Compartmentalization of metabolic pathways in Candida albicans : a matter of transport
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Chapter 3

The role of *Candida albicans* peroxisomal and mitochondrial carnitine acetyl-transferases in intracellular acetyl unit transport

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Abstract

Carnitine acetyl-transferase (Cat2) is essential for growth on non-fermentable carbon sources like fatty acids, ethanol and acetate in the yeast *Candida albicans*. The function of Cat2 is to reversibly link acetyl units to carnitine to enable transport over the peroxisomal or mitochondrial membranes. Growth on fatty acids requires acetyl unit transport from peroxisomes to mitochondria where it enters the tricarboxylic (TCA) cycle, while growth on acetate or ethanol generates cytosolic acetyl-CoA that has to be transported to mitochondria for breakdown in the TCA cycle and to peroxisomes to generate C4 compounds in the glyoxylate cycle. We have shown previously that the *CAT2* gene encodes the peroxisomal and mitochondrial isozymes and that a *cat2* knockout strain looses almost all Cat activity (Chapter 2). Here we describe the phenotypes of strains exclusively expressing either the peroxisomal (perCAT2) or mitochondrial Cat2 (mitCAT2). The perCAT2 strain is, like the *cat2* knockout, unable to grow on oleate, acetate, ethanol and citrate and shows reduced growth on lactate indicating that mitochondrial Cat2 is indispensable during growth on these non-fermentable carbon sources. The mitCAT2 strain that lacks a peroxisomal Cat2 is still able to grow on acetate, ethanol and citrate, but cannot grow on fatty acids, despite its very high β-oxidation activity. How this strain is able to shuttle acetyl units from peroxisomes to mitochondria is currently unclear, but we discuss possible alternative, carnitine-independent, pathways. The accumulation of (iso)citrate in the mitCAT2 strain suggests a transport defect of this metabolite from mitochondria to peroxisomes, a phenotype that might be correlated with the aberrant peroxisomal morphology in the mitCAT2 strain.
Introduction

Compartmentalization is one of the main characteristics of eukaryotic cells and separation of metabolic pathways to different organelles is thought to convey an advantage over the uni-compartmental system of bacteria. However, the consequence of compartmentalization is that the various pathways at the different locations must be connected through the transport of metabolites over the organellar membranes. Acetyl-CoA is a central metabolite that is the product and substrate of many pathways that partake in central carbon metabolism. During growth on glucose, acetyl-CoA is produced in the mitochondria where it can directly enter the tricarboxylic acid (TCA) cycle to be oxidized to CO₂ and H₂O (18). However, during growth on other carbon sources like fatty acids, ethanol or acetate, acetyl-CoA is produced in different locations in the cell requiring shuttling of acetyl-CoA between compartments. Utilization of ethanol or acetate as sole carbon source results in cytosolic production of acetyl-CoA, while during growth fatty acids acetyl-CoA is produced in peroxisomes, the sole site of β-oxidation of fatty acids in most yeasts. Acetyl-CoA cannot cross the organelar membranes without the aid of the carrier molecule carnitine (34). Acetyl units are reversibly bound to carnitine by carnitine acetyl-transferases (Cat) forming acetyl-carnitine, which can be transported over the membrane. On the other side of the membrane the reverse reaction catalyzed by Cat takes place, converting acetyl-carnitine to acetyl-CoA and carnitine. We have shown previously that for acetyl-CoA transport the opportunistic fungal pathogen Candida albicans is strictly dependent on the carrier molecule carnitine and the activity of the major carnitine acetyl-transferase (Cat2) while growing on oleic acid, ethanol or acetate (Chapters 2 and 4). This is in contrast with Saccharomyces cerevisiae, which has two parallel pathways for acetyl-CoA transport depending either on Cat2 or on peroxisomal citrate synthase (Cit2). Moreover this fungus cannot synthesize carnitine (34, 35).

The major carnitine acetyl transferase, Cat2, of both C. albicans and S. cerevisiae is encoded by a single gene of which the translation products are dually localized to mitochondria and peroxisomes (Chapter 2 and 7). Elgersma et al. showed that S. cerevisiae CAT2 has two in frame start codons and that transcription initiation is dependent on carbon source. The longer transcript contains a mitochondrial targeting signal (MTS) and its translation product is targeted to mitochondria. The shorter transcript does not contain the MTS and encodes the peroxisomal Cat that is targeted to peroxisomes via its C-terminal peroxisomal targeting signal (PTS1). However, in yeast-two-hybrid experiments it was shown that the S. cerevisiae Cat2 has a PTS1-dependent and -independent interaction with the PTS1-receptor Pex5 and that the enzyme can be localized to peroxisomes via an internal targeting signal (7, 17). Expression of the peroxisomal Cat2 is carbon source dependent: the shorter peroxisomal Cat2 transcript is only detectable in oleate-grown but not in glycerol- or acetate-grown cells (7).

It is thought that the peroxisomal Cat2 is involved in export of acetyl-CoA produced during β-oxidation and that the main function of the mitochondrial Cat2 is to release acetyl-CoA from acetyl-carnitine in the mitochondria. Growth on fatty acids or C2 carbon sources like ethanol and acetate also requires a functional glyoxylate cycle. This
microorganism-specific shunt bypasses the decarboxylation steps of the TCA cycle thereby enabling gluconeogenesis by linking acetyl-CoA (C2) to glyoxylate (C2) forming malate (C4), a reaction catalyzed by malate synthase (Mls1). The glyoxylate cycle, therefore, is the second pathway besides the TCA cycle that is dependent on acetyl-CoA supply. Since Mls1 and the second key enzyme of the glyoxylate cycle, isocitrate lyase (Icl1), are peroxisomal in \textit{C. albicans} (26), growth on C2 carbon sources requires import of acetyl-CoA into peroxisomes. Whether this import is carnitine-dependent is not known. Interestingly, the localization of Mls1 in \textit{S. cerevisiae} is carbon source dependent: the enzyme is cytosolic when cells are growing on ethanol or acetate, but peroxisomal when grown on oleate (11).

Enzymes that might play a role in the transport of acetyl units produced in the cytosol are Yat1 (or Ctn1) and Yat2 (or Ctn3) that have homology with carnitine acetyltransferases (32). However, both \textit{C. albicans} and \textit{S. cerevisiae} \textit{cat2} null mutants display very little (< 5% in \textit{S. cerevisiae}, undetectable in \textit{C. albicans}; Chapter 2 and reference 15) residual Cat activity, suggesting that Yat1 and Yat2 may not encode true carnitine acetyltransferases (see the Addendum to this chapter).

Although the essential role of Cat2 during growth on non-fermentable carbon sources has been firmly established (Chapter 2 and (43), our understanding of the flow of acetyl-CoA between compartments and the exact role of Cat2 in this process is rudimentary. To investigate the function of the peroxisomal and mitochondrial Cat2 in more detail, we constructed strains that exclusively express one of both isozymes. We show that mitochondrial Cat2 is indispensable during growth on non-fermentable carbon sources and that this isozyme contributes to growth on lactate. The function of the peroxisomal Cat2 on the other hand is less clear, as a mutant that lacks the peroxisomal Cat2 (mitCAT2) is still able to grow on ethanol and acetate and displays β-oxidation activity, but does not grow on rich oleate medium. Based on the phenotypic analysis of the Cat2 mutants and current literature we present a hypothetical model explaining the flow of acetyl units between compartments in \textit{C. albicans}.

Materials and Methods

\textit{Media and culture conditions}

\textit{C. albicans} strains were grown at 28°C unless otherwise stated. For routine non-selective culturing of \textit{C. albicans} strains YPD + Uri (2% bactopeptone, 1% yeast extract, 2% glucose and 80 μg/ml uridine) was used. \textit{C. albicans} transformants were selected and grown on minimal solid medium containing 0.67% Yeast Nitrogen Base (YNB) w/o amino acids (DIFCO), 2% glucose and amino acids as needed (20 μg/ml arginine, 20 μg/ml histidine, 80 μg/ml uridine). Growth curves were performed in liquid YNB medium with glucose (2%), oleic acid/Tween 80 (0.12%/0.2%), sodium acetate (2% with 0.5% 1 M potassium phosphate buffer pH 6.0) or ethanol (2%). For enzyme assays or measurements of metabolites, strains were pregrown for 16 hours on minimal glucose medium (YNB with 2% glucose), inoculated at OD_{600} 0.2 in YNB 0.3% glucose medium and grown for 8 hours. Finally, the strains were inoculated at OD_{600} 0.005 into rich oleate
medium (YPO; 2% bactopeptone, 1 % yeast extract, 0.12%/0.2% oleic acid/Tween 80) or rich acetate medium (YPA; 2% bactopeptone, 1% yeast extract, 2% sodium acetate) and grown for 16 hours.

Table I. Strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Species/Name</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCY2</td>
<td>S. cerevisiae Y2H</td>
<td>MAT, gal4, gal80, URA3::GAL1-lacZ lys2-801amber, his3-200, trpl-63 leu2 ade2-101::Ura</td>
<td>(4)</td>
</tr>
<tr>
<td>CSN76</td>
<td>C. albicans wildtype auxotroph</td>
<td>arg4/arg4 his1 his1 ura3::imm434/ura3::imm434 iro1::imm434/iro1::imm434</td>
<td>(23)</td>
</tr>
<tr>
<td>CSN76-P</td>
<td>wildtype prototroph</td>
<td>ura3::imm434/ura3::imm434::URA3 iro1::imm434/iro1::imm434::IRO1</td>
<td>Chapter 2</td>
</tr>
<tr>
<td>CEM28</td>
<td>cat2Δ/Δ</td>
<td>arg4Δ/ARG4 his1Δ/HIS1 ura3Δ::imm434/ura3Δ::imm434::URA3 iro1Δ::imm434/iro1Δ::imm434::IRO1</td>
<td>Chapter 2</td>
</tr>
<tr>
<td>CEM38</td>
<td>cat2Δ/Δ + URA3</td>
<td>arg4Δ/ARG4 his1Δ/his1Δ ura3Δ::imm434/ura3Δ::imm434::URA3 iro1Δ::imm434/iro1Δ::imm434::IRO1</td>
<td>Chapter 2</td>
</tr>
<tr>
<td>CEM38</td>
<td>cat2Δ/Δ + CAT2</td>
<td>arg4Δ/ARG4 his1Δ/his1Δ ura3Δ::imm434/ura3Δ::imm434::URA3::CAT2 iro1Δ::imm434/iro1Δ::imm434::IRO1 cat2Δ::CdHIS1 cat2Δ::ARG4</td>
<td>Chapter 2</td>
</tr>
<tr>
<td>CEM38</td>
<td>cat2Δ/Δ + perCAT2</td>
<td>arg4Δ/ARG4 his1Δ/his1Δ ura3Δ::imm434/ura3Δ::imm434::URA3::perCAT2 iro1Δ::imm434/iro1Δ::imm434::IRO1 cat2Δ::CdHIS1 cat2Δ::ARG4</td>
<td>This study</td>
</tr>
<tr>
<td>CEM38</td>
<td>cat2Δ/Δ + mitCAT2</td>
<td>arg4Δ/ARG4 his1Δ/his1Δ ura3Δ::imm434/ura3Δ::imm434::URA3::mitCAT2 iro1Δ::imm434/iro1Δ::imm434::IRO1 cat2Δ::CdHIS1 cat2Δ::ARG4</td>
<td>This study</td>
</tr>
<tr>
<td>CEM16</td>
<td>fox2Δ/Δ + URA3</td>
<td>arg4Δ/ARG4 his1Δ/his1Δ ura3Δ::imm434/ura3Δ::imm434::URA3 iro1Δ::imm434/iro1Δ::imm434::IRO1 fox2Δ::HIS1 fox2Δ::ARG4</td>
<td>(27)</td>
</tr>
</tbody>
</table>

**Spot test**

Cells were pregrown on minimal glucose medium, transferred to 0.3% glucose medium, spun down, washed with water twice and resuspended to a concentration of about 2.7 x 10^7 cells/ml (OD_600_ = 1.0) and serially diluted (1:10 dilutions). Four microliters of each dilution was spotted onto agar plates. Plates contained 0.67% YNB with glucose (2%), oleic acid/Tween 80 (0.12%/0.2%), ethanol (2%), sodium acetate (2% with 0.5% potassium phosphate buffer pH 6.0), lactate (2%, to pH 4.5 with NaOH) or citrate as a carbon source. The pictures were taken after 3 – 5 days of incubation at 28°C.
Table II. Plasmids used in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Vector-insert</th>
<th>Purpose</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pgGAD</td>
<td>pgGAD-empty</td>
<td>Y2H, Gal4 transactivation domain</td>
<td>Clontech</td>
</tr>
<tr>
<td>pGBT9</td>
<td>PGBT10-empty</td>
<td>Y2H, Gal4 DNA-binding domain</td>
<td>Clontech</td>
</tr>
<tr>
<td>pGAD-TR</td>
<td>pgAD with tryptophan marker</td>
<td>Y2H, transcription activation</td>
<td>This study</td>
</tr>
<tr>
<td>pGBT10-L</td>
<td>pGBT10 with leucine maker</td>
<td>Y2H, DNA binding</td>
<td>This study</td>
</tr>
<tr>
<td>pPC</td>
<td>Design MCS</td>
<td></td>
<td>(4)</td>
</tr>
<tr>
<td>21.29</td>
<td>Gal4-DB/TPR</td>
<td>Insertion of CaPEX5 for Y2H</td>
<td>(6)</td>
</tr>
<tr>
<td>pTi252</td>
<td>Gal4-CaPEX5-DB/TPR</td>
<td>Y2H</td>
<td>This study</td>
</tr>
<tr>
<td>pKa01</td>
<td>pGAD-TR-CaPEX5</td>
<td>Y2H</td>
<td>This study</td>
</tr>
<tr>
<td>pKa07</td>
<td>pGAD-TR-CaPEX5_N376D</td>
<td>Y2H</td>
<td>This study</td>
</tr>
<tr>
<td>pKa02</td>
<td>pGBT10-L-CaMDH1-3</td>
<td>Y2H</td>
<td>This study</td>
</tr>
<tr>
<td>pKa15</td>
<td>pGBT10-L-ICL1</td>
<td>Y2H</td>
<td>This study</td>
</tr>
<tr>
<td>pKa55</td>
<td>pGBT10-L-CAT2</td>
<td>Y2H</td>
<td>This study</td>
</tr>
<tr>
<td>pKa56</td>
<td>pGBT10-L-CAT2APTsi</td>
<td>Y2H</td>
<td>This study</td>
</tr>
<tr>
<td>pLUBP</td>
<td>pLUBP-empty</td>
<td>URA3 complementation</td>
<td>(29)</td>
</tr>
<tr>
<td>pKa30</td>
<td>pLUBP-CAT2</td>
<td>CAT2 complementation</td>
<td>Chapter 2</td>
</tr>
<tr>
<td>pKa32</td>
<td>pLUBP-perCAT2</td>
<td>Peroxosomal Cat2 complementation</td>
<td>This study</td>
</tr>
<tr>
<td>pKa36</td>
<td>pLUBP-mitCAT2</td>
<td>Mitochondrial Cat2 complementation</td>
<td>This study</td>
</tr>
</tbody>
</table>

Strains and plasmids

*C. albicans* strains used in this study are listed in Table I and are derivatives of SN76 (23). Plasmids used in this study are listed in Table II. Primers are listed in Table III. The marker of the *S. cerevisiae* yeast-2-hybrid plasmids containing the Gal4 transactivating domain (pgAD; Clontech) and Gal4 DNA-binding domain (pGBT9; Clontech) were swapped to create pgAD-TR with tryptophan (TR) and pGBT10-L with leucine (L) as selectable markers. The multiple cloning site (MCS) of plasmid pPC was introduced in pgAD-TR by using double-stranded oligo’s MCS-pGAD-F and MCS-pGAD-R and in pGBT10-L by PCR with double-stranded oligo’s MCS-pGBT-F and MCS-pGBT-R. A PCR product containing the full length *C. albicans* PEX5 gene was obtained with CaPEX5-F-ATG and CaPEX5-R-STOP and cloned BglII/SphI into pSP73 and sequenced. The insert was cloned BglII/SalI into plasmid 21.29 (6), resulting in pTi252. The insert was cloned BglII/SacII into pgAD-TR resulting in pKa01. Primers KS59 and KS60 were used for site-directed mutagenesis on pKa01 to introduce the N376D mutation in PEX5, resulting in plasmid pKa07. The *C. albicans* MDH1-3 gene was fused to the Gal4 DNA-binding domain by cloning the PCR product obtained with primers CaMDH12_F_ATG and CaMDH12_R_STOP into pGBT10-L using restriction sites BamHI and XhoI, resulting in pKa02. The *C. albicans* ICL1 gene was cloned Smal/BglII into pGBT10-L using primers KS80 and KS81, resulting in pKa15. PCRs were performed with primers KS191 & KS192 and KS191 & KS193 to PCR the *C. albicans* CAT2 gene with and without the 3’ PTS1 sequence. Both PCR products were cloned BamHI/SpeI into pGBT10-L, creating pKa55 and pKa56. Construction of the *C. albicans* prototrophic SN76 (wild type) and cat2Δ/Δ strains have been described previously (Chapter 2). Plasmid pKa30, containing the full-length CAT2 gene and a 800 bp promoter region was used to complement the cat2 null strain (Chapter 2). To create a construct that would exclusively express peroxosomal Cat2, site-directed mutagenesis was performed on pKa30 with primers KS132 and
Peroxisomal and mitochondrial carnitine acetyl-transferase

KS133 to mutate the first ATG of the CAT2 gene into a stop codon (M001*), resulting in pKa32. To create a construct that would exclusively express mitochondrial Cat2p, a PCR was performed with primers KS128 and KS129 to amplify the CAT2 promoter and gene without the C-terminal 3 amino acids encompassing the PTS1. The second ATG that is presumed to initiate translation of the peroxisomal Cat2, was changed by site-directed mutagenesis to an alanine (M023A) with primers KS130 and KS131, resulting in pKa36. CEM28 (cat2Δ/Δ) was transformed with plasmids pKa32 and pKa36, resulting in strains CKS59 (cat2Δ/Δ + perCAT2) and CKS61 (cat2Δ/Δ + mitCAT2).

Table III. Primers used in this study

<table>
<thead>
<tr>
<th>Primer/Purpose</th>
<th>5'-3' sequence</th>
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<tbody>
<tr>
<td>MCS-pGAD-F</td>
<td>CTAGCGTCGACCCCCGGATCCCGGAATTCAGTCTACTAGTAGGCCT</td>
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<tr>
<td></td>
<td>GAGCTTCCCGGGGT</td>
</tr>
<tr>
<td>MCS-pGAD-R</td>
<td>TCGAACCGGCGAGCTCAGGCTTACTAGTAGTCTGAATTCCGGAT</td>
</tr>
<tr>
<td></td>
<td>CCCGGGCTCGACG</td>
</tr>
<tr>
<td>MCS-pGBT-F</td>
<td>AATTGTGACCCCGGGATCCCGGAATTCAGTCTACTAGTAGGCCTG</td>
</tr>
<tr>
<td></td>
<td>AGCTTCCCGGGGT</td>
</tr>
<tr>
<td>MCS-pGBT-R</td>
<td>TCGAACCGGCGAGCTCAGGCTTACTAGTAGTCTGAATTCCGGAT</td>
</tr>
<tr>
<td></td>
<td>CCCGGGCTCGAC</td>
</tr>
<tr>
<td>CaPEX5-F-ATG</td>
<td>gaAGATCTcaGGATCCatgctttgtttgtgtaggg</td>
</tr>
<tr>
<td>CaPEX5-R-STOP</td>
<td>agatGCATGCGcgcggACTAGTtagaaactaatctggaactctg</td>
</tr>
<tr>
<td>KS59, F_pex5_SD</td>
<td>atgaacctggcaatagtttatcaggaaggtagtgaatgt</td>
</tr>
<tr>
<td>KS60, R_pex5_SD</td>
<td>gcattatacacctcttgtgatataactaatggaactatgcat</td>
</tr>
<tr>
<td>CaMDH12_F_ATG</td>
<td>gcgGAATCTCaggttgaaggttctagtagcag</td>
</tr>
<tr>
<td>CaMDH12_R_STOP</td>
<td>ccgccTCCGAGttcaaataggttacgacg</td>
</tr>
<tr>
<td>KS80, F_Icl1_Smal</td>
<td>agtCCCGGGAtggtcattcactcactcactgatcac</td>
</tr>
<tr>
<td>KS81, R_Icl1_BglII</td>
<td>cgcAGATCTtaggtgaactgtttctaggttcggtcctt</td>
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<tr>
<td>KS191, F_Cat2_BamH1</td>
<td>cggGAATCTagttttatgaacttccaaacaa</td>
</tr>
<tr>
<td>KS192, R_Cat2_Spel</td>
<td>cgACTAGTttttattttcgttctagtcggttccag</td>
</tr>
<tr>
<td>KS193, R_Cat2ΔPTS1_Spel</td>
<td>cgACTAGTttttattttcgttctagtcggttccag</td>
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<td>KS128, CAT2_F_Stul</td>
<td>TGGAGCTCTGTTGATTTAATACTCCGGCATGT</td>
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<td>KS129, CAT2_R_ΔPTS1_KpnI</td>
<td>TGGAGCTCTGTTGATTTAATACTCCGGCATGT</td>
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<td>KS130, CAT2_M023A_F</td>
<td>CTCAACAAGTTCCAATCTATAATCGGCGAGTTGGGAAACTTTC</td>
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<td>KS131, CAT2_M023A_R</td>
<td>GAATTTTTCTCTCTCCTACTGCGCCGGATTAGTTGGGAAACTTTC</td>
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<td>KS132, CAT2_M001*_F</td>
<td>GTATACATATTTTGTTGATTTAATACTGAGTTGGGAAACTTTC</td>
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<tr>
<td>KS133, CAT2_M001*_R</td>
<td>GATACTATTTTGTTGATTTAATACTGAGTTGGGAAACTTTC</td>
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</tbody>
</table>

Transformation

C. albicans was transformed using a modified lithium acetate protocol (38). The heat shock was carried out at 44°C for 15 minutes.

Yeast-two-hybrid interactions

The constructed Y2H plasmids were transformed to the S. cerevisiae two-hybrid reporter
strain PCY2 and transformants were selected on minimal glucose plates supplemented with 30 µg/ml lysine, 20 µg/ml adenine, 20 µg/ml uracil and 20 µg/ml histidine. Interactions were assayed by staining with X-gal and quantified by determining β-galactosidase activity (17). Total β-galactosidase activity was determined by the formula: 

$$\frac{1000 \times OD_{420}}{PxVxt}.$$ 

P = protein (mg/ml), V = volume (ml), t = time (min).

**Subcellular fractionation and density gradient analysis**

The subcellular fractionation of *C. albicans* strains was performed as previously described (Chapter 2) and the gradient fractions were analyzed for the presence of enzymatic activity of the peroxisomal marker 3-hydroxyacyl-CoA dehydrogenase (3HAD) (39), the mitochondrial marker Fumarase (33) and carnitine acetyl transferase (Cat) (7).

**Mass-spec measurements of metabolites**

For acyl-CoA measurements strains were grown for 16 hours on YPO and washed twice with water. Acyl-CoA measurements were performed as described by Hammond et al. (10) with some modifications that were described previously (Chapter 2). For carnitine and acetyl-carnitine measurements, cells were grown for 16 hours on YPO or YPA without or with 2 mM carnitine and washed twice with water. 20 OD units were spun down, taken up in 500 µl PBS and 200 µl glassbeads were added after which the tubes were vortexed for 20 minutes at 4°C. The supernatant was transferred to a new tube and the glassbeads were washed with an additional 200 µl PBS that was also transferred. The pooled supernatants were centrifuged twice at high speed to remove cell debris and whole cells, resulting in the final lysates. The protein concentration of the lysates was determined by the method of Bradford using BSA as a standard (2). Determination of carnitine and acetyl-carnitine by LC-MS-MS was performed as previously described (28). For the determination of (iso)citrate, succinate, fumarate and lactate, cells were inoculated in YNB 2% glucose and grown overnight. The next morning the culture was diluted in YNB 0.3% glucose to an OD<sub>600</sub> of 0.15, grown for 6-8 hours, then inoculated in YPO at an OD<sub>600</sub> of 0.002 and grown overnight. After 16 hours of growth the cells were collected by centrifugation (10 min at 4,000 g), washed 3 x with 25 ml distilled water and collected again by centrifugation (10 min at 4,000 g) and converted to spheroplasts in approximately 45 minutes (Chapter 2). Spheroplasts were harvested by centrifugation at 2,300 g for 8 min at 4°C and the pellet was resuspended in 1.5 ml buffer V (10 mM MOPS, 10% w/v glycerol, 5 mM DTT, 0.9% NaCl, pH 7.4). Spheroplasts were sonicated 2 times for 30 seconds to amplitude 10 while on ice and left on ice for 30 seconds in between sessions. The homogenate was centrifuged twice at 20,000 g at 4°C to remove debris, nuclei and whole cells. Levels of organic acids (iso)citrate, succinate, fumarate and lactate were determined by conventional mass spectrometry following extraction with ethyl acetate/diethyl ether. The acids were separated as their methoxime/trimethylsilyl esters (1).

**Fatty acid β-oxidation measurements**

β-oxidation activity in intact cells was measured essentially as described before (34) except that the cells were resuspended at an OD<sub>600</sub> of 1. Production of CO₂ and incorporation of radiolabel into acid-soluble material were followed with time.
Electron microscopy

Oleate-induced cells were fixed with 2% (wt/vol) formaldehyde, and ultrathin sections were prepared as described previously (12). Immunolabeling was performed using antibodies directed against *S. cerevisiae* 3-ketoacyl-CoA thiolase and gold-conjugated protein A.

Isocitrate lyase enzyme assay

Preparation of cell-free extracts and enzyme assays were performed essentially as described previously (5), except that extracts were freshly prepared and the assays were carried out in a UVIKON 820 double beam spectrophotometer (Kontron) at room temperature.

Immunoblotting

Protein extracts were separated on a 10% SDS-polyacrylamide gel and blotted to nitrocellulose membrane using a semi-dry system. Antibodies used were directed against *S. cerevisiae* thiolute (Thiol), catalase (Cta1), malate synthase (Mls1) (19), peroxisomal membrane protein 35 (Pmp35/Ant1) (37), citrate synthase (Cit1) (36), hexokinase (Hxk1), isocitrate dehydrogenase (Idh1) (a kind gift of H. van der Spek, FNWI), glucose 6-phosphate dehydrogenase (Zwf1) (Sigma), *Ashbya gossypii* isocitrate lyase (Icl1) (21). α-Thiol, α-Cta1, α-Icl1, α-Mls1 and α-Cit1 antibodies were previously tested for specific cross-reactivity with the corresponding *C. albicans* proteins (Chapter 2, references 26, 27). In this study, α-Ant1 (1:2000), α-Hxk1 (1:1000), α-Zwf1 (1:500) and α-Idh1 (1:1000) antibodies were tested for cross-reactivity with *C. albicans* proteins. Each antibody detected a band of the predicted molecular weight in a total cell lysate.

Results

*C. albicans* Cat2 interacts with CaPex5 in a PTS1-dependent manner

To address the question whether peroxisomal targeting of *C. albicans* Cat2 is dependent on its (putative) PTS1 (-AKLCOOH), we designed a two-hybrid experiment. The *C. albicans* PTS1 receptor Pex5 was cloned into a GAL4 transcription activation (TA) domain plasmid (pGAD-TR) and the full-length CAT2 was cloned into a GAL4 DNA binding (DB) domain plasmid (pGBT10-L). As controls the PTS1 protein MDH1-3 (-SKLCOOH) and the peroxisomal protein Icl1 were also cloned into pGBT10-L. The latter protein, Icl1, lacks a consensus PTS1, but is targeted to peroxisomes in a Pex5-dependent manner (26). Plasmids were co-transformed to *S. cerevisiae* yeast-2-hybrid strain PCY2 and expression of the reporter LacZ was quantified by performing a β-galactosidase assay (Fig. 1). Cat2 and MDH1-3 showed a clear interaction with Pex5, while Icl1 exhibited a very weak interaction with the PTS1 receptor. Deletion of the three C-terminal amino acids of Cat2 (CAT2∆PTS1) resulted in a complete loss of Pex5 interaction. To further investigate the nature of the interaction between Pex5 and Cat2, a mutation that was shown to disrupt Pex5-PTS1 interaction in *S. cerevisiae* (16) was made in *C. albicans* Pex5 (CaPEX5N376D). CaPEX5N376D did not interact with the PTS1 protein Mdhl-3, showing that the N376D mutation completely disturbs PTS1 interaction. β-galactosidase was only slightly reduced for the CaPEX5N376D – Icl1 interaction, suggesting that *C. albicans*
Icl1 is able to interact with Pex5 in a PTS1-independent manner, as has previously been shown for Castor bean Icl1\textsubscript{∆PTS1} (25), and that folding of the mutant Pex5 is not grossly affected. No interaction was observed between CaPex5\textsubscript{N376D} and Cat2. These results show that the interaction between Pex5 and Cat2 is strictly dependent on the Cat2 PTS1 and suggest that peroxisomal import of Cat2 can be abolished by deletion of the three C-terminal amino acids.

**Mitochondrial and peroxisomal Cat2**

In *S. cerevisiae*, dual localization of Cat2 to peroxisomes and mitochondria is regulated on transcriptional level resulting in two proteins that either contain or lack the N-terminal mitochondrial targeting sequence (MTS), while both proteins harbor the C-terminal PTS1. Because of the sequence similarity between *S. cerevisiae* and *C. albicans* and the two conserved in frame ATGs, we assumed that transcriptional regulation of the *C. albicans* CAT2 gene would be comparable. Two constructs were designed using the *C. albicans* wild type Cat2 complementation construct, containing a 800 bp promoter region and the full length gene, as a template (Chapter 2). The peroxisomal Cat2 construct (perCAT2) was created by mutation of the first ATG into a stop codon (M001\**) to block translation initiation of the Cat2 form containing the MTS. To create the mitochondrial Cat2 (mitCAT2), the three C-terminal amino acids –AKL\textsubscript{COOH} (DB-CaCAT2\textsubscript{∆PTS1}), the PTS1 protein MDH1-3 (DB-MDH1-3) or the peroxisomal protein Icl1 that lacks a predicted PTS1 (DB-ICL1). The empty DB plasmid was taken along as a control. Asterisks: no activity detectable.

![Figure 1. *C. albicans* Cat2 interacts with Pex5 in a PTS1-dependent manner](image)

\[\text{β-galactosidase activity in *S. cerevisiae* strain PCY2 co-expressing DNA binding (DB) and transcription activation (TA) domain fusions. The TA domain was fused to the *C. albicans* PTS1-receptor Pex5 (TA-CaPex5) or Pex5 with the N376D mutation (TA-CaPex5\textsubscript{N376D}). Putative PTS1 proteins were fused to the DB domain: full length *C. albicans* Cat2 (DB-CaCAT2), Cat2 without the three C-terminal amino acids –AKL\textsubscript{COOH} (DB-CaCAT2\textsubscript{∆PTS1}), the PTS1 protein MDH1-3 (DB-MDH1-3) or the peroxisomal protein Icl1 that lacks a predicted PTS1 (DB-ICL1). The empty DB plasmid was taken along as a control. Asterisks: no activity detectable.}\]
no Cat2 activity was detected above the background in the cat2 null strain (Chapter 2). Localization of Cat2 activity in the wild type, perCat2 and mitCat2 strains was investigated by subcellular fractionation and Nycodenz density gradient analysis. The wild type strain showed a dual distribution of Cat2 activity between peroxisomes and mitochondria (Fig. 2B). Cat2 activity in the perCAT2 strain colocalized with the peroxisomal marker 3HAD, but a second Cat peak is visible at the top of the gradient, presumably representing lysed peroxisomes (Fig. 2C). A single Cat2 activity peak is seen in the gradient of the mitCAT2 strain that shows a strict co-localization with the mitochondrial marker Fumarase (Fig. 2D). These experiments strongly suggest that Cat2 localizes to the designated organelles in the perCAT2 and mitCAT2 strains.

**Figure 2: Subcellular distribution of Cat activity in wild type, perCAT2 and mitCAT2 strains**

(A): Total Cat activity in homogenates of the wild type, cat2Δ/Δ, cat2Δ/Δ + CAT2, peroxisomal Cat2 (perCAT2) and mitochondrial Cat2 (mitCAT2) strain grown on rich glucose and rich oleate medium. All strains showed higher activity on oleate compared to glucose, except for the cat2Δ/Δ in which no activity could be detected as indicated by the asterisk. (B): Distribution of Cat activity, the peroxisomal marker 3HAD and the mitochondrial marker Fumarase in a Nycodenz density gradient of the wild type strain, the perCAT2 strain (C) and the mitCAT2 strain (D). In each experiment an organellar pellet fraction was loaded on the gradient.

**Growth phenotype of the perCAT2 and mitCAT2 strains**

The individual contribution of the peroxisomal and mitochondrial Cat2 isoymes to growth on various carbon sources was studied by spot assays and growth curves. Serial dilutions of all strains were spotted on minimal YNB plates with glucose, oleate, lactate, citrate, acetate or ethanol as sole carbon source (Fig. 3A). In all tested conditions, the perCAT2 showed a similar growth defect as the cat2 null mutant: no growth was seen on oleate, citrate, acetate and ethanol and intermediate growth was observed on lactate.
These data suggest that mitochondrial Cat2 is essential for metabolizing these carbon sources. The mitCAT2 strain grew like wild type on lactate and showed reduced growth on citrate and acetate and strongly reduced growth on oleate. Growth of the mitCAT2 strain on ethanol was also strongly reduced compared to the wild type, but a few colonies did appear after several days. A survival assay was conducted by plating individual cells of each strain on glucose, oleate, acetate or ethanol and calculating cell survival on non-fermentable carbon sources relative to glucose (number of colonies on medium X/number of colonies on glucose x 100). The mitCAT2 strain had a 30%, 52% and 20% survival on oleate, acetate and ethanol, respectively. Growth of colonies on the ethanol plates took about 7 days compared to 3 days for the wild type (data not shown). This survival assay shows that although the mitCAT2 strain is capable of growing on oleate, acetate and ethanol, the cell survival rate and the growth rate are (much) lower compared to the wild type. To investigate this intriguing growth phenotype of the mitCAT2 in more detail, we performed growth curves on minimal glucose, oleate, acetate and ethanol medium. Growth on glucose was comparable for all strains (Fig. 3B), while only the wild type and complemented strain grew on liquid minimal oleic acid medium (Fig. 3C). The reason for this slightly different growth phenotype of the mitCAT2 on oleate plates and in liquid oleate medium is unclear. Initiation of growth of the mitCAT2 strain was delayed on acetate and ethanol compared to the wild type, but after a lag phase of 30 and 100 hours, respectively, a near wild type growth rate was observed on both carbon sources (Fig. 3D and E).
These results show that a mitochondrial localized Cat2 is essential for growth on most non-fermentable carbon sources tested, except lactate. The peroxisomal Cat2 on the other hand is dispensable while growing on lactate, citrate, ethanol or acetate. Growth on (liquid) oleate medium however does require peroxisomal Cat2.

Figure 4: Uncoupling of growth and β-oxidation activity in the mitCAT2 strain
(A): Levels of acetyl-CoA and butyryl-CoA (C4-CoA) in cells grown on rich oleate medium. (B): β-oxidation of labeled oleic acid in cell grown on rich oleate medium measured by production of labeled CO2. (C): Production of acid soluble intermediates from labeled oleic acid during β-oxidation. (D): Growth of the CAT2 strains on rich oleate acid medium: only the wild type and complemented strain reach the maximum OD_{600}.

The mitCAT2 strain displays high β-oxidation activity
As an indicator of deficient intracellular transport of acetyl units, we determined the accumulation of acetyl-CoA and C4-CoA in cells grown on rich oleate medium. Acetyl-CoA and C4-CoA levels were found to be elevated in the cat2 null mutant, the perCAT2 and mitCAT2 compared to the wild type and complemented strain, indicating a transport defect (Fig. 4A). To directly determine the ability of the different strains to transport acetyl-CoA, generated during fatty acid β-oxidation, from peroxisomes to mitochondria, we measured the amount of CO2 that is produced by the mitochondrial TCA cycle and the acid soluble counts representing carbon metabolism intermediates (34). Cells were pre-grown overnight on rich oleate medium, washed twice and then incubated with 1-^{14}C-labeled oleic acid. Samples were taken after 1, 2 and 4 hours of incubation and the amount of produced labeled CO2 and acid soluble counts were determined for each time point (Fig. 4B and C). CO2 production was undetectable in the cat2 null mutant and the perCAT2 strain, while the mitCAT2 strain showed a high CO2 production that was 2-3 fold higher compared to the wild type and complemented strain. Similarly, acid soluble counts were very high in the mitCAT2 strain (2-3 fold higher than wild type), but low in the cat2 null and perCAT2 strains. While these data indicate that the mitCAT2 strain

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can efficiently \(\beta\)-oxidize fatty acids, the growth assays on minimal oleic acid medium show that fatty acids cannot be used by this strain as sole carbon and energy source. To determine whether ATP is generated during fatty acid \(\beta\)-oxidation in the mitCAT2 strain that can be used to support growth, we analyzed the strain on rich oleate medium containing yeast extract and peptone (Fig. 4D). The mitCAT2 strain reached an OD\(_{600}\) of about 5 after 24 hours of incubation, a value that is comparable to that of the cat2 null and perCAT2 strains, while the wild type and complemented strains reached an OD\(_{600}\) of about 15. Together, these results show that in the absence of peroxisomal Cat2 the mitCAT2 strain is capable of transporting peroxisomal acetyl units to the mitochondria where it is broken down in the TCA cycle, but that this strain is not able to generate biomass from the oxidized fatty acids.

![Image](image_url)

**Figure 5: All CAT2 strains display an aberrant peroxisomal morphology**
Characterization of peroxisomal morphology by immuno electron microscopy. Thin sections of the wild type, cat2 null mutant, complemented strain, perCAT2 and mitCAT2 strains were incubated with \(\alpha\)-thiolase antibodies and protein A-conjugated gold particles to label peroxisomes. The 6th panel shows details of peroxisomal morphology in the mitCAT2 strain.

**Aberrant peroxisomal morphology in the different Cat2 strains**
We and others have previously shown that defects in fatty acid \(\beta\)-oxidation can result in aberrant peroxisome morphology (3, 8, 9, 26, 30, 31). To determine peroxisome morphology, the Cat2 mutant strains were grown on rich oleate medium and analyzed by immunoelectron microscopy. Peroxisomes were labeled with a cross-reacting antibody directed against *S. cerevisiae* thiolase, a known peroxisomal matrix protein. Peroxisomes in the cat2 null strain were more prevalent, larger and more irregularly shaped than peroxisomes in wild type cells, a phenotype that was even more apparent in the perCAT2 strain (Fig. 5). Peroxisomes of the mitCAT2 seemed to be even more affected as their size and shape were very irregular. Long protrusions or “needles” were
often visible in the mitCAT2 strain and the very small round peroxisomal profiles seen in the mutant strains might be cross-sections of such protrusions.

**Characterization of peroxisomal functions of the Cat2 strains**

Because increased protein import into peroxisomes has been suggested to cause aberrant morphology (31), we determined protein abundance of peroxisomal, mitochondrial and cytosolic markers to see whether they were affected in the Cat2 strains (Fig. 6A). In all Cat2 mutants the levels of catalase (Cta1) and the glyoxylate cycle enzymes isocitrate lyase (Icl1) and malate synthase (Mls1) were slightly elevated, while the levels of the β-oxidation enzyme thiolase (Thiol) and peroxisomal the membrane adenine nucleotide transporter (Ant1) appeared to be much higher in the mutants compared to wild type and complemented strains. Mitochondrial TCA cycle enzymes citrate synthase (Cit1) and isocitrate dehydrogenase (Idh1) were also clearly more abundant in all the Cat2 mutants, while the levels of the cytosolic markers hexokinase (Hxk1) and glucose-6-phosphate dehydrogenase (Zwf1) were similar in all strains. Although the increased levels of peroxisomal proteins could contribute to the increased peroxisomal volume observed in all Cat2 mutants, it does not explain the morphological differences between the cat2 null, perCAT2 and mitCAT2 strains as all tested markers are similarly elevated in all mutants. Previously, it was reported that aberrant peroxisome morphology might affect transport of metabolites over the peroxisomal membrane (26). As the mitCAT2 strain is able to

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**Figure 6. Abundance of peroxisomal and mitochondrial marker proteins in the CAT2 strains and levels of metabolites in the mitCAT2 strain**

(A): Immunoblot showing levels of peroxisomal, mitochondrial and cytosolic markers proteins in the wild type, cat2 null perCAT2 and mitCAT2 strains. In each lane equal amounts of total protein was layered. (B): Icl1 activity in wild type, cat2 null, perCAT2 and mitCAT2 strains. (C): Levels of intermediates in the wild type and mitCAT2 strain. Metabolites measured were isocitrate/citrate, succinate, fumarate and lactate.
β-oxidize but not grow on fatty acids and has aberrant peroxisomes we wanted to test whether the underlying problem in this strain is a (peroxisomal) transport defect. A previous experiment (Fig. 6A) already revealed that the enzymes of the β-oxidation, the glyoxylate cycle and TCA cycle are abundantly expressed in the mitCAT2 strain and that the TCA cycle is active in this mutant (Fig. 4B and C). We tested the activity of the peroxisome-localized Icl1 that converts isocitrate to glyoxylate and succinate and found that Icl1 activity in all Cat2 mutants was at least as high as wild type activity (Fig. 6B). Next, we determined the levels of the TCA/glyoxylate cycle intermediates (iso)citrate (isocitrate and citrate were detected as one peak in our mass spec analysis) succinate, fumarate and lactate by mass spectrometry (Fig. 6C). Succinate, fumarate and lactate were lower in the mitCAT2 strain than in the wild type strain, while the levels of (iso)citrate were more than 3 fold higher in the mitCAT2 strain. The strongly elevated (iso)citrate levels in conjunction with a functional TCA and glyoxylate cycle (Icl1) may suggest that the mitCAT2 strain is unable to grow on fatty acids because (iso)citrate cannot enter the peroxisomal glyoxylate cycle due to a transport defect. This transport defect maybe caused by the aberrant peroxisomal morphology as was also reported for the C. albicans fox2 null strain (26).

Discussion

To unravel the mechanism of carnitine-dependent acetyl unit transport between organelles we studied the function of the dually localized Cat2 of C. albicans. We constructed strains that exclusively express either the peroxisomal Cat2 (perCAT2) or the mitochondrial Cat2 (mitCAT2) and studied their phenotypes. Below we discuss the possible functions of both Cat2 proteins in cells growing on different carbon sources and present a hypothetical model of carnitine-dependent transport between the different compartments of the yeast cell (Fig. 7).

Import of acetyl units into mitochondria

Activity of the mitochondrial Cat2 is very likely to be associated with the (intra)-mitochondrial conversion of acetyl-carnitine to acetyl-CoA, which feeds into the TCA cycle that generates reducing equivalents that can be used for ATP synthesis. Acetyl-CoA can be produced directly in the mitochondria from cytosolic pyruvate by the pyruvate dehydrogenase complex (Pda). Therefore, growth on glucose does not require acetyl unit transport and likewise acetyl unit transport is not strictly required during growth on lactate. In contrast, the mitochondrial Cat2 is suggested to be required during growth on oleate, ethanol, acetate and citrate. The phenotype of the perCAT2 strain that lacks the mitochondrial Cat2 supports this hypothesis, as this strain cannot grow on oleate, acetate, ethanol or citrate and displays intermediate growth on lactate (Fig. 3).

Export of acetyl units from peroxisomes

The activity of the peroxisomal Cat2 is thought to be required for the export of acetyl units produced during β-oxidation of fatty acids. Peroxisomal Cat2 can link the acetyl units to carnitine and acetyl-carnitine can cross the membrane and be transported further to the mitochondria. In contrast to S. cerevisiae, C. albicans does not have an alternative,
Peroxisomal and mitochondrial carnitine acetyl-transferase citrate synthase-dependent, export pathway as it lacks the peroxisomal isoenzyme Cit2 (Chapter 2). The mitCAT2 strain lacking peroxisomal Cat2 activity (Fig. 2D) is unable to utilize fatty acids as carbon and energy source either in minimal or rich medium (Fig. 3C and 4D), indicating that the peroxisomal Cat2 activity is indispensable under these conditions. Surprisingly, the mitCAT2 is able to β-oxidize fatty acids efficiently (Fig. 4B and C). The employed β-oxidation assay measures the production of labeled CO₂ from 1-¹⁴C-labeled oleic in whole cells and therefore requires a functional peroxisomal β-oxidation, transport of acetyl units from peroxisomes to mitochondria and a functional TCA cycle where CO₂ is produced. The mitCAT2 strain shows a very high production of CO₂ and incorporation of label in acid soluble intermediates. The suggestion that the mitCAT2 can efficiently oxidize fatty acids is supported by the clearance of turbid (rich) oleate medium after 24 hours of incubation (data not shown). These results strongly suggest that under these conditions acetyl units are exported from peroxisomes, even in the absence of peroxisomal Cat2. Possible ways to export acetyl units independently of peroxisomal Cat2 are conversion of acetyl-CoA to acetate by a peroxisomal thiosesterase or a putative peroxisomal acetyl-CoA hydrolase (Fig. 7, dashed box 1).

Figure 7. Hypothetical model showing the inter-organellar flow of acetyl units in C. albicans
In mammals, peroxisomal thioesterases and carnitine-acyl/acetyl-transferases were shown to be differentially expressed and suggested to provide complementary systems for exporting β-oxidation products from peroxisomes (40). While *S. cerevisiae* has a single peroxisomal thioesterase (Tes1) (20), the *C. albicans* genome encodes 5 putative thioesterases, of which four have a likely PTS1 (–AKL<sub>COOH</sub> or –SRL<sub>COOH</sub>). Thioesterases hydrolyse acyl-CoAs to the corresponding free fatty acids. Several studies have suggested however that peroxisomes may also contain acetyl-CoA thioesterase activity (14, 24, 42) and a acetyl-CoA thioesterase referred to as ACOT12 has been detected in isolated peroxisomes of rat and mouse (22, 41). It remains to be investigated if one of the *C. albicans* isoenzymes is localized to peroxisomes and has acetyl-CoA thioesterase activity. An other possibility to produce acetate from acetyl-CoA is posed by the enzyme acetyl-CoA hydrolase (Ach1), however this protein is predicted to be cytosolic as it lacks obvious targeting signals. The produced acetate might be able to cross the peroxisomal membrane by active transport or diffusion. In plants, transport of acetate in the other direction (from cytosol to peroxisomes) was shown to be dependent on the Comatose transporter (Cts) that is also the acyl-CoA transporter in plants (13). Pxa1 and Pxa2 are the yeast orthologues of Cts and therefore these transporters might also play a role in acetate transport. Upon arrival in the cytosol, acetate can be converted to acetyl-CoA by one of the cytosolic acetyl-CoA synthases (Acs1 or Acs2), linked to carnitine by a putative cytosolic carnitine acetyl-transferase and transported to mitochondria. If true, it is remarkable that this carnitine-independent export of C2 units appears to be very efficient as can be deduced form the high CO<sub>2</sub> production measured in the β-oxidation assay (Fig. 4B).

**Uncoupling of β-oxidation and glyoxylate cycle in the mitCAT2 strain**

One of the most striking observations is that the mitCAT2 strain is still able to β-oxidize fatty acids, but cannot use these fatty acids for energy conservation and biomass formation and thus for growth. Our data suggest that the TCA cycle and glyoxylate cycle are functional in this strain and that isocitrate or citrate accumulates when cells are incubated in rich oleate medium (Fig. 4 and 6). As (iso)citrate produced in the mitochondrial TCA cycle must be transported to peroxisomes to feed the glyoxylate cycle, the accumulation of (iso)citrate suggest a transport defect of this metabolite, either its export out of the mitochondria or its import into peroxisomes. An increase in size and a changed peroxisomal morphology has been previously connected to transport deficiencies in *C. albicans* (26) and we speculate that the aberrant peroxisomal morphology of the mitCAT2 strain might cause a similar transport defect. As a consequence, (iso)citrate cannot enter the peroxisomal glyoxylate cycle thereby blocking the synthesis of C4 units for gluconeogenesis (Fig. 7, marked 3). If citrate cannot be shuttled into the glyoxylate cycle due to a peroxisomal transport defect resulting in an accumulation of citrate (Fig. 6), there might be an increased flow of citrate through the TCA cycle resulting in increased decarboxylation, which would explain the increased CO<sub>2</sub> production in the β-oxidation assay (Fig. 4B).

**Transport of acetyl-CoA from cytosol to peroxisomes**

During growth of *C. albicans* on non-fermentable carbon sources other then fatty acids, such as ethanol and acetate, acetyl-CoA is produced in the cytosol and needs to
be imported into peroxisomes where the key enzymes of the glyoxylate cycle, Mls1 and Icl1, are localized (26). This situation is different from that in *S. cerevisiae* where Mls1 is cytosolic during growth on acetate or ethanol, but peroxisomal during growth on oleic acid (11). We hypothesize that in *C. albicans* the putative carnitine acetyl-transferases Yat1 and Yat2 are involved in generating acetyl-carnitine in the cytosol that can be transported to peroxisomes and/or mitochondria (Fig. 7, dashed box 2), a suggestion that is based on mutant phenotypes and their sequence homology to Cat2. However, since no Cat activity can be measured in a cat2 null strain (Chapter 2) it remains to be seen whether Yat1 and Yat2 are true carnitine acetyl-transferases (see Addendum to this Chapter). Alternatively acetate may be transported over the peroxisomal membrane and converted to acetyl-CoA by Acs1 or Acs2 (Fig. 7, dashed box 1), although these proteins are predicted to be cytosolic due to absence of obvious targeting signals. This alternative pathway may explain the unexpected observation that the mitCAT2 strain lacking peroxisomal Cat2 is able to grow on ethanol, acetate and citrate, although with some delay (Fig. 3). However, growth of the mitCAT2 strain does not start immediately, but only after a large number of hours (depending on the carbon source), suggesting some kind of metabolic adaptation. What this metabolic adaptation entails is currently unclear, but the fact that the strain, once started, achieves wild type growth rates on ethanol and acetate, suggests very efficient generation of peroxisomal acetyl units, which cannot be easily explained by diffusion of acetate over the peroxisomal membrane. Clearly, further work is required to resolve this issue.

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