

# Molecular characterization of carnitine-dependent transport of acetyl-CoA from peroxisomes to mitochondria in *Saccharomyces cerevisiae* and identification of a plasma membrane carnitine transporter, Agp2p

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**In *Saccharomyces cerevisiae*,  $\beta$ -oxidation of fatty acids is confined to peroxisomes. The acetyl-CoA produced has to be transported from the peroxisomes via the cytoplasm to the mitochondrial matrix in order to be degraded to CO<sub>2</sub> and H<sub>2</sub>O. Two pathways for the transport of acetyl-CoA to the mitochondria have been proposed. The first involves peroxisomal conversion of acetyl-CoA into glyoxylate cycle intermediates followed by transport of these intermediates to the mitochondria. The second pathway involves peroxisomal conversion of acetyl-CoA into acetylcarnitine, which is subsequently transported to the mitochondria. Using a selective screen, we have isolated several mutants that are specifically affected in the second pathway, the carnitine-dependent acetyl-CoA transport from the peroxisomes to the mitochondria, and assigned these CDAT mutants to three different complementation groups. The corresponding genes were identified using functional complementation of the mutants with a genomic DNA library. In addition to the previously reported carnitine acetyl-CoA transferase (CAT2), we identified the genes for the yeast orthologue of the human mitochondrial carnitine acylcarnitine translocase (YOR100C or CAC) and for a transport protein (AGP2) required for carnitine transport across the plasma membrane.**

**Keywords:** acetyl-CoA/ $\beta$ -oxidation/carnitine/fatty acids/peroxisomes

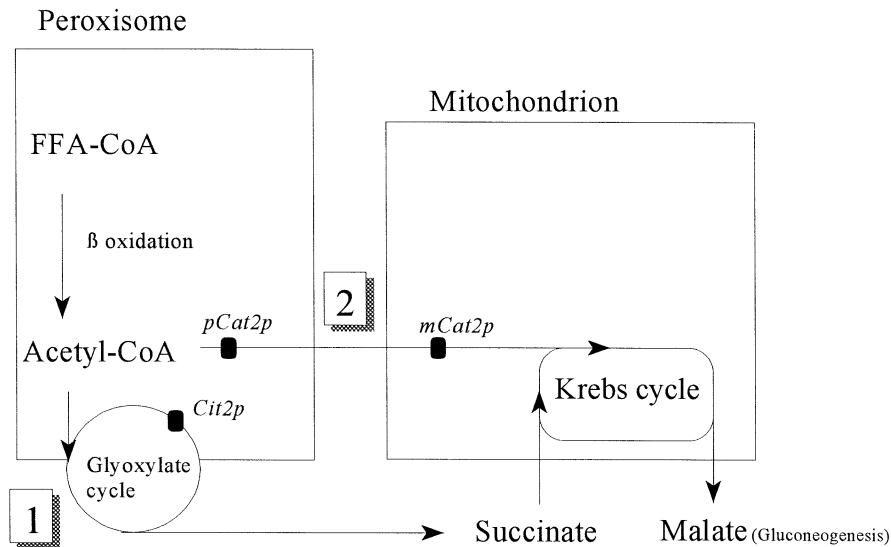
## Introduction

The  $\beta$ -oxidation of fatty acids in mammalian cells takes place in both mitochondria and peroxisomes. Long-chain fatty acids are oxidized primarily in mitochondria whereas very-long-chain fatty acids and certain branched-chain fatty acids are handled primarily by peroxisomes (Schulz, 1991; Seedorf *et al.*, 1994; Dieuaide-Noubhani *et al.*, 1996; Leenders *et al.*, 1996; Jiang *et al.*, 1997; Wanders *et al.*, 1997). The importance of peroxisomal fatty acid  $\beta$ -oxidation is emphasized by the existence of inherited diseases in man (e.g. X-linked adrenoleukodystrophy) that are caused by an impairment in peroxisomal  $\beta$ -oxidation (Wanders *et al.*, 1995).

It is generally accepted that mammalian fatty acid  $\beta$ -oxidation in peroxisomes is incomplete and only involves chain shortening of fatty acids to produce acetyl-CoA and/or propionyl-CoA plus medium-chain acyl-CoAs. These are then transported to the mitochondria as carnitine esters, where they are further oxidized to CO<sub>2</sub> and H<sub>2</sub>O (Bieber, 1988; Osmundsen *et al.*, 1991; Reddy and Mannaerts, 1994) as shown convincingly for pristanic acid (Verhoeven *et al.*, 1998).

In contrast to mammals, degradation of fatty acids in yeast takes place exclusively in peroxisomes (Kunau *et al.*, 1995). The acetyl-CoA produced has to be transported from the peroxisomes to the mitochondria for complete oxidation to CO<sub>2</sub> and H<sub>2</sub>O. Two pathways for the transport of acetyl units have been identified (van Roermund *et al.*, 1995). In the first, acetyl-CoA enters the peroxisomal glyoxylate cycle to produce succinate, which is subsequently transported to the mitochondria, probably via the putative dicarboxylate carrier, Acr1p (Palmieri *et al.*, 1997). The second pathway involves the intraperoxisomal conversion of acetyl-CoA into acetylcarnitine, which is catalysed by carnitine acetyltransferase (Cat2p). The peroxisomal and mitochondrial Cat2p of *Saccharomyces cerevisiae* are encoded by a single gene CAT2 (Elgersma *et al.*, 1995) and are responsible for >95% of the total carnitine acetyltransferase activity in oleate-grown yeast cells (Kispal *et al.*, 1993). The existence of two pathways for the transport of acetyl units from peroxisomes to mitochondria, which are acting in parallel, became clear with the finding that disruption of *either* the CIT2 gene, encoding the peroxisomal glyoxylate cycle enzyme citrate synthase (Cit2p), *or* the CAT2 gene did not affect growth of yeast on oleate, whereas a mutant with *both* genes disrupted ( $\Delta$ cit2/cat2) failed to grow on oleate, due to the inability to oxidize this fatty acid (van Roermund *et al.*, 1995).

Based on these findings, we developed a selective screen for the isolation of mutants that are specifically defective in the carnitine-dependent acetyl-unit transport from peroxisomes to mitochondria (CDAT mutants). In this paper we report the isolation and characterization of various mutants, which could be assigned to three distinct complementation groups. The gene mutated in the first complementation group is the CAT2 gene, which codes for both the peroxisomal and mitochondrial Cat2p proteins. The genes affected in the remaining two complementation groups were identified by functional complementation of the mutants. In complementation group 2, a previously uncharacterized gene was mutated, known as YOR100c. This gene encodes a member of the mitochondrial carrier family, which appears to be the orthologue of the human mitochondrial carnitine acylcarnitine translocase. In complementation group 3 the AGP2 gene was mutated.



**Fig. 1.** Schematic representation of the two pathways for the transport of acetyl units from peroxisomes to mitochondria in *S.cerevisiae*. Pathway 1, the glyoxylate cycle; pathway 2, the carnitine-dependent acetyl-CoA transport.

Although previously reported to be a general amino acid permease, we provide evidence that Agp2p is required for the uptake of L-carnitine from the medium into the yeast cell.

## Results

### **Isolation of CDAT mutants defective in the carnitine-dependent transport of acetyl-CoA from peroxisomes to mitochondria**

Oxidation of straight-chain fatty acids in yeast is confined to peroxisomes and generates acetyl-CoA as the end product. Our previous studies have shown that transport of acetyl-CoA from peroxisomes to mitochondria may proceed via two independent pathways (Figure 1) namely, the glyoxylate cycle (pathway 1) or the carnitine-dependent pathway (pathway 2). Fatty acid oxidation and growth on oleate, which requires  $\beta$ -oxidation, were completely normal if either of the two pathways were blocked. However, mutants in which *both* pathways were blocked showed deficient fatty acid oxidation and impaired growth on oleate, indicating that the two pathways function in parallel.

In order to identify the components required for the carnitine-dependent acetyl-CoA transport from peroxisomes to mitochondria, we first deleted the gene coding for Cit2p, the peroxisomal citrate synthase, thereby blocking the glyoxylate cycle. The resulting  $\Delta$ *cit2* mutant, which was still able to grow on oleate (Figure 1), was subsequently mutagenized by ethyl methyl sulfonate (EMS) treatment followed by the isolation of mutants that no longer grew on oleate (see Materials and methods). In principle, these mutants could be affected either in peroxisome biogenesis (*pex* mutants; Distel *et al.*, 1996) or fatty acid  $\beta$ -oxidation.

In order to distinguish between these two possibilities, a construct expressing green fluorescent protein (GFP) with the C-terminal peroxisomal targeting signal PTS1 was transformed into the  $\Delta$ *cit2* cells prior to mutagenesis.

Of the 99 mutants deficient for oleate growth, 21 appeared to have GFP-PTS1 mislocalized to the cyto-

**Table I.** Complementation analysis of CDAT mutants

CDAT mutants	CIT2	CAT2	YOR100C	AGP2
<i>CDAT-1</i>	++	-	++	-
<i>CDAT-2</i>	++	++	-	-
<i>CDAT-3</i>	++	-	-	++
<i>CDAT-4</i>	++	++	-	-
<i>CDAT-5</i>	++	-	++	-
<i>CDAT-6</i>	++	-	-	++
<i>CDAT-7</i>	++	-	++	-
<i>CDAT-8</i>	++	++	-	-
<i>CDAT-9</i>	++	-	++	-
<i>CDAT-10</i>	++	++	-	-
$\Delta$ <i>cit2/cat2</i>	++	++	-	-
$\Delta$ <i>cit2/cac</i>	++	-	++	-
$\Delta$ <i>cit2/agp2</i>	++	-	-	++
Wild-type	++	++	++	++

CDAT mutants transformed with the genes coding for citrate synthase (*CIT2*), carnitine acetyltransferase (*CAT2*), carnitine acylcarnitine translocase (*YOR100C*) or the amino acid permease (*AGP2*) were able (+) or unable (-) to grow on oleate-containing plates. As positive and negative controls wild-type cells and  $\Delta$ *cit2/cat2*,  $\Delta$ *cit2/cac* and  $\Delta$ *cit2/agp2* deletion mutants were used.

plasm. Since this is the expected phenotype for *pex* mutants, these mutants were excluded from further analysis.

To distinguish between mutants affected in  $\beta$ -oxidation and in carnitine-dependent transport, we transformed the remaining 78 mutants with the *CIT2* gene. Ten mutants regained the ability to grow on oleate plates, which made them candidates for mutants with a specific defect in the carnitine-dependent transport of acetyl-CoA from peroxisomes to mitochondria (Table I). This was also supported by the observation that these 10 mutants displayed impaired  $\beta$ -oxidation activities when measured in intact cells, whereas  $\beta$ -oxidation was normal in lysates (not shown).

### **Characterization of the CDAT mutants**

**Carnitine acetyltransferase (*CAT2*).** Earlier studies have established that Cat2p, which is located both in peroxi-

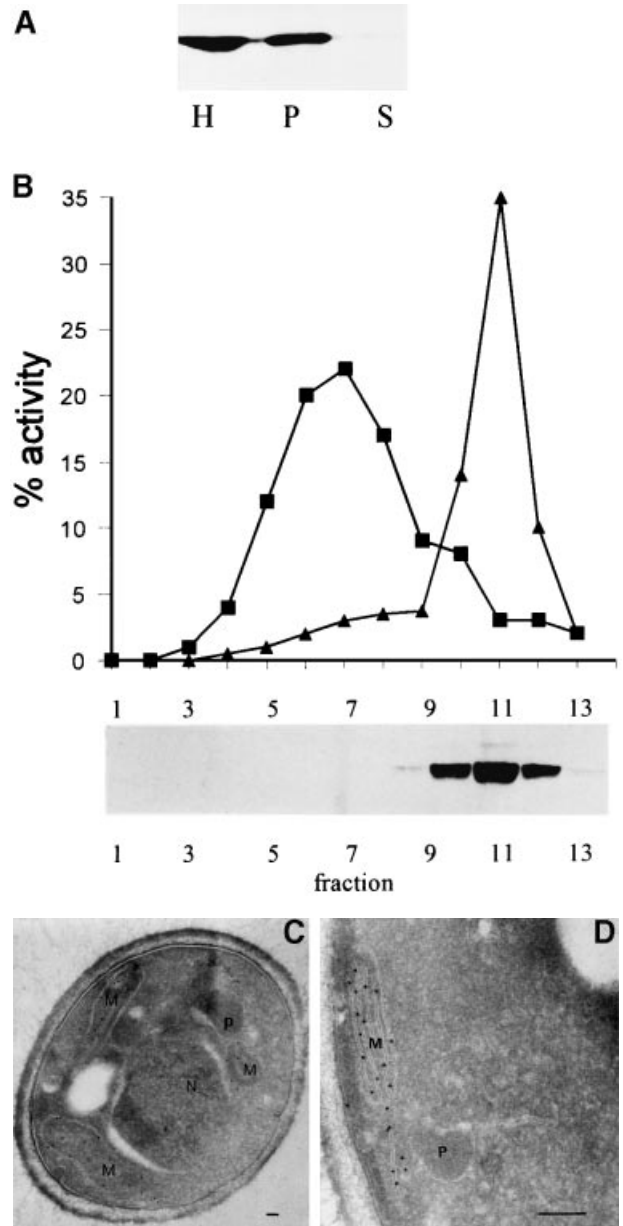
somes and mitochondria (Elgersma *et al.*, 1995), is involved in the transport of acetyl-CoA from the peroxisomal matrix to mitochondria. It was therefore anticipated that some of the CDAT mutants would be mutated in the *CAT2* gene. In order to identify these mutants, we transformed the 10 CDAT mutants with the wild-type *CAT2* gene (Table I) and found restoration of growth on oleate in four mutant strains (*CDAT-2*, *-4*, *-8* and *-10*).

**Cloning of *YOR100C*.** The genes affected in the remaining six mutants were identified by functional complementation, i.e. restoration of growth on oleate following transformation with a genomic DNA library. Four mutant strains (*CDAT-1*, *-5*, *-7* and *-9*) appeared to be affected in *YOR100C*, which has previously been reported to code for an oleate-inducible mitochondrial protein (Karpichev and Small, 1998). This gene was identified in the yeast genome sequencing project and predicted to encode a protein with six transmembrane segments and with strong sequence homology to members of the mitochondrial carrier family. Highest homology was observed with the gene products of the *Drosophila DMCOLT 2* (Y12495) gene (36%) and the *Caenorhabditis elegans DIF1* gene (36%), and with the human mitochondrial carnitine acylcarnitine translocase described by Indiveri *et al.* (1997) (CACT; 34%). The structural relationship between Hs-CACT and the gene product of *YOR100C* is also clear from the similarities in their hydrophobicity profiles (data not shown), suggesting that we identified the yeast orthologue of the human mitochondrial carnitine acylcarnitine translocase. To verify that all four mutants were affected in *YOR100C*, we sequenced the *YOR100C* open reading frames (ORFs) amplified by PCR from the mutants, and found distinct mutations (results not shown).

Inspection of the 5'-region of *YOR100C* revealed the presence of a putative oleate response element (ORE). These OREs are found in a number of oleate-inducible yeast genes including the genes coding for  $\beta$ -oxidation enzymes, indicating that the protein product of *YOR100C* plays a role in fatty acid  $\beta$ -oxidation.

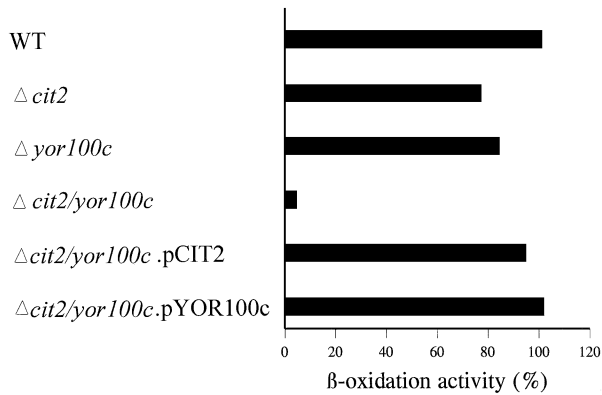
**Localization and characterization of Yor100cp**

To study the subcellular localization of Yor100cp in oleate-grown cells, we epitope-tagged the protein at its N-terminus with the NH-tag (see Materials and methods). This construct was able to complement the  $\Delta$ *cit2/yor100c* mutant indicating that the NH-tag does not interfere with the protein's function. After subcellular fractionation of the oleate-grown transformant, we found NH-Yor100cp to be present exclusively in the organellar pellet (Figure 2A). To separate the peroxisomes from the mitochondria, the organellar pellet was further fractionated by Nycodenz density gradient centrifugation. Immunoblot analysis of the gradient fractions using the NH-antibodies revealed that NH-Yor100cp co-localized with the mitochondrial marker indicating a localization in mitochondria (Figure 2B). This was confirmed by immunoelectron microscopy of oleate-induced cells expressing the NH-Yor100cp from a single copy plasmid. Figure 2C and D shows exclusive labelling of the mitochondrial inner membrane (cristae). This result indicates that the product of the *YOR100C* gene is a mitochondrial inner membrane protein.



**Fig. 2.** Identification of Yor100cp as a mitochondrial inner membrane protein in *S.cerevisiae*. (A) Biochemical fractionation of wild-type cells expressing NH-Yor100cp. Oleate-grown cells were fractionated by differential centrifugation of a homogenate (H) into a 17 000 g pellet (P) and supernatant (S). (B) The 17 000 g pellet (P) was further fractionated by Nycodenz equilibrium density gradient centrifugation (fractions 1–13). NH-Yor100cp was detected by immunoblot analysis. Mitochondrial and peroxisomal matrix markers are fumarase (FUM) and 3-hydroxyacyl-CoA dehydrogenase (3HAD), respectively. Fraction 1 is at the bottom of the gradient. (C) Immunogold electron micrograph showing association of NH-Yor100cp with the mitochondria. (D) Detail of immunogold electron micrograph showing association of NH-Yor100cp with the mitochondrial membrane (cristae). Cells were labelled with 10 nm gold particles. Scale bar, 0.2  $\mu$ m. Abbreviations: m, mitochondrion; p, peroxisome; n, nucleus.

In order to investigate the function of Yor100cp, we disrupted the *YOR100C* gene in wild-type cells and in the  $\Delta$ *cit2* mutant, by replacing *YOR100C* with the *KAN* gene (see Materials and methods). The resulting deletion mutant grew normally on glucose and glycerol. In contrast to the deletion mutants created from the wild-type strain, which grew normally, growth of  $\Delta$ *cit2/yor100c* cells on oleate



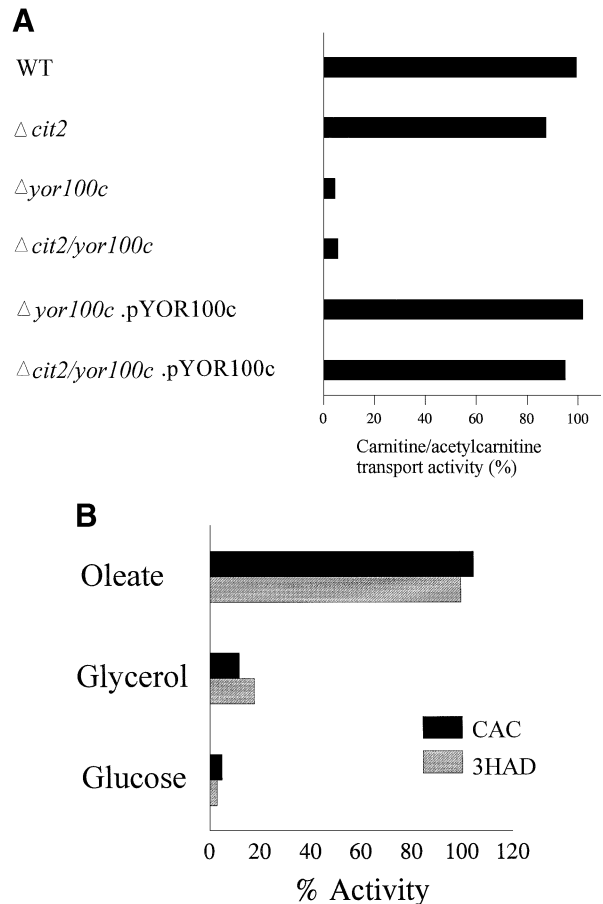
**Fig. 3.** β-oxidation of oleate is reduced in Δ*cit2*–*yor100c* cells. Wild-type and mutant cells grown on oleate were incubated with [1-<sup>14</sup>C]oleate and β-oxidation was measured (see Materials and methods). The β-oxidation rates in wild-type cells were taken as reference (100%) and are expressed as the sum of [1-<sup>14</sup>C]CO<sub>2</sub> and water-soluble β-oxidation products produced.

was strongly impaired. To establish whether Yor100cp functions as the yeast carnitine acylcarnitine translocase as predicted from its similarity to the human protein, we studied the oxidation of 1-<sup>14</sup>C-labelled oleate in wild-type cells and deletion mutants. As shown in Figure 3, oxidation of [1-<sup>14</sup>C]oleate was strongly impaired only in the Δ*cit2*–*yor100c* mutant but was normal in the other mutant strains. The ability to β-oxidize fatty acids could be restored by transformation with either the *YOR100C* or the *CIT2* gene, indicating that the β-oxidation defect of Δ*cit2/yor100c* cells on oleate is reversible.

If the block in β-oxidation of oleate in Δ*cit2/yor100c* mutants is indeed caused by a defect in the transport of acetyl-CoA, or better still acetylcarnitine (Figure 1), from the peroxisomes to mitochondria as a result of the absence of the gene products of *YOR100C* and *CIT2*, this would be reflected in a defective import of acetylcarnitine into the mitochondria. This was tested by incubation of spheroplasts prepared from oleate-grown wild-type, Δ*yor100c*, Δ*cit2* and Δ*cit2*–*yor100c* cells for 10 min with [1-<sup>14</sup>C]acetylcarnitine in the presence of low concentrations of digitonin. As shown in Figure 4A, the oxidation of [1-<sup>14</sup>C]acetylcarnitine to [1-<sup>14</sup>C]CO<sub>2</sub> was strongly impaired in the Δ*yor100c* and Δ*cit2/yor100c* mutants, whereas oxidation was normal in wild-type and Δ*cit2* cells. Oxidation was again normal in Δ*cit2/yor100c* or Δ*yor100c* cells that were transformed with the *YOR100C* gene.

Figure 4B shows the carnitine acetylcarnitine translocase activities measured in wild-type cells grown on glucose, glycerol and oleate. The results show that the activity was repressed by glucose, derepressed by glycerol and induced by oleate, which illustrates that expression of *YOR100C* is similar to that of the β-oxidation enzymes such as 3-hydroxyacyl-CoA dehydrogenase (3HAD), as already predicted from the presence of the ORE box in the *YOR100C* promoter.

Taken together, our experiments show that the gene product of *YOR100C* is a member of the mitochondrial carrier family, which is induced on oleate, involved in the transport of acetyl-CoA from peroxisomes to mitochondria and functions as a carnitine acylcarnitine translocase in the mitochondrial inner membrane of *S.cerevisiae*.



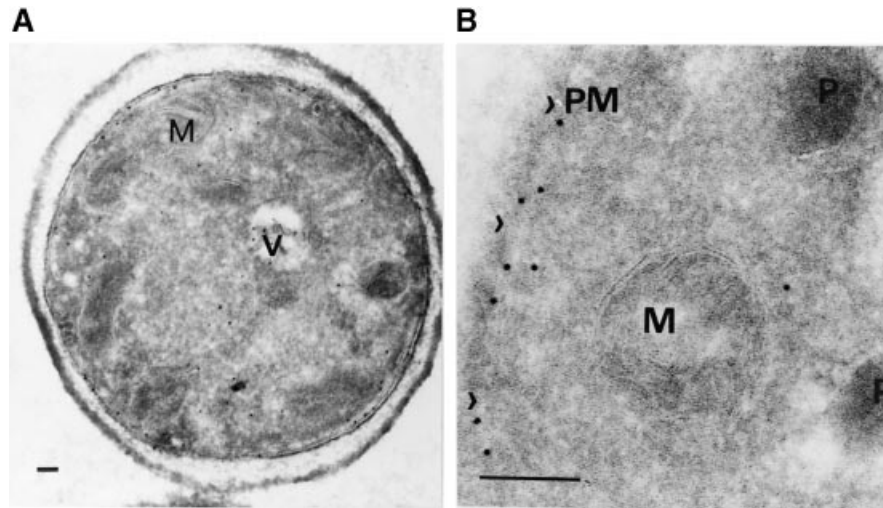
**Fig. 4.** (A) Carnitine acylcarnitine translocase activity is reduced in Δ*cit2*–*yor100c* and Δ*yor100c* cells. Wild-type cells and mutant cells grown on oleate were incubated with [1-<sup>14</sup>C]acetylcarnitine and the carnitine acylcarnitine translocase activity was measured by quantification of the amount of [1-<sup>14</sup>C]CO<sub>2</sub> produced (see Materials and methods). Rates are expressed relative to the rate in wild-type cells. (B) Carnitine acylcarnitine translocase activity is induced by oleate. Wild-type cells grown on glucose, glycerol and oleate were incubated with [1-<sup>14</sup>C]acetylcarnitine for 10 min and carnitine acylcarnitine translocase activity was measured. The rates in oleate-grown cells were taken as reference (100%).

#### Cloning of the *AGP2* gene, encoding a member of the amino acid permease family

Functional complementation of the remaining two mutant strains (*CDAT-3* and *CDAT-6*) identified the YBR132C ORF as the affected gene (Table I). YBR132C is identical to the previously reported *AGP2* (André, 1995), a gene that codes for a 596 amino acid protein with 12 potential transmembrane domains and which belongs to the family of amino acid permeases. Proteins belonging to this family are assumed to function as plasma membrane proton-symporters. Inspection of the 5'-region of the *AGP2* gene revealed the presence of a putative ORE (Karpichev and Small, 1998).

#### Localization and characterization of *Agp2p*

To verify that *Agp2p* is required for oleate growth, we made a gene deletion of *AGP2* in wild-type and Δ*cit2* cells. Growth of the resulting deletion mutants was unaffected on rich glucose, glycerol or oleate media, except for the Δ*cit2/agp2* double mutant, which showed strongly



**Fig. 5.** (A) Immunogold electron micrograph showing association of Agp2p-HA with the plasma membrane and ER membranes. (B) Detail of immunogold electron micrograph showing predominant association of Agp2p-HA with the plasma membrane, but not with peroxisomes and mitochondria. Cells were labelled with 10 nm gold particles. Scale bar, 0.2  $\mu$ m. Abbreviations: m, mitochondrion; p, peroxisome; n, nucleus; pm, plasma membrane.

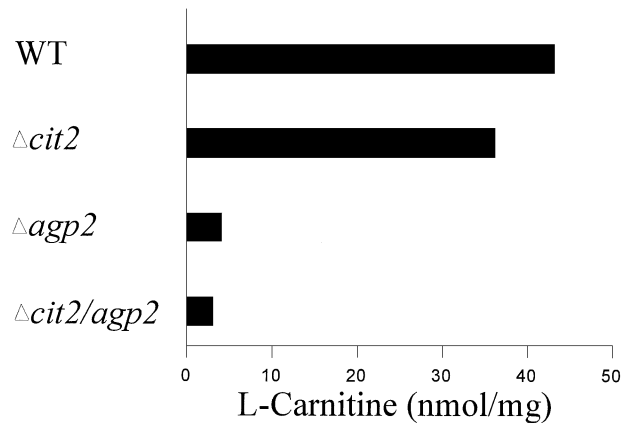
impaired growth on oleate, the same phenotype as observed for the original mutants.

To study the subcellular localization of the *AGP2* gene product, Agp2p, we introduced the HA-tag at the C-terminus of the protein. This did not affect the function of the protein as demonstrated by the fact that this construct functionally complemented the  $\Delta$ *cit2/agp2* double mutant. Using subcellular fractionation experiments, we studied the localization of Agp2p-HA in cells grown overnight in rich oleate medium. A total cellular extract was compared with homogenates prepared from spheroplasts by gentle osmotic lysis and fractionated by successive differential centrifugation steps into a 2500 g pellet (P1), a 17 000 g pellet (P2), a 100 000 g pellet (P3) and a supernatant fraction (S). All fractions were analysed for the presence of marker enzymes for various subcellular compartments, including mitochondria, peroxisomes, endoplasmic reticulum (ER) and plasma membrane. Agp2p-HA co-localized with all fractions and thus behaved differently to one of the marker enzymes (data not shown), suggesting that the protein has different locations inside the cell. This is in line with the observations of Ljungdahl *et al.* (1992), who indicated that Agp2p is localized in the plasma membrane and the ER.

To confirm the intracellular localization of Agp2p, we performed immunoelectron microscopy of oleate-induced cells expressing Agp2p-HA from a single copy plasmid. Figure 5 shows prominent labelling of the plasma membrane in addition to labelling of ER membranes and vacuoles in oleate-induced yeast cells.

#### **Agp2p functions as a carnitine transporter**

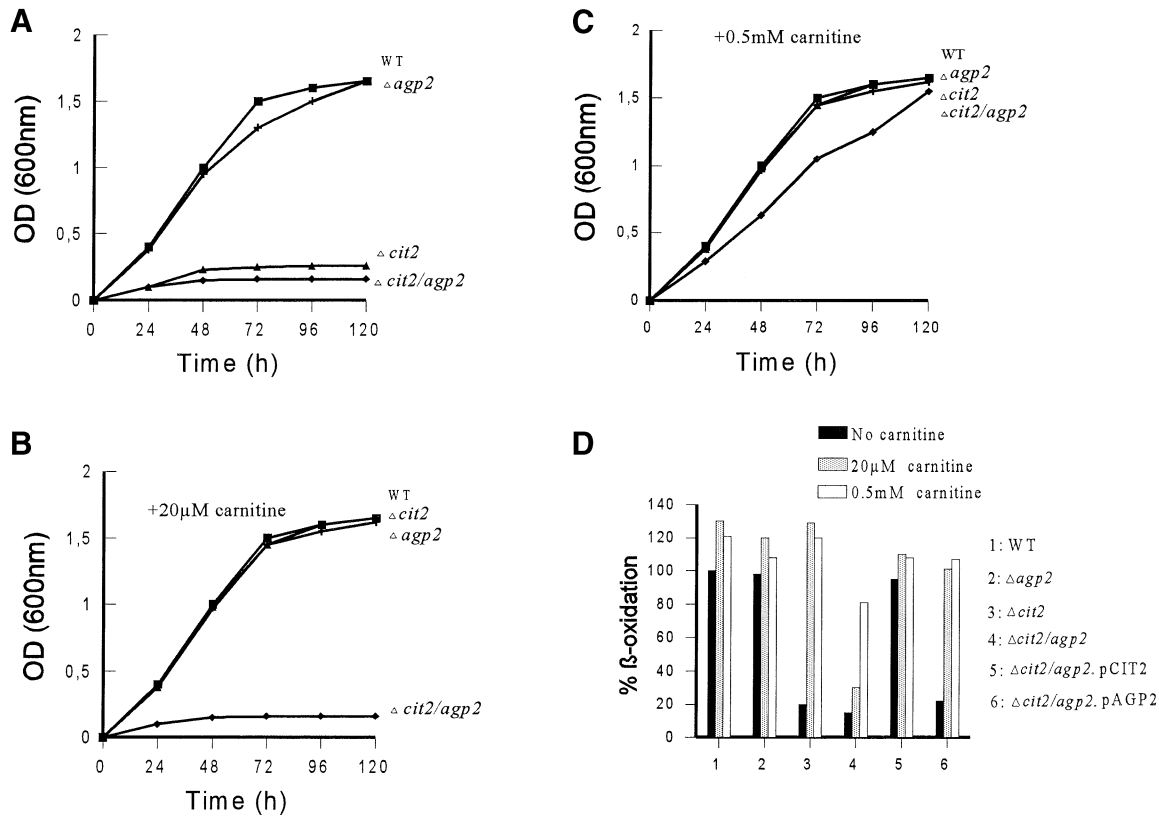
In order to investigate the role of Agp2p in the carnitine-dependent acetyl-CoA transport from peroxisomes to mitochondria, we measured the total intracellular carnitine levels in  $\Delta$ *agp2* and  $\Delta$ *cit2/agp2* cells (Figure 6) and found a profound decrease. Based on the prediction that Agp2p functions as a transporter in the plasma membrane, this observation strongly suggested that Agp2p is required for the uptake of carnitine from the medium. This would imply that the wild-type *S.cerevisiae* strain used for this



**Fig. 6.** Total intracellular carnitine levels are decreased in  $\Delta$ *agp2* cells. The intracellular carnitine levels were measured in wild-type and mutant cells grown on oleate-containing medium (see Materials and methods).

study is not capable of synthesizing L-carnitine. Indeed, when we tested the  $\Delta$ *cit2* strain on minimal oleate medium, we only observed growth after the addition of 20  $\mu$ M L-carnitine (Figure 7B) but not in the absence of carnitine (Figure 7A), indicating that this strain is not capable of *de novo* carnitine synthesis. Growing these cells in the presence of high concentrations of L-carnitine (Figure 7C) could compensate for the defect in oleate growth introduced by the deletion of *AGP2* in  $\Delta$ *cit2* cells. In agreement with these results, incubating the cells with high concentrations of L-carnitine (Figure 7D) could also restore the impaired oxidation of oleate in  $\Delta$ *cit2/agp2* cells.

To provide direct evidence for the function of Agp2p as a carnitine transporter, we measured the uptake of L-[1-<sup>14</sup>C]carnitine in wild-type and  $\Delta$ *agp2* cells transformed with or without *AGP2*. Uptake of L-[1-<sup>14</sup>C]carnitine was blocked completely in  $\Delta$ *agp2* cells and increased significantly after transformation with the *AGP2* gene (Figure 8A). Incubation of whole cells with 10  $\mu$ M of the SH-reagent *N*-ethylmaleimide (NEM) led to a complete block of carnitine uptake (Figure 8A). At these low concentrations,



**Fig. 7.** (A–C) Growth of wild-type cells and mutant cells on minimal oleate medium containing no (A), 20 μM (B) or 500 μM L-carnitine (C). The following symbols were used: (■) wild-type cells, (+)  $\Delta agp2$  cells, (▲)  $\Delta cit2$  cells, (◆)  $\Delta cit2/agp2$ . (D) Oleate β-oxidation is reduced in  $\Delta cit2/agp2$  cells. Wild-type and mutant cells grown on oleate were incubated with [ $^{14}$ C] oleate without carnitine or with 20 μM or 500 μM carnitine and β-oxidation were measured as described in Materials and Methods. The β-oxidation rates in wild-type cells were taken as reference (100%) and are expressed as the sum of [ $^{14}$ C] CO<sub>2</sub> and water-soluble β-oxidation products produced.

NEM is practically membrane impermeable, which confirms that Agp2p is functionally active in the plasma membrane of *S.cerevisiae*.

The uptake of L-[ $^{14}$ C]carnitine was particularly high in cells at pH 5.0 (Figure 8B), which suggests that Agp2p functions as a carnitine-H<sup>+</sup> symporter. Carnitine uptake was not sodium dependent since replacement of sodium with potassium did not affect the uptake (Figure 8C).

The concentration dependence of L-carnitine transport was examined to estimate the half-saturation concentration ( $K_m$ ) of Agp2p-mediated L-carnitine transport. The half-saturation concentration and the maximum transport activity ( $V_{max}$ ) were estimated to be 5 μM and 21.2 nmol/mg/10 min, respectively (Figure 8D).

The specificity of Agp2p-mediated L-carnitine transport was examined in terms of the inhibitory effect on the initial uptake of L-[ $^{14}$ C]carnitine. As is evident from Figure 8C, structurally analogous compounds including D-carnitine and acetylcarnitine reduced L-carnitine uptake at 10 μM. Accordingly, the structural requirement of Agp2p-mediated L-carnitine transport is rather strict. Amino acids including lysine, serine, leucine and threonine did not affect the uptake of L-carnitine.

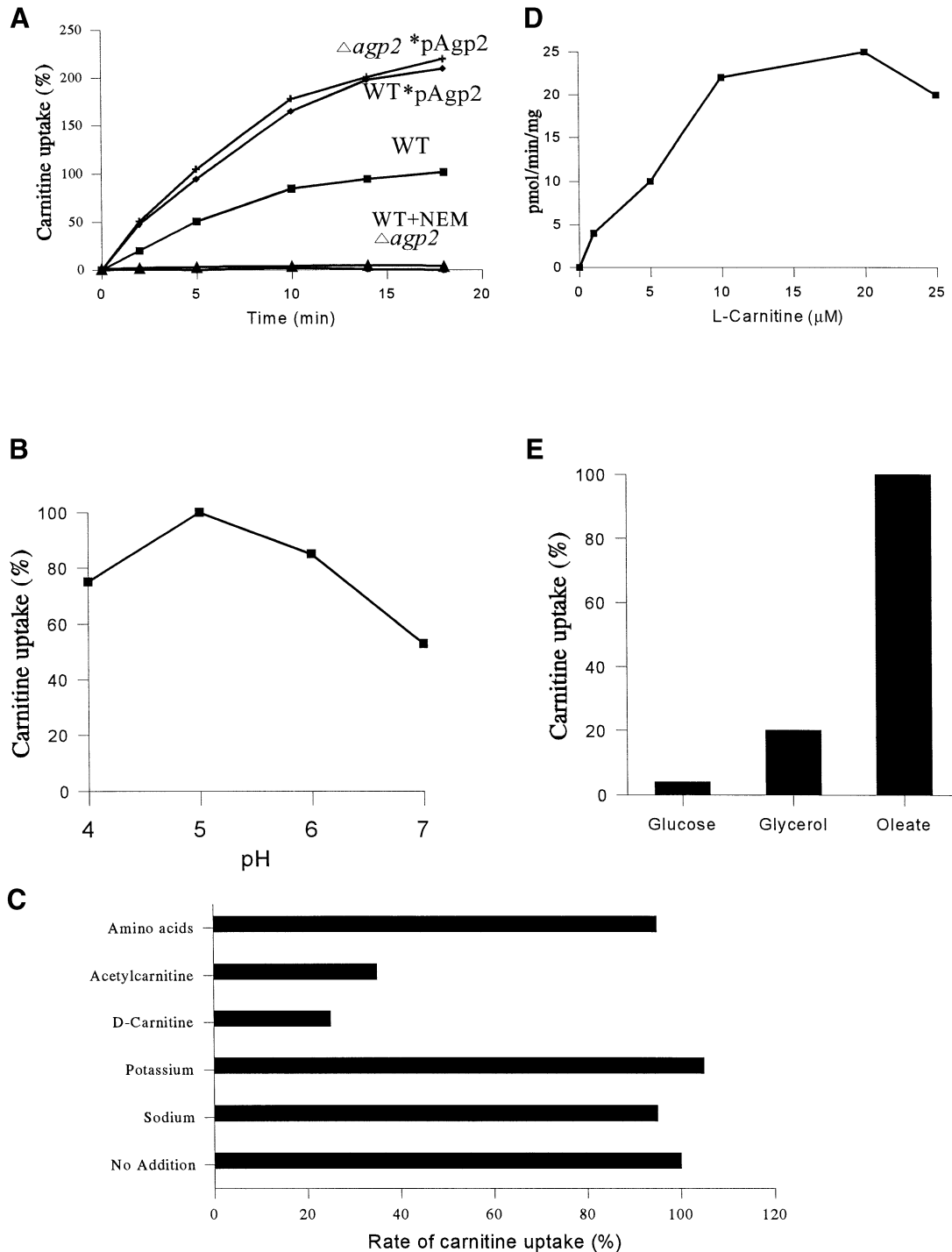
Figure 8E shows the uptake of L-carnitine in wild-type cells grown on glucose, glycerol and oleate. The results show that L-carnitine uptake was repressed by glucose, derepressed by glycerol and induced by oleate, which indicates that the expression of Agp2p is similar to that of β-oxidation enzymes. Although this was also predicted

from the presence of the ORE box in the *AGP2* promoter, it cannot be excluded at this point that the activity of Agp2p is also regulated at the protein level. Taken together, these data indicate that L-carnitine uptake is functionally linked to fatty acid oxidation.

## Discussion

Peroxisomes are the exclusive site of fatty acid β-oxidation in *S.cerevisiae*. Since the peroxisomal membrane is impermeable to small molecules, the question arises as to how the acetyl-CoA produced in peroxisomes is transported to the mitochondria for final oxidation to CO<sub>2</sub> and H<sub>2</sub>O. Our earlier studies (Elgersma *et al.*, 1995; van Roermund *et al.*, 1995) have shown that there are only two ways in which acetyl-CoA can leave the peroxisome (Figure 1). It was the purpose of this study to resolve the structure of pathway 2, which catalyses the carnitine-dependent transport of acetyl-CoA from peroxisomes to mitochondria. To this end, a negative selection screen was developed based on mutagenesis of the  $\Delta cit2$  mutant in which pathway 1 is blocked (Figure 1). The 10 CDAT mutants identified in this screen were found to represent three different genes. The first gene was *CAT2*, which codes for both the peroxisomal and mitochondrial carnitine acetyltransferase activity in oleate-grown cells (Elgersma *et al.*, 1995).

In addition to the four *cat2* mutants, four mutants were affected in the *YOR100C* gene, which we demonstrated



**Fig. 8.** Carnitine uptake is reduced in  $\Delta agp2$  cells. (A) Wild-type and mutant cells grown on oleate were incubated with L-[1- $^{14}$ C]-carnitine and L-carnitine uptake was measured (see Materials and methods). The uptake rates in wild-type cells were taken as reference (100%). The following symbols were used: (■) wild-type cells, (▲)  $\Delta agp2$  cells, (+)  $\Delta agp2.pAGP2$  cells, (◆) wild-type.pAGP2 cells and (○) wild-type cells incubated with 10  $\mu$ M NEM. (B) pH optimum of L-carnitine uptake in oleate-induced wild-type cells. (C) D-carnitine and acetylcarnitine reduced the L-carnitine uptake in oleate-induced wild-type cells. Amino acids including lysine, serine, leucine and threonine did not affect the uptake. The uptake rates in wild-type cells were taken as reference (100%). Cells were incubated for 10 min with L-[1- $^{14}$ C]-carnitine. (D) Concentration dependence of L-carnitine transport in oleate-induced wild-type cells. (E) L-carnitine uptake is induced by oleate. Wild-type cells grown on glucose, glycerol and oleate were incubated with L-[1- $^{14}$ C]-carnitine for 10 min and L-carnitine uptake was measured. The uptake rates in wild-type cells were taken as reference (100%).

to code for the carnitine acylcarnitine translocase, a member of the mitochondrial carrier family. This carrier catalyses the exchange between acylcarnitine and free carnitine and is present in the mitochondrial inner membrane of many eukaryotic species. That the *YOR100C*

gene indeed codes for the functional orthologue of the human CACT has recently been confirmed by the functional complementation of the  $\Delta cit2/yor100c$  mutant with the human CACT cDNA (unpublished results). Following the rules for yeast gene nomenclature, we therefore propose

to rename the *YOR100C* gene into *CAC* (carnitine acylcarnitine carrier) and the encoded protein Caccp.

Although we also predicted the existence of a similar carrier involved in the export of acetylcarnitine in the peroxisomal membrane, so far we have not identified candidate mutants. Several possibilities can be put forward to explain this. First, mutants affected in a putative peroxisomal carnitine acylcarnitine translocase may not be able to grow on oleate after transformation with the *CIT2* gene. Secondly, the peroxisomal membrane may be permeable to acetylcarnitine. Thirdly, the *YOR100C* (*CAC*) gene may code for both the mitochondrial and peroxisomal carnitine acylcarnitine translocase, analogous to the carnitine acetyltransferase (*CAT2*) gene (although the results of our localization experiments using a tagged version of Yor100cp do not support this). Finally, our selection screen may not be saturated or may fail to identify these mutants for other, unknown, reasons (e.g. functional redundancy of carriers capable of acetylcarnitine export).

The third gene identified in our screen is ORF YBR132C (*AGP2*), which codes for Agp2p, a protein of 596 amino acids. Agp2p contains 12 potential transmembrane domains and is related to Put4p, Alp1p, Lyp1p, Can1p and Gap1p, which are all members of the family of amino acid permeases. Based on sequence similarity, Nelisson *et al.* (1997) and André (1995) described Agp2p as one of the 18 members of this family with an unknown function. Members of this family are initially inserted into the ER membrane (Green *et al.*, 1989; Green and Walter *et al.*, 1992) and subsequently translocated to the plasma membrane via the yeast secretory pathway. Degradation of such carriers occurs after uptake in the vacuole. Immunogold electron microscopy studies confirmed that Agp2p is located primarily in the plasma membrane, but also in the ER and the vacuole, which is in agreement with the findings of Ljungdahl *et al.* (1992).

Furthermore, our results show that Agp2p is induced by growth on oleate, regulates the carnitine level inside the cells and is essential for oleate growth only when the peroxisomal glyoxylate cycle is inactive as a result of the disruption of *CIT2* (Figure 1). This indicates that intracellular carnitine is required for the export of acetyl-CoA to mitochondria by allowing the intraperoxisomal conversion of acetyl-CoA to acetylcarnitine. Interestingly, blocking of the glyoxylate cycle activity in wild-type cells results in an oleate-growth-deficient phenotype only during growth on minimal oleate (without carnitine), whereas there is normal growth if L-carnitine (20  $\mu$ M) is added (Figure 7B) or on rich oleate medium that already includes ~20  $\mu$ M carnitine, suggesting that these cells are incapable of *de novo* synthesis of carnitine.

Primary carnitine deficiency in man is caused by a deficiency of the active transport of carnitine across the plasma membrane, which is catalysed by a Na<sup>+</sup>/carnitine transporter (Tein *et al.*, 1996). Recently, Tamai *et al.* (1998) cloned the human organic cation transporter, OCTN2, which is a sodium-ion-dependent, high-affinity carnitine carrier, and demonstrated that mutations in its gene are responsible for primary carnitine deficiency.

Most adult tissues, including skeletal muscle, kidney, placenta and heart, show high expression of OCTN2 and have been reported to take up carnitine via a sodium

ion-dependent, carrier-mediated transport mechanism (Rebouche, 1977; Molstad *et al.*, 1978; Vary and Neely, 1982; Bremer, 1983; Stieger *et al.*, 1995; Prasad *et al.*, 1996; Tein *et al.*, 1996). Tissues that have apparently low-affinity carnitine transporters, such as liver, brain and intestine, showed low expression of OCTN2 (Tamai *et al.*, 1998). Surprisingly, the carnitine transporter we identified did not show any sequence similarity to the human OCTN2 or to any human proteins currently present in the databases. Furthermore, in contrast to OCTN2, the carnitine transport by Agp2p is Na<sup>+</sup> independent.

In conclusion, we identified three genes that are involved in the carnitine-dependent acetyl-CoA transport from peroxisomes to mitochondria in *S.cerevisiae*: *CAT2*, *YOR100C* (*CAC*) and *AGP2*. The identification of these three proteins contributes to a better understanding of the communication between peroxisomes and mitochondria, and sheds new light on the physiological and biochemical functions of carnitine.

## Materials and methods

### Yeast strains and culture conditions

The wild-type strain used in this study was *S.cerevisiae* BJ1991 (Mat  $\alpha$ , *leu2*, *trp1*, *ura3-251*, *prb1-1122*, *pep4-3*, *gal2*). The *Δfox1* and *Δcit2* mutants have been described previously (Voorn-Brouwer *et al.*, 1993; Elgersma *et al.*, 1995). Yeast transformants were selected and grown on minimal medium containing 0.67% yeast nitrogen base without amino acids (YNB-WO; Difco), supplemented with 0.3% glucose and amino acids (20  $\mu$ g/ml) as needed. Liquid rich media used to grow cells for DNA isolation, growth curves, subcellular fractionation,  $\beta$ -oxidation assays, immunogold electron microscopy and enzyme assays were composed of 0.5% potassium phosphate buffer pH 6.0, 0.3% yeast extract, 0.5% peptone and either 3% glycerol or 0.12% oleate/0.2% Tween-40. Before shifting to these media, the cells were grown on minimal 0.3% glucose medium for at least 24 h. Minimal oleate medium contains YNB-WO supplemented with all amino acids and 0.12% oleate/0.2% Tween-40.

### Mutant selection

*Δcit2* cells were transformed with a GFP-PTS1 expression construct (Hettema *et al.*, 1998) and grown on 0.3% glucose medium. Aliquots containing approximately  $5 \times 10^8$  cells were treated with 1.5–3% EMS as described by Lawrence (1991). The EMS treatment was stopped by adding 10% (w/v) sodium thiosulfate. Survival in independent experiments varied between 44 and 68%. After washing, the EMS-treated cells were allowed to recover by growing them for 4 h in 10 ml of minimal medium containing 2% glucose and the appropriate amino acids (20  $\mu$ g/ml). Following mutagenesis, cells were grown on plates containing 2% glucose and subsequently replica-plated onto glycerol and oleate plates. Using this method 99 mutants were selected that were deficient in oleate growth but capable of glycerol growth.

### Cloning, sequencing and disruption of the *YOR100C* and *AGP2* genes

The impaired growth of *CDAT-1* and *CDAT-3* cells on oleate plates was used for cloning of the *YOR100c* and the *AGP2* genes, respectively, by functional complementation with an *S.cerevisiae* genomic library. The transformants were selected on plates containing glucose and subsequently replica-plated onto glycerol or oleate plates. Different plasmids were selected for further characterization (*pCDAT1.1*, *pCDAT1.2*, *pCDAT1.3* or *pCDAT3.1*, *pCDAT3.2*, *pCDAT3.3*). Complementing plasmids were rescued in *Escherichia coli* and retransformed to *CDAT-1* and *CDAT-3* cells to confirm linked complementation. The genomic inserts of the complementing plasmids were sequenced by the dideoxy-chain-termination method. The obtained nucleotide and predicted amino acid sequences were compared with the *S.cerevisiae* Genome Database, which led to the identification of *YOR100C* and *YBR132c* (*AGP2*) as the genes involved.

To construct *Δyor100c* and *Δagp2* deletion mutants, the entire *YOR100C* or *AGP2* ORF was replaced by the *kanMX4* marker gene



(Wach, 1996). The PCR-derived construct for disruption comprised of the *kanMX4* gene flanked by short regions of homology (50 bp) corresponding to the *YOR100C* and *AGP2* 3' and 5'-noncoding region. pKan was used as template with the *YOR100C* primers (5'-GTA-TAATTCCTTTAGTCGAAATAGATATATTTCAAGCGCATATATA-GGCCGTACGCTGCAGTGCAG) and (5'-ACTCCATGCGTTACA-AATATGAACGCTTCGACAACAACGCCAAGGAAACATCGATGA-ATTCGAGCTCG) and the *AGP2* primers (5'-TTCAGGAGTAAGGGT-AGTGTAGTTACACCATACTGGTATTGATATTATCCGTACGCTGCAGTGCAG) and (5'-TCTGACAATAAATTTGGAGGCAGTCA-ATGTAATTTGTGAATATAACGACATCGATGAATTCGAGCTCG). The resulting PCR fragments were introduced into *S.cerevisiae* wild-type BJ1991 cells and *Δcit2* mutant cells. G418-resistant clones were selected by growth on YPD plates containing 200 mg/l G418 (Wach, 1996).

**Subcellular fractionation and Nycodenz gradients**

Subcellular fractionation was performed as described by Van der Leij *et al.* (1992). Organelle pellets were layered on top of 15–35% Nycodenz gradients (12 ml), with a cushion of 1.0 ml of 50% Nycodenz solutions containing 5 mM MES pH 6.0, 1 mM EDTA, 1 mM KCl and 8.5% sucrose. The sealed tubes were centrifuged for 2.5 h in a vertical rotor (MSE 8 × 35) at 19 000 r.p.m. at 4°C. Gradients were analysed for enzyme activity of various marker enzymes as described below.

In addition, 150 µl of each fraction from the Nycodenz gradient was used for precipitation in a 2 ml Eppendorf tube together with 1350 µl of 11% (w/v) trichloroacetic acid (TCA). After being left overnight at 4°C, samples were centrifuged for 15 min at 12 000 r.p.m. at 4°C. The pellet obtained was resuspended in 100 µl Laemmli sample buffer and used for SDS-PAGE analysis.

**Preparation of extracts**

Cells were harvested, washed twice in water and extracts were prepared in a buffer containing 200 mM Tris-HCl pH 8.0, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT) and 10% glycerol (v/v) by disrupting the cells with glass beads on a vortex. Cell debris was removed by centrifugation for 1 min at 13 000 r.p.m. in an Eppendorf centrifuge.

**Western blotting**

Proteins were separated on 12% SDS-polyacrylamide gels and transferred onto nitrocellulose filters in transfer buffer (25 mM Tris, 190 mM glycine, 20% methanol). The blots were blocked by incubation in phosphate-buffered saline (PBS), with 1% bovine serum albumin (BSA). The same buffer was used for incubation with the primary antibodies and with IgG-coupled alkaline phosphatase. The blots were stained in buffer composed of 100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM MgCl<sub>2</sub> plus BCIP and NBT following the manufacturer's instructions (Boehringer Mannheim).

**Electron microscopy**

Oleate-induced cells were fixed with 2% paraformaldehyde (w/v) and 0.5% glutaraldehyde (w/v). Ultra-thin sections were prepared as described by Gould *et al.* (1990).

**NH- and HA-epitope tagging and antibodies**

For epitope tagging of proteins two different epitopes were used. The first was the NH-epitope with the sequence MQDLPNDNSTAGGS, which corresponds to the N-terminus of the mature haemagglutinin protein and is recognized by a polyclonal antiserum. To introduce the NH-tag, an oligonucleotide adaptor encoding the NH-epitope was ligated into the *Sac1-BamHI* site of the single-copy catalase A (CTA1) expression plasmid as described by Elgersma *et al.* (1996).

The second tag was the HA-epitope with the sequence YDVPDY-ASLKGE\*, which is recognized by the monoclonal antibody 12CA5. To introduce the HA-epitope tag at the C-terminus of proteins, an oligonucleotide adaptor encoding the HA-epitope was ligated into the *Pst1-HindIII* site of the single-copy catalase A (CTA1) expression plasmid as described by Elgersma *et al.* (1996).

**Total carnitine measurements**

Twenty millilitres of oleate-grown cells (OD = 1.5) were washed twice and disrupted by vigorously vortexing for 30 min at 4°C with ~200 µl glass beads in an end volume of 400 µl. Cell debris (75 µl) was mixed and subsequently deproteinized with 500 µl acetonitrile and centrifuged (12 000 r.p.m., 15 min). The resulting supernatant was dried under nitrogen at 45°C and subsequently derivatized in 100 µl butanol-HCl

for 15 min at 60°C. Samples were dried under nitrogen at 45°C and redissolved in 140 µl acetonitril. Free carnitine was measured as described by Vreken *et al.* (1999).

**Enzyme assays**

β-oxidation assays in intact cells were performed as described previously by Van Roermund *et al.* (1998). The carnitine acylcarnitine translocase activity was measured in spheroplasts prepared from wild-type or mutant cells grown on oleate. Activity measurements were performed in a medium containing 1.2 M sorbitol, 50 mM KP<sub>1</sub> pH 7.5, 1 mM EDTA, 200 000 d.p.m. [1-<sup>14</sup>C] acetylcarnitine (5 µM) and digitonized (20 µg/ml) spheroplasts (100 µg protein). This digitonine concentration selectively permeabilizes the plasma membrane, as demonstrated by complete release of the cytosolic marker enzyme phosphoglucose isomerase (PGI), whereas intracellular membranes of mitochondria and peroxisomes remain intact (Verleur *et al.*, 1997). Reactions were allowed to proceed for 10 min at 28°C, and subsequently stopped by the addition of 100 µl 1.3 M perchloric acid. Radiolabelled CO<sub>2</sub> was trapped overnight in 500 µl of 2 M NaOH.

3-hydroxyacyl-CoA dehydrogenase activity was measured on a Cobas-Fara centrifugal analyser by monitoring the acetoacetyl-CoA-dependent rate of NADH consumption at 340 nm (Wanders *et al.*, 1992). Fumarase activity was measured on a Cobas-Fara centrifugal analyser by monitoring APADH production at 365 nm. The reaction was started with 10 mM fumarate in an incubation mixture of 100 mM Tris pH 9.0, 0.1% Triton-X-100, 4 U/ml malate dehydrogenase (Boehringer) and 1 mM APAD for 5 min at 37°C. Protein concentrations were determined by the bicinchoninic acid method described by Smith (1985).

**Acknowledgements**

We are grateful to Dr H.Waterham for critical reading of the manuscript and participation in the final stages of this work, to L.IJlst for stimulating discussions, to Dr Y.Elgersma for providing the genomic DNA library, to G.E.Mochtar for technical assistance and to Dr P.van der Sluijs for the NH-epitope and NH-antiserum.

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Received May 12, 1999; revised September 10, 1999;  
accepted September 13, 1999