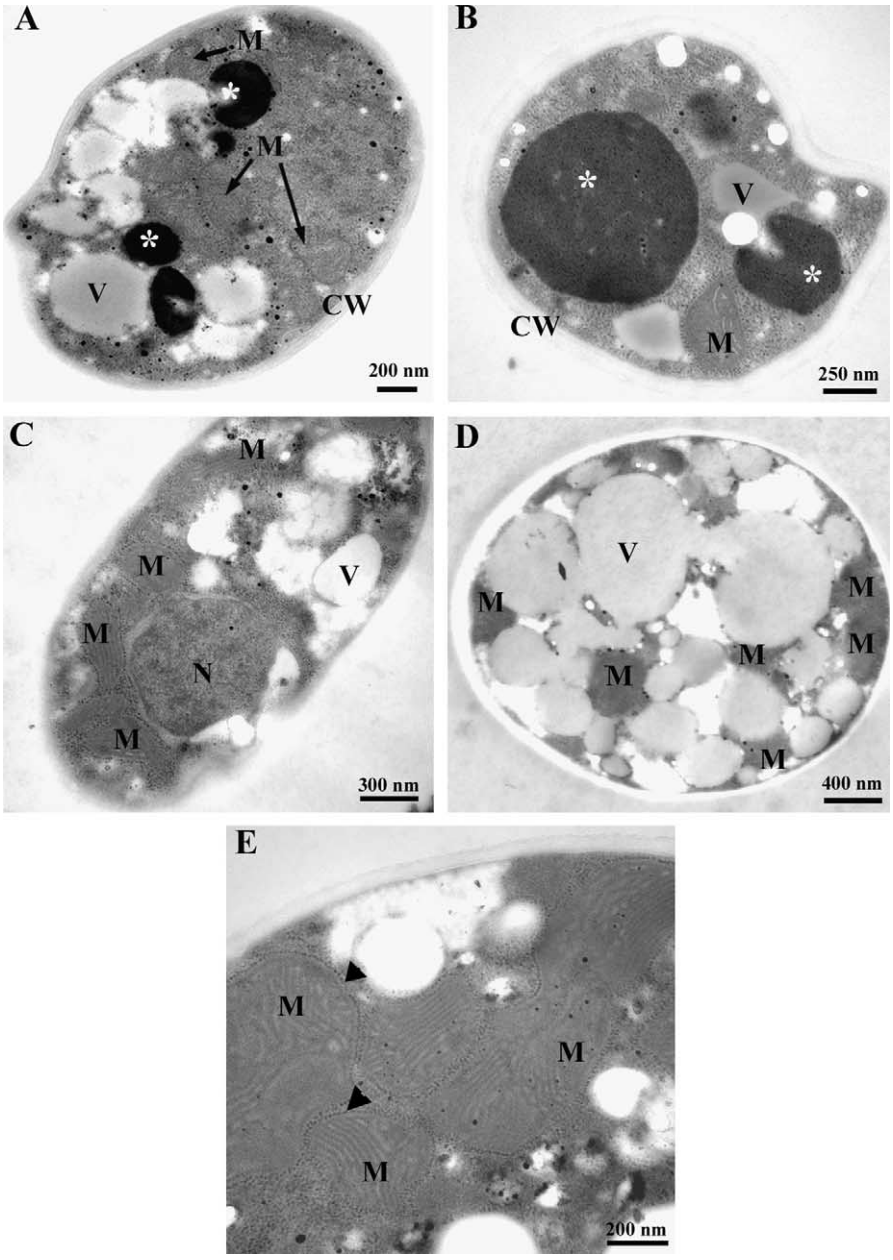

Fig. 2. Ultrathin sections of *Sp. pararoseus* grown without (A, B) and with (C, D, E) MeRi 5%. Micrographs were taken of 2% paraformaldehyde/3%glutaraldehyde-fixed, metaperiodate-treated, reduced osmium post-fixed and EPON embedded cells. *Abbreviations*: CW: cell wall, M: mitochondria, N: nucleus, Mb: microbody, V: vacuole. * indicates electron dense inclusions in the cytosol.

Table 2
Levels of β-oxidation enzyme activity measured in cell-free extracts obtained from *Sporidiobolus* sp. yeasts cultivated without (non-induced) or with (induced) ricinoleic acid methyl ester

	ACO	ACDH	ECH	HAD	Thiolase
Sp. ruinenii					
Non-induced	0.16 ± 0.06	0.28 ± 0.01	38.6 ± 3.2	2.6 ± 0.55	0.98 ± 0.3
Induced	0.79 ± 0.24	0.50 ± 0.05	40.7 ± 2.4	3.4 ± 0.25	8.3 ± 0.45
Sp. pararoseus					
Non-induced	0.03 ± 0.01	1.4 ± 0.4	6.2 ± 1.2	1.1 ± 0.18	1.8 ± 0.3
Induced	0.06 ± 0.02	8.9 ± 0.3	24.4 ± 1.8	3.6 ± 0.2	7.2 ± 0.32

ACO: Acyl-CoA oxidase, ACDH: Acyl-CoA dehydrogenase, ECH: Enoyl-CoA hydratase, HAD: Hydroxy-acyl-CoA dehydrogenase. Enzyme activities are expressed in nkat/mg protein and values are means ± SD for three determinations.

observed previously [5], associated with the development of a very large mitochondrial apparatus in *Sporidiobolus pararoseus* after cultivation on fatty acids, provide strong evidence to back the hypothesis of a compartmentalization of β-oxidation, primarily in the mitochondrion in this yeast. This is a new finding in yeasts, because to date, this β-oxidation strategy has mainly been observed in mammalian cells. For this reason, *Sporidiobolus pararoseus* appears to be peculiar in the yeast family and thus a useful micro-organism in lower eukaryotes, to enable the more detailed investigation of β-oxidation at a molecular level.

Acknowledgements

The authors wish to thank Charles Schneider (INRA Dijon, URLEG) who developed customized programs for the image analysis software.

References

[1] Baltazar, M.F., Dickinson, F.M. and Ratledge, C. (1999) Oxidation of medium-chain acyl-CoA esters by extracts of *Aspergillus niger*: enzymology and characterization of intermediates by HPLC. Microbiology – UK 145, 271–278.

[2] Maggio-Hall, L.A. and Keller, N.P. (2004) Mitochondrial β-oxidation in *Aspergillus nidulans*. Mol. Microbiol. 54, 1173–1185.

[3] Kunau, W.-H., Büne, S., Moreno de la Garza, M., Kionka, C., Mateblowski, M., Schultz-Borchard, U. and Thieringer, R. (1988) Comparative enzymology of β-oxidation. Biochem. Soc. Trans. 16, 418–420.

[4] Trotter, P. (2001) The genetics of fatty acid metabolism. Ann. Rev. Nut. 21, 97–119.

[5] Blin-Perrin, C., Mollé, D., Dufossé, L., Le-Quéré, J.-L., Viel, C., Mauvais, G. and Feron, G. (2000) Metabolism of ricinoleic acid into γ-decalactone: β-oxidation and long chain acyl intermediates of ricinoleic acid in the genus *Sporidiobolus* sp. FEMS Microbiol. Lett. 188, 69–74.

[6] Feron, G., Dufossé, L., Pierard, E., Bonnarme, P., LeQuéré, J.-L. and Spinnler, H.E. (1996) Production, identification, and toxicity of γ-decalactone and 4-hydroxydecanoic acid from *Sporidiobolus* spp. Appl. Environ. Microbiol. 62, 2826–2831.

[7] Kawaguchi, A., Yoshimura, T. and Okuda, S. (1981) A new method for the preparation of Acyl-CoA thioesters. J. Biochem. 89, 337–339.

[8] Broadway, N.M., Dickinson, F.M. and Ratldege, C. (1992) Long-chain acyl-CoA ester intermediates of β-oxidation of mono- and di-carboxylic fatty acids by extracts of *Corynebacterium* sp. strain 7E1C. Biochem. J. 285, 117–122.

[9] Norwood, D.L., Bus, C.A. and Millington, D.S. (1990) Combined high-performance liquid chromatographie-continuous-flow fast atom bombardment mass spectrometric analysis of acylcoenzyme A compounds. J. Chromatograph. 527, 289–301.

[10] Sass, E., Blachinsky, E., Karniely, S. and Pines, O. (2001) Mitochondrial and cytosolic isoforms of yeast fumarase are derivatives of a single translation product and have identical amino termini. J. Biol. Chem. 276, 46111–46117.

[11] Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

[12] Shimizu, S., Yasui, K., Tani, Y. and Yamada, H. (1979) Acyl-CoA oxidase from *Candida tropicalis*. Biochem. Biophysic. Res. Commun. 91, 108–113.

[13] Ramsay, R.R. and Tubbs, P.K. (1975) The mechanism of fatty acid uptake by heart mitochondria: an acyl-carnitine exchange. FEBS Lett. 54, 21–25.

[14] Binstock, J.F. and Schulz, H. (1981) Fatty acid oxidation complex from *Escherichia coli*. Methods Enzymol. 71, 403–411.

[15] Lück, H. (1965) Catalase In: Methods of Enzyme Analysis (Bergmeyer, H.U., Ed.), pp. 895–897. Academic Press, New-York and London.

[16] Stitt, M. (1984) In: Fumarase. Methods of enzymatic analysis (Bergmeyer, H.E., Ed.), vol. IV, pp. 359–362. Verlag Chemie, Weinheim.

[17] Kim, K.S., Rosenkrantz, M.S. and Guarente, L. (1986) *Saccharomyces cerevisiae* contains two functional citrate synthase genes. Mol. Cell Biol., 1936–1942.

[18] Wright, R. (2000) Transmission electron microscopy of yeast. Microsc. Res. Technol. 51, 496–510.

[19] Luft, J.H. (1961) Improvments in epoxy resin embedding methods. J. Biophys. Biochem. Cytol. 9, 409.

[20] Wolf, H.J., Christiansen, M. and Hanson, R. (1980) Ultrastructure of methanotrophic yeasts. J. Bacteriol. 141, 1340–1349.

[21] Tanaka, A., Osumi, M. and Fukui, S. (1982) Peroxisomes of alkane-grown yeast: fundamental and practical approach. Annals New York Academy of Sciences 386, 183–199.

[22] Veenhuis, M., Mateblowski, M., Kunau, W.-H. and Harder, W. (1987) Proliferation of microbodies in *Saccharomyces cerevisiae*. Yeast 3, 77–84.

[23] Titorenko, V.I., Chan, H. and Rachubinski, R.A. (2000) Fusion of small peroxisomal vesicles in vitro reconstructs an early step in the in vivo multistep peroxisome assembly pathway of *Yarrowia lipolytica*. J. Cell Biol. 148, 29–43.

[24] Feron, G., Dufossé, L., Mauvais, G., Bonnarme, P. and Spinnler, H.E. (1997) Fatty acid accumulation in the yeast *Sporidiobolus salmonicolor* during batch production of γ-decalactone. FEMS Microbiol. Lett. 149, 17–24.

[25] Veenhuis, M., Zwart, K.B. and Harder, W. (1981) Biogenesis and turnover of peroxisomes involved in the concurrent oxidation of methanol and methylamine in *Hansenula polymorpha*. Arch. Microbiol. 129, 35–41.

[26] Keyhani, E. (1973) Ribosomal granules associated with outer motochondrial membrane in aerobic yeast cells. J. Cell Biol. 58, 480–484.

[27] Thieringer, R. and Kunau, W.-H. (1991) The β-oxidation system in catalase-free microbodies of the filamentous fungus *Neurospora crassa*. Purification of a multifunctional protein possessing 2-enoyl-CoA hydratase, L-3-hydroxyacyl-CoA dehydrogenase, and 3-hydroxyacyl-CoA epimerase activities. J. Biol. Chem. 266, 13110–13117.

[28] Fukui, S. and Tanaka, A. (1979) Peroxisomes of alkane and methanol grown yeasts. J. Appl. Biochem. 1, 171–201.

[29] Osumi, M., Miwa, N., Teranishi, Y., Tanaka, A. and Fukui, S. (1974) Ultrastructure of *Candida* yeasts grown on n-alkanes: appearance of microbodies and its relationship to high catalase activity. Arch. Microbiol. 99, 181–201.

[30] Kamasawa, N., Ohtsuka, I., Kamada, Y., Ueda, M., Tanaka, A. and Osumi, M. (1996) Immunoelectron microscopic observation of the behaviors of peroxisomal enzymes inducibly synthesized in an *n*-alkane-utilizable yeast cell, *Candida tropicalis*. Cell. Struct. Funct. 21, 117–122.

[31] Kionka, C. and Kunau, W.-H. (1985) Inducible β-oxidation pathway in *Neurospora crassa*. J. Bacteriol. 161, 153–157.