Intracellular Acetyl Unit Transport in Fungal Carbon Metabolism

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Intracellular acetyl unit transport in fungal carbon metabolism

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Abstract
Acetyl-CoA is a central metabolite in carbon and energy metabolism. Because of its amphiphilic nature and bulkiness, acetyl-CoA cannot readily traverse biological membranes. In fungi two systems for acetyl unit transport have been identified: a shuttle dependent on the carrier carnitine and a (peroxisomal) citrate synthase-dependent pathway. In the carnitine-dependent pathway carnitine acetyl-transferases (Cats) exchange the CoA group of acetyl-CoA for carnitine, thereby forming acetyl-carnitine, which can be transported between subcellular compartments. Citrate synthase catalyzes the condensation of oxaloacetate and acetyl-CoA to form citrate that can be transported over the membrane. Since essential metabolic pathways like fatty acid β-oxidation, the tricarboxylic acid (TCA) cycle and the glyoxylate cycle are physically separated into different organelles, shuttling of acetyl units is essential for growth of fungal species on various carbon sources like fatty acids, ethanol, acetate or citrate. In this review we summarize the current knowledge on the different systems of acetyl transport that are operational during alternative carbon metabolism, with special focus on two fungal species: *Saccharomyces cerevisiae* and *Candida albicans*. 
Introduction

*Candida albicans* is an opportunistic commensal fungus that is part of the normal human microflora. The fungus has colonized the oral cavity of most infants by the age of one month (43) and resides in the gastrointestinal tract in the majority of adults (30). Opportunistic infections by *C. albicans* include invasion of oral and vaginal mucosal surfaces, but alterations in host immunity or physical perturbation, for example through surgery, can cause invasion of the fungus into the bloodstream resulting in life threatening systemic infection. *C. albicans* seemingly promotes a sustainable commensal lifestyle by negatively regulating its own population size in the gastrointestinal tract (54). During commensal and pathogenic stages, the fungus encounters different niches in the body that vary widely with respect to physiology, pH, type of immunological defense and availability of nutrients (3). To be able to survive and develop an infection *in vivo* *C. albicans* needs to adapt to these changing environments. One important aspect of this adaptation is the ability of *C. albicans* to use a wide variety of carbon sources. Microarray studies have revealed that in plasma and tissue glucose is the major carbon source (10, 50), whereas during phagocytosis by macrophages or neutrophils fatty acid β-oxidation and the glyoxylate cycle are highly upregulated (9, 10, 25), suggesting that in the latter conditions the fungus metabolizes alternative carbon sources such as fatty acids. Independent of the carbon source that cells grow on, the C2 unit acetyl-CoA is a central metabolite that serves as the substrate or product of various metabolic pathways. Acetyl-CoA is essential for the production of ATP, as it is used to replenish the mitochondrial tricarboxylic acid (TCA) cycle with C2 units and therefore acetyl-CoA supply in the mitochondrial matrix is essential for growth. However, growth on carbon sources other than glucose leads to the production of acetyl-CoA in peroxisomes or the cytosol and therefore requires transport of acetyl units over (the) organelar membrane(s). In this review we describe the current knowledge on pathways of alternative carbon metabolism in *C. albicans*, with special focus on the role of carnitine-dependent acetyl unit transport, and highlight major differences in carbon metabolism between this pathogenic fungus and the non-pathogenic model yeast *Saccharomyces cerevisiae*. 
Glucose metabolism

During growth on glucose, fungi can employ two major strategies for energy production: oxidative respiration or non-oxidative fermentation. Both respiration and fermentation employ glycolysis as the central pathway, but the former is more energetically efficient than the latter as more NAD+ can be produced per molecule glucose in the presence of oxygen. Most eukaryotes are obligate or facultative aerobes and therefore predominantly employ respiration in the presence of oxygen, while the fermentation pathway is only used in the absence of oxygen. However, it is well established that *S. cerevisiae* exhibits a unique dependence on the fermentation pathway and therefore ferments sugars to ethanol instead of using respiration, even under aerobic conditions (18). *C. albicans*, on the other hand is a facultative aerobe and predominantly oxidizes glucose via pyruvate to carbon dioxide through the TCA cycle. Glucose is taken up by the cell and phosphorylated by hexokinase (Hxk1), a reaction that requires ATP, to trap the molecule in the cell. The formed glucose-6-phosphate can enter the catabolic glycolysis where it is degraded to pyruvate, which can be transported over the mitochondrial membrane. The pyruvate dehydrogenase complex (Pda), which is associated with the mitochondrial membrane, converts pyruvate into acetyl-CoA that is released in the mitochondrial matrix. Therefore, during growth on glucose acetyl-CoA is directly synthesized in the mitochondrial matrix and no transport of acetyl-CoA over the mitochondrial membrane is required (Fig. 1B). In the absence of added sources of acetyl-CoA there is, however, a strict requirement for the production of cytosolic acetyl-CoA, which is essential as a building block for the biosynthesis of fatty acids. In *S. cerevisiae* and *C. albicans* cytosolic acetyl-CoA is synthesized via the pyruvate-acetaldehyde-acetate pathway (Fig. 1B). Of the two acetyl-CoA synthetases that have been identified in these yeast species (ACS1 and ACS2), ACS2 seems to play a major role in the generation of cytosolic acetyl-CoA when glucose is the carbon source (4, 51).

Transcription factors Tye7 and Gal4 were shown to be the major regulators of expression of genes involved in glycolysis in *C. albicans* (2). Tye7 seems to be the main transcription factor that determines the flux between energy storage and energy production at the glucose-6-phosphate branch point, independently of the carbon source. Gal4 on the other hand bound more targets involved in pyruvate metabolism and binding was carbon source dependent. The *C. albicans* tye7 null and a tye7/gal4 null strains showed severe growth defects during growth on
fermentable carbon sources when respiration was inhibited or oxygen was limited. In addition, the strains showed severe virulence defects in both the *Galleria mellonella* and mouse intravenous model showing that proper transcriptional regulation of glycolysis is essential during infection. The authors hypothesize that the virulence defect of the *tye7* null and *tye7*/*gal4* null strains is the result of growth defects due to a low oxygen environment during invasive infection (2).

**Fatty acid β-oxidation**

In mammals, very long chain fatty acids undergo shortening by the peroxisomal β-oxidation, while shorter fatty acids are directly imported into mitochondria (20). In yeasts like *S. cerevisiae* and *C. albicans* fatty acid β-oxidation is exclusively peroxisomal (20). After import into the peroxisome, acyl-CoA is oxidized completely to acetyl-CoA units in four enzymatic steps performed by three different enzymes. The *C. albicans* genome encodes multiple isozymes for some of the enzymatic steps of β-oxidation (two acyl-CoA oxidases, one multifunctional enzyme and three 3-ketoacyl-CoA thiolases), a complexity that resembles the human β-oxidation pathway (three acyl-CoA oxidases, two multifunctional enzymes and three 3-ketoacyl-CoA thiolases) but not that of *S. cerevisiae* (one acyl-CoA oxidase, one multifunctional enzyme and one 3-ketoacyl-CoA thiolase). The increased number of β-oxidation isozymes in *C. albicans* relative to *S. cerevisiae* might indicate that this fungus has undergone an adaptation towards a more sophisticated fatty acid metabolism compared to *S. cerevisiae*, allowing it to handle a wider variety of fatty acid substrates.

Transcriptional activation of β-oxidation genes in *S. cerevisiae* is regulated by the Pip2/Oaf1 heterodimer that binds to the upstream oleate-response element (ORE) (40). *C. albicans* does not encode orthologs of the Pip2/Oaf1 transcription factors but expresses a Zn$_2$-Cys$_6$ transcription factor called Ctf1 that binds a CCTCGG motif that is present in the majority of β-oxidation and glyoxylate cycle genes (16, 37). Ctf1 is an ortholog of transcription factors FarA and FarB of the filamentous fungus *Aspergillus nidulans*. These two transcription factors were shown to be essential for oxidation of short chain fatty acids (FarB) or short and long chain fatty acids (FarA) (16) and are part of a large family of proteins with orthologs in multiple fungi. As FarA/FarB/Ctf1 orthologs are found in many other fungi with the notable exception of *S.*
cerevisiae (16), regulation of β-oxidation genes by this family of transcription factors seems to be a conserved trait and the Pip2/Oaf1 system is most likely unique to S. cerevisiae and its closest relatives. Other relevant differences in the regulatory network for non-fermentable carbon metabolism between S. cerevisiae and C. albicans are the Cat8 and Adr1 transcription factors that regulate the expression of glyoxylate cycle and gluconeogenesis enzymes in S. cerevisiae but do not seem to play similar roles in C. albicans (15, 37, 45).

**The glyoxylate cycle**

The products of the peroxisomal β-oxidation are shortened fatty acids and acetyl units and the latter need to be transported to the mitochondrial TCA cycle for ATP generation. Unlike mammals, fungi can grow on fatty acids as sole carbon source as they not only are able to generate energy from acetyl (C2) units, but also can synthesize sugars from C2. The pathway that is responsible for the generation of C4 from C2 units is the glyoxylate cycle, which consists of two key enzymes: isocitrate lyase (Icl1) and malate synthase (Mls1). The glyoxylate cycle is unique to plants and microorganisms such as E. coli and fungi and is essential for growth on non-fermentable carbon sources like fatty acids, ethanol or acetate as sole carbon source. Disruption or mutation of Icl1 or Mls1 leads to a complete growth defect on non-fermentable carbon sources (14, 26, 29). The glyoxylate cycle essentially is a shunt of the TCA cycle that acts on isocitrate, thereby excluding the decarboxylation steps of the TCA cycle catalyzed by isocitrate dehydrogenase (Idh1) and α-ketoglutarate dehydrogenase (α-Kgdh) (**Fig. 1B**). The glyoxylate cycle was shown to be essential for virulence in C. albicans (26) and the group of Lorenz also mapped the upregulation of C. albicans genes involved in alternative carbon metabolism during phagocytosis by macrophages (25). The upregulation of the β-oxidation machinery during phagocytosis suggested an important role of this metabolic pathway in virulence; however, C. albicans fox2 null strains that are unable to β-oxidize fatty acids exhibit no gross virulence defects (33, 36). In support of these findings, carnitine acetyl-transferase (cat2) null strains that are unable to use carnitine for transport of acetyl units between compartments also do not display any virulence defects (48, 55). Therefore, the exact role of the glyoxylate cycle and the contribution of alternative carbon metabolism to C. albicans pathogenesis remain unclear.
The subcellular distribution of the (key) glyoxylate cycle enzymes varies between organisms (reviewed in (22) (Fig. 2A and B). In *C. albicans* Icl1 and Mls1 are compartmentalized in peroxisomes (32), as is the case in plants and fungi, with the exception of *S. cerevisiae* where Icl1 is cytosolic and Mls1 is localized to the peroxisomes or cytosol depending on the growth conditions (21, 28). Aconitase (Aco), the glyoxylate/TCA enzyme that converts citrate to isocitrate, has been reported to be extra-mitochondrial in plants (5) and yeast (38). The *C. albicans* genome encodes two aconitase enzymes, Aco1 and Aco2, both of which have a predicted mitochondrial targeting sequence (MTS), but Aco1 additionally has a putative peroxisomal targeting sequence (PTS1; -SKY_COOH). We determined the subcellular distribution of the aconitases experimentally and found a dual localization. The majority of the signal detected with an α-Aco1 antibody co-fractionated with the mitochondria and some associated with the peroxisomes (our unpublished data). The presence of Aco1 in peroxisomes implies transport of citrate from mitochondria to peroxisomes where it is converted to isocitrate and can feed into the glyoxylate cycle. Final proof for a peroxisomal localization of Aco1 and transport of citrate over the peroxisomal membrane in *C. albicans*, however, awaits further experiments.

Other non-fermentable carbon sources that can be utilized by fungi are ethanol and acetate. After uptake these substrates are converted in the cytosol to acetyl-CoA (Fig. 1B). Because fungi possess the glyoxylate cycle, ethanol or acetate can be utilized not only as energy source but also as building blocks for higher order carbohydrates. To accomplish this, cytosolic acetyl units need to be transported to both the mitochondrial TCA cycle and the peroxisomal glyoxylate cycle, the mechanism of which will be discussed below. Therefore growth of fungi on ethanol or acetate as sole carbon source is dependent on the glyoxylate cycle enzymes Icl1 and Mls1 (14, 26, 29) but also on transport of acetyl units between subcellular compartments (47, 48, 55).

**Carnitine-dependent and -independent transport of acetyl units**

Because of its amphiphilic nature and bulkiness, acetyl-CoA cannot freely cross biological membranes (1, 41, 52). Two major pathways have been identified that allow the export acetyl units generated by peroxisomal β-oxidation: i) a carnitine shuttle in which carnitine-acetyl transferases reversibly link the carrier carnitine to the acetyl unit of acetyl-CoA forming acetyl-carnitine that can cross the membrane, and ii) through the action of peroxisomal citrate synthase
that catalyzes the condensation of acetyl-CoA with oxaloacetate to form citrate, which can be transported over the peroxisomal membrane. The relative importance of the two modes of acetyl unit transport varies from species to species. In plants only the citrate synthase pathway is operational (34), whereas most fungi use the carnitine shuttle (Fig. 2A). A notable exception is *S. cerevisiae* for which it has been shown that both pathways function in parallel (Fig. 2B) (50).

The genomes of *C. albicans* and *S. cerevisiae* encode three putative carnitine acetyl-transferases: Cat2, Yat1 and Yat2 (also called CTN2, CTN1 and CTN3 in *C. albicans*) (35). Cat2 is the major carnitine acetyl-transferase that has a dual localization to peroxisomes and mitochondria in both organisms (6, 48). A dual localization of Cat2 has also been shown for *Candida tropicalis* (19) and is predicted for most other fungi (our unpublished observations), suggesting that the carnitine shuttle is operational in most of these species. However, BLAST analysis of the sequenced fungal genomes has revealed that most fungal species seem to lack a predicted peroxisomal citrate synthase (48). Consistent with this, we have shown experimentally that *C. albicans* peroxisomes do not contain citrate synthase and, therefore, this organism is completely dependent on Cat2 for the transport of acetyl units (48, 55) (Fig. 2A). The dependence of *C. albicans* on carnitine is also emphasized by the finding that this organism expresses a functional carnitine biosynthesis pathway (46), while *S. cerevisiae* is dependent on Cit2 activity in the absence of sufficient carnitine (53). Phylogenetic analysis suggests that in *S. cerevisiae* the mitochondrial and peroxisomal Cit originate from a recent gene duplication (12), again emphasizing that yeast is likely to represent the outlier amongst the fungi in the way acetyl units are exported from peroxisomes.

Peroxisomal Cat2 generates acetyl-carnitine inside the organelle, which can subsequently be transported across the membrane. Upon arrival in the mitochondria, the acetyl units are released from acetyl-carnitine by the reverse reaction catalyzed by mitochondrial Cat2 (Fig. 1B). Although this metabolic scheme indicates that peroxisomal and mitochondrial Cat2 are equally important for acetyl unit transport from peroxisomes to mitochondria during growth on fatty acids, this does not seem to be the case. Remarkably, the study of *C. albicans* mutant strains that specifically lack either the mitochondrial Cat2 (perCAT2 strain) or the peroxisomal Cat2 (mitCAT2 strain) showed that peroxisomal Cat2 is not essential for fatty acid β-oxidation. The
high levels of β-oxidation in the mitCAT2 strain (measured by CO₂ production in whole cells) suggests that acetyl units are still exported from the peroxisomal matrix and enter the mitochondrial TCA cycle, even in the absence of peroxisomal Cat2 (47). A carnitine-independent route for exporting acetyl units has been demonstrated in mammals (24). Here, it was shown that peroxisomal thioesterases convert acetyl-CoA to acetate that is able to cross the peroxisomal membrane. We hypothesize that a similar pathway may exist in C. albicans. The C. albicans genome encodes four thioesterases that are predicted to be peroxisomal, a situation that again is more comparable to humans (three thioesterases) than to S. cerevisiae (a single thioesterase). In addition, transport of acetyl units from peroxisomes to mitochondria in the absence of peroxisomal CAT2 was shown to be dependent on cytosolic carnitine acetyltransferase Yat1 (Fig. 1B). This dependence suggests that transport of acetyl-CoA over the mitochondrial membrane and/or feeding into the TCA cycle in C. albicans requires formation of acetyl-carnitine in the cytosol (47). This is interesting, since experiments in S. cerevisiae suggest that acetate can be transported over the mitochondrial membrane under acid pH conditions (8).

Surprisingly, while the mitCAT2 strain displays very high β-oxidation activity, it is unable to grow on fatty acids as sole carbon source. This uncoupling of fatty acid oxidation and growth points towards a defect of the glyoxylate cycle and more specifically a peroxisomal import defect of (iso)citrate when oleate is the carbon source but not when the mutant cells are grown on acetate or ethanol (47). During growth on acetate and ethanol, acetyl-CoA is produced in the cytosol and needs to be transported to the peroxisomal glyoxylate cycle and the mitochondrial TCA cycle. We have shown in C. albicans that growth on ethanol and acetate is dependent on the mitochondrial Cat2, but not strictly on the peroxisomal Cat2. This again suggests that acetate might be capable of crossing the peroxisomal membrane but not the mitochondrial (inner) membrane (47). This is fundamentally different in S. cerevisiae as a cit2Δ/cat2Δ does not display any β-oxidation activity in intact cells, while normal β-oxidation takes place in total lysates of the cit2Δ/cat2Δ strain when membrane barriers are absent. These results strongly suggest that S. cerevisiae is unable to export acetyl units when both Cit2 and Cat2 are absent and support the notion that in yeast acetyl-CoA (or acetate) cannot cross the peroxisomal membrane (52) (Fig. 2B).
The carnitine acetyl-transferase homologues Yat1 and Yat2 localize to the cytosol in *C. albicans* (47), while *S. cerevisiae* Yat1 localizes to the outer mitochondrial membrane (44) and Yat2 localizes to the cytosol (11) (Fig. 2). In *S. cerevisiae* disruption of either *YAT1* or *YAT2* in the *cit2Δ/cat2Δ* background results in the same growth phenotype as the *cit2Δ/cat2Δ* strain: all double knockouts are unable to grow on oleate, ethanol or acetate (49) (Fig. 2C). It is currently unclear why all three carnitine acetyl-transferases are essential for growth on non-fermentable carbon sources in a strain lacking peroxisomal citrate synthase. Because of their homology to carnitine acetyl-transferases and their observed subcellular localization, both *C. albicans* Yats were initially hypothesized to function as carnitine acetyl-transferases acting on cytosolic acetyl-CoA derived from ethanol and acetate (Fig. 1B and 2A). However, the growth phenotypes of *C. albicans YAT1* and *YAT2* disruption strains are quite different: a *yat1* null strain grows like wild type on oleate but is unable to grow on ethanol or acetate, while a *yat2* null strain does not display any growth defect (Fig. 2C) (35, 55). In addition, we recently showed that in *C. albicans* Yat1 is a genuine cytosolic carnitine acetyl-transferase, but Yat2 does not contribute to acetyl-carnitine formation during growth on oleate or acetate (47). From these findings the picture emerges that in *C. albicans* Yat1 handles the conversion of cytosolic acetyl units and Yat2 is not a functional carnitine acetyl-transferase. Establishing the true function of Yat2 remains an interesting challenge in the field.

**Carnitine transporters**

Acetyl-carnitine is transported over the peroxisomal membrane in fungi, but specific peroxisomal acetyl-carnitine transporter protein(s) have thus far not been identified. Acetyl-carnitine and acyl-carnitine transport over the mitochondrial membranes on the other hand has been well characterized in humans, where peroxisomal β-oxidation acts on very long chain fatty acids and shortened long chain acyl units are transported to the mitochondrial matrix as acyl-carnitine for further oxidation. Long chain fatty acids taken up by the cells are first activated in the cytosol to acyl-CoAs. The enzyme carnitine palmitoyl-transferase I (CPTI) associated with the outer mitochondrial membrane then links carnitine to the acyl unit of acyl-CoA. Next, acyl-carnitine is transported over the outer mitochondrial membrane by diffusion or an unknown transporter and over the inner mitochondrial membrane via the specific carrier carnitine-acylcarnitine translocase (CACT). CPTII in the mitochondrial matrix links the acyl unit to
intramitochondrial CoA, forming acyl-CoA that can enter mitochondrial fatty acid β-oxidation. CACT is also able to transport acetyl-carnitine (reviewed by (42). The *S. cerevisiae* gene Yor100c (CRC1) was identified as the ortholog of human CACT (31, 53) and the human carrier was shown to functionally complement a *S. cerevisiae cit2Δ/crc1Δ* mutant (17). Based on sequence similarity, the likely *C. albicans* CACT/CRC1 ortholog is *orf19.2599*. Besides carnitine-mediated transport between organelles, carnitine can also be taken up from the extracellular medium. *S. cerevisiae* Agp2, a transporter that is a member of the amino acid permease family, was shown to localize to the plasma membrane where it is responsible for the uptake of carnitine from the extracellular medium (53). We identified the *C. albicans orf19.4679* as the most likely Agp2 ortholog based on blast analysis, but a disruption mutant was still able to import carnitine (our unpublished observations). The screen performed by Van Roermund et al. (53) aimed at isolation of *S. cerevisiae* mutants affected in acetyl unit transport from peroxisomes to mitochondria failed to identify a peroxisomal acyl/acetyl-carnitine carrier. A possible explanation for this observation is that recent evidence suggests that the peroxisomal membrane contains pore-forming proteins that enable transfer of small molecules (MW less than 400 Da) across the membrane (1, 13, 39, 41). This finding may suggest that a small molecule like acetyl-carnitine does not require a specific transporter but can pass the peroxisomal membrane through a pore-forming channel. Characterization of metabolite transport over the peroxisomal membrane remains a big challenge for the future.

**Acknowledgements**

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**References**


52. van Roermund, C. W., Y. Elgersma, N. Singh, R. J. Wanders, and H. F. Tabak. 1995. The membrane of peroxisomes in Saccharomyces cerevisiae is impermeable to NAD(H) and acetyl-CoA under in vivo conditions. Embo J 14:3480-3486.


Figure legends

Figure 1. Central carbon metabolism in C. albicans
A. Morphology of peroxisomes and mitochondria in glucose-grown C. albicans cells expressing GFP-tagged 3-ketoacyl-CoA thiolase (POT1/orf19.7520). Peroxisomes were visualized by GFP fluorescence and mitochondria by MitoTracker Red staining.

Pdc: pyruvate decarboxylase, perCat2: peroxisomal Cat2, Scs: succinyl-CoA synthetase, Sdh: succinate dehydrogenase, Tes: thioesterase, Yat1: carnitine acetyl-transferase. Putative carriers/transporters are marked by ●. Question marks indicate uncertainty about conversions or means of transport.

**Figure 2. Acetyl unit transport pathways in fungi**

Schematic representation of acetyl unit transport pathways in *C. albicans* (A) and *S. cerevisiae* (B). Growth phenotypes of deletion mutant strains on different carbon sources (C). Phenotypes of *S. cervisiae* strains with a disrupted *CIT2* gene were assessed in media containing L-carnitine (*). Abbreviations: Aco1: aconitase, β-ox: β-oxidation of fatty acids, Cit2: peroxisomal citrate synthase, Icl1: isocitrate lyase, mitCat2: mitochondrial Cat2, Mls1: malate synthase, perCat2: peroxisomal Cat2, TCA cycle: tricarboxylic acid cycle, Yat1/Yat2: carnitine acetyl-transferase, Glu: glucose, Cit: citrate, Eth: ethanol, Ac: acetate, Ol: olate. +: growth, +/-: weak growth, -: no growth.
Figure 1

A. Images of peroxisomes and mitochondria.

B. Diagram illustrating the metabolic pathways between peroxisomes and mitochondria.

Key metabolic terms include:
- β-oxidation
- acetyl-CoA
- acyl-CoA
- acyl-carnitine
- carnitine shuttle
- glyoxylate cycle
- Glucose
- NAD
- NADH
- Gluconeogenesis
- Fatty acid biosynthesis

Various enzymes and pathways are depicted, such as Acetyl-CoA, Malonyl-CoA, and metabolic intermediates like succinate and oxaloacetate.
Figure 2

A. *C. albicans*/most fungi

B. *S. cerevisiae*

C. *C. albicans* mutant strain:

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S. cerevisiae mutant strain:

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