Carnitine acetyltransferases are required for growth on non-fermentable carbon sources but not for pathogenesis in *Candida albicans*

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Carbon starvation is a significant stress encountered by the opportunistic fungal pathogen *Candida albicans*, and mutations in several pathways required to assimilate non-fermentable carbon sources attenuate virulence. These pathways – β -oxidation, the glyoxylate cycle and gluconeogenesis – are compartmentalized in the fungal cell between the peroxisome, mitochondria and cytosol; thus, the cell must transport key intermediates between these organelles. Transport of acetyl-CoA, a particularly important intermediate of carbon metabolism, is catalysed by membrane-associated carnitine acetyltransferases (CATs). We report here the characterization of the three predicted CAT genes in *C. albicans*, *CTN1*, *CTN2* and *CTN3*. Strains lacking *CTN1* or *CTN2* were unable to grow on ethanol or acetate as sole carbon source; additionally, citrate was utilized poorly ($\Delta ct n2$) or not at all ($\Delta ct n1$) and the $\Delta ct n2$ mutant failed to grow on fatty acids as well. In contrast, deletion of *CTN3* had no observable phenotype. All three genes were upregulated in the presence of non-fermentable carbon sources and after macrophage phagocytosis. *CTN1* and *CTN3* were able to complement the corresponding *Saccharomyces cerevisiae* $\Delta yat1$ and $\Delta yat2$ mutants. However, these mutants had no obvious attenuation in virulence in a mouse model of disseminated candidiasis, in contrast to other carbon metabolism mutants. These findings extend our understanding of nutrient stress *in vivo* and *in vitro* and the contribution of metabolic pathways to virulence in *C. albicans*.

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INTRODUCTION

The polymorphic yeast *Candida albicans* is the most common fungal pathogen of humans and it can infect virtually any tissue and organ within the body. The most serious manifestation, disseminated haematogenous candidiasis, has a mortality rate of ~40% (Wenzel, 1995; Wisplinghoff *et al.*, 2004). *C. albicans* is also, however, a ubiquitous and harmless part of our normal microbiota. The transition from commensal to pathogen generally results from changes in the host innate immune system. Therefore, the interaction between this fungal pathogen and phagocytic cells is a key to disease development.

We focus on the interaction between macrophages and *C. albicans*. *In vitro*, *C. albicans* is able to survive phagocytosis by differentiating into elongated hyphae that eventually disrupt the macrophage. An extensive microarray analysis of the *Candida*–macrophage interaction revealed a very complex programme of gene expression in response to phagocytosis in this fungus; a substantial part of this programme was the upregulation of specific metabolic pathways and stress response systems (Lorenz *et al.*, 2004).

Expression analysis of neutrophil–*C. albicans* interactions revealed a similarly complex response, but one that was quite different in terms of the pathways and processes induced (Fradin *et al.*, 2003; Rubin-Bejerano *et al.*, 2003). Thus it is clear that *C. albicans* elaborates a very sophisticated response to immune cell contact.

The transcript profiles of phagocytosed cells resemble those of cells starved for carbon, with particular induction of pathways to utilize alternative or non-fermentable carbon sources (Lorenz *et al.*, 2004). Like other fungi, *C. albicans* uses glucose as the preferred carbon source, but can utilize a variety of non-fermentable carbon sources such as ethanol, acetate, citrate, lactate and oleate. These alternative carbon sources are assimilated through three main metabolic pathways: β -oxidation, the glyoxylate cycle and gluconeogenesis. We and others have shown that disabling these pathways through the mutation of key enzymes causes varying degrees of attenuation of virulence in a mouse model of disease (Barelle *et al.*, 2006; Lorenz & Fink, 2001; Piekarska *et al.*, 2006; Ramírez & Lorenz, 2007). These studies make it clear that carbon starvation is a relevant and important stress encountered by *C. albicans* during infection; as a result we are interested in further defining metabolic requirements *in vivo*.

Abbreviation: CAT, carnitine acetyltransferase.

A key intermediate in alternative carbon metabolism is acetyl-CoA, which is produced by the breakdown of ethanol, fatty acids and some amino acids, and is used by the glyoxylate and TCA cycles. Some of these processes are compartmentalized in the fungal cell, occurring in the peroxisome, mitochondria or cytosol, necessitating intracellular transport of acetyl-CoA. This is facilitated by carnitine acetyltransferases (CATs) that catalyse the transfer of the acetyl group from acetyl-CoA to carnitine followed by the transport of acetylcarnitine across the membrane. This process is referred to as the 'carnitine shuttle' (Swiegers *et al.*, 2001) and is important during growth on non-fermentable carbon sources. *Saccharomyces cerevisiae* has three CAT genes, *YAT1*, *YAT2* and *CAT2* (Swiegers *et al.*, 2001). *C. albicans* also has three predicted CAT genes, *CTN1*, *CTN2* and *CTN3* (Prigneau *et al.*, 2004); these genes are significantly upregulated by macrophage phagocytosis (Lorenz *et al.*, 2004; Prigneau *et al.*, 2004).

Here we describe the characterization of the *CTN* genes in *C. albicans*. To this end, we generated deletion mutants in the *CTN1*, *CTN2* and *CTN3* genes and tested their growth *in vitro* and *in vivo*. Δ *ctn1* and Δ *ctn2* mutants had an overlapping spectrum of growth defects in the presence of alternative carbon sources: the Δ *ctn1* strain could not utilize acetate, ethanol or citrate while the Δ *ctn2* mutant did not grow on acetate, ethanol or oleate and grew poorly on citrate. The Δ *ctn3* mutant had no obvious phenotype, as previously reported (Prigneau *et al.*, 2004). These phenotypes are more severe than the *S. cerevisiae* CAT mutants,

reinforcing recent findings that there are significant differences in carbon metabolism between the two species. We verified that all three genes were induced in the macrophage, as well as on some non-fermentable carbon sources. We also showed that *C. albicans* *CTN1* and *CTN3* complement the corresponding *S. cerevisiae* mutants. None of these mutants, however, affected virulence in a mouse model of disseminated candidiasis. These findings extend our understanding of alternative carbon source utilization pathways in *C. albicans* and their role in pathogenesis.

METHODS

Strain construction. Strains are listed in Table 1. The *C. albicans* Δ *ctn1*, Δ *ctn2* and Δ *ctn3* mutants were constructed using the SAT1-flipper cassette (Reuß *et al.*, 2004). Briefly, 600 bp fragments flanking the gene of interest were cloned between *KpnI* and *XhoI* (5') or *SacI* and *SacI* (3') sites in pSFS1. The resulting plasmid was linearized with *KpnI* and *SacI* and the disruption cassette was used to transform strain SC5314 by electroporation (Reuß *et al.*, 2004) with selection on YPD plates containing 100 µg nourseothricin ml⁻¹. Correct integration was confirmed by PCR, and excision of the Nour^R cassette was induced by growth in YCB-BSA at 30 °C for 24 h. Proper excision was confirmed by PCR. The second allele was deleted in the same manner. Mutants were complemented by reintroducing the ORF at its native locus. The upstream fragment of the disruption construct was replaced with the complete ORF; the transforming and selecting were the same as for the disruptions. The double mutant Δ *ctn1* Δ *ctn3* was constructed by sequentially deleting the two genes.

The *S. cerevisiae* mutants Δ *yat1*, Δ *yat2*, Δ *cat2* and Δ *cit2* were obtained from the haploid collection made by the *Saccharomyces* Genome

Table 1. Strains

Strain	Genotype	Source or reference
<i>C. albicans</i>		
SC5314	Wild-type	Fonzi & Irwin (1993)
MRC6	Δ <i>fox2::hisG</i> / Δ <i>fox2::hisG</i> <i>ura3/ura3</i> <i>RPS10/rps10::Clp10-URA3</i>	Ramírez & Lorenz (2007)
MLC48	SC5314 <i>ctn1::FRT/ctn1::FRT</i>	This study
MLC49	SC5314 <i>ctn1::FRT/ctn1::FRT</i>	This study
MLC50	SC5314 <i>ctn3::FRT/ctn3::FRT</i>	This study
MLC51	SC5314 <i>ctn3::FRT/ctn3::FRT</i>	This study
MLC53	SC5314 <i>ctn1::FRT/ctn1::FRT</i> , <i>ctn3::FRT/ctn3::FRT</i>	This study
MLC56	SC5314 <i>ctn1::FRT/ctn1::FRT</i> , <i>ctn3::FRT/ctn3::FRT</i>	This study
MLC58	SC5314 <i>ctn1::FRT/ctn1::FRT::CTN1</i>	This study
MLC60	SC5314 <i>ctn3::FRT/ctn3::FRT::CTN3</i>	This study
HZC30	SC5314 <i>ctn2::FRT/ctn2::FRT</i>	This study
HZC31	SC5314 <i>ctn2::FRT/ctn2::FRT</i>	This study
HZC32	SC5314 <i>ctn2::FRT/ctn2::FRT::CTN2</i>	This study
<i>S. cerevisiae</i>		
BY4741	<i>MATa/his3/leu2/met17/ura3</i>	Brachmann <i>et al.</i> (1998)
	BY4741 <i>yat1</i>	Winzeler <i>et al.</i> (1999)
	BY4741 <i>yat2</i>	Winzeler <i>et al.</i> (1999)
	BY4741 <i>cat2</i>	Winzeler <i>et al.</i> (1999)
	BY4741 <i>cit2</i>	Winzeler <i>et al.</i> (1999)
	BY4741 <i>cit2/yat1</i>	This study
	BY4741 <i>cit2/yat2</i>	This study
	BY4741 <i>cit2/cat2</i>	This study

Deletion Project (Winzeler *et al.*, 1999). $\Delta yat1 \Delta cit2$, $\Delta yat2 \Delta cit2$ and $\Delta cat2 \Delta cit2$ double mutants were constructed by introducing $\Delta cit2::Nour^R$ into $\Delta yat1$, $\Delta yat2$ and $\Delta cat2$ strains using a PCR disruption approach (Wach *et al.*, 1994) using the *Nour* resistance cassette from pAG25 (Goldstein & McCusker, 1999). Correct double mutants were confirmed by PCR.

Plasmid construction. To make *S. cerevisiae* overexpression plasmids, the *ScYAT1*, *ScYAT2* and *ScCAT2* ORFs were PCR amplified and cloned between *EcoRI* and *HindIII* sites in pRS426-GPD, a high-copy vector with the constitutive *GPD1* promoter (Mumberg *et al.*, 1995); *CaCTN1* and *CaCTN3* were cloned between *BamHI* and *Sall* sites; *CaCTN2* was cloned between *BamHI* and *XhoI* sites. Plasmids used in this study are listed in Table 2.

In vitro growth assays. To test the growth of *C. albicans* strains on different carbon sources, the strains were grown in YPD to mid-exponential phase. Cells were collected by centrifugation and washed with water. They were transferred to a 96-well plate at OD₆₀₀ ~0.5 and serially diluted fivefold. They were spotted using a multiprong pin replicator onto solid YNB medium containing 2% glucose, potassium acetate, ethanol, sodium citrate, lactate or oleate as the sole carbon source, and incubated at 30 °C for 2–7 days, depending on the carbon source.

For the *S. cerevisiae* complementation assays, mutant strains $\Delta yat1 \Delta cit2$, $\Delta yat2 \Delta cit2$ and $\Delta cat2 \Delta cit2$ were transformed with the pRS426-GPD plasmids. Transformants were patched to YNB medium containing 2% glucose and amino acids to satisfy growth requirements. Plates were incubated at 30 °C for 3 days and replica plated to media containing 2% glucose, or 2% ethanol plus 10 µg carnitine ml⁻¹, and incubated at 30 °C for 3–10 days.

Northern analysis. SC5314 cells were grown in YNB with 2% glucose overnight, collected by centrifugation, washed twice with water, resuspended in YNB with 2% glucose, acetate, oleate or lactate and grown for 1 h. Cells were collected by centrifugation and frozen in dry ice-ethanol. Total RNA was extracted using the hot acid phenol method as described by Ausubel *et al.* (1993). Fifteen nanograms of each sample was separated on a 1% MOPS-agarose gel with formaldehyde and then transferred to a nylon membrane. Gene-specific probes were amplified by PCR and were labelled with [³²P]dCTP using the RadPrime DNA labelling system (Invitrogen). Probes were purified using Roche Quick Spin columns. Blots were incubated in pre-hybridization solution containing 5 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate), 50% formamide, 5 × Denhardt's solution, 0.1% SDS and 100 µg ml⁻¹ single-stranded DNA for 2 h at 42 °C followed by hybridization overnight. rRNA was used as a loading control.

RT-PCR. Cells (4×10^7) of the murine macrophage line J774A were plated in a 750 ml tissue culture flask in RPMI with 10% FBS and incubated overnight at 37 °C with 5% CO₂. Overnight cultures of strain SC5314 were diluted 1:50 in YPD and grown for ~4 h at 30 °C. Cultures were collected by centrifugation, washed in PBS and counted using a haemocytometer; 10^8 *C. albicans* cells were added to the flasks containing macrophages (to give a *C. albicans* : macrophage ratio of approx. 2:1) and incubated for 1 h. For control cultures, 2×10^8 *C. albicans* cells were inoculated to tissue culture flasks in 50 ml RPMI plus 10% serum. Cells were collected by scraping the flasks with rubber scrapers in ice-cold water and transferring to 50 ml conical tubes. Cells were centrifuged at 3000 r.p.m. at 4 °C, washed twice with ice-cold water, transferred to microfuge tubes, and frozen on dry ice. Total RNA was isolated by hot phenol extraction (Ausubel *et al.*, 1993). First-strand cDNA was synthesized according to the Invitrogen SuperScriptII RT protocol. The cDNA product was diluted 1:10 twice and 2 µl of each dilution was used as template in the PCR reaction. Primers were designed to amplify a ~300 bp internal fragment of *CTN1*, *CTN2* or *CTN3*, or 18S rRNA as a control.

Filamentation assay. *C. albicans* strains from fresh YPD plates were struck to single colonies on medium 199 (pH 7.0), or YP medium containing 3% glycerol or 10% serum. Plates were incubated at 37 °C for 4 days. Colony morphology was examined under a Zeiss dissecting scope. Images were assembled in Adobe Photoshop.

In vivo virulence assays. Mouse virulence assays were carried out as described previously (Ramírez & Lorenz, 2007). Adult, female, outbred ICR mice (21–25 g) were obtained from Harlan. Cultures of *C. albicans* were grown in YPD and serially passaged twice by overnight growth at 30 °C. Cells were collected by centrifugation, washed and resuspended in PBS. Mice were infected through tail vein injections with 10^6 *C. albicans* yeast-form cells in 100 µl PBS. The group size was 10 mice per strain. Infected mice were monitored for signs of infection and euthanized when moribund according to approved protocols. Survival data were analysed with Prism3 (Graphpad Software) using the log rank test. All animal assays were conducted in accordance with protocols approved by the University of Texas Health Science Center Animal Welfare Committee.

RESULTS

Carbon metabolic pathways are compartmentalized into several organelles in eukaryotes, including the mitochondria and peroxisome, as well as the cytosol, and several 'shuttles' facilitate the intracellular transport of various intermediates. Acetyl-CoA, the most important of these

Table 2. Plasmids

Plasmid	Description	Resource or reference
pSFS1	<i>FRT/P_{SAP2} FLP/SAT1/FRT</i>	Reuß <i>et al.</i> (2004)
pAG25	natMX4	Goldstein & McCusker (1999)
pRS426-GPD		Mumberg <i>et al.</i> (1995)
pHZ110	pRS426-GPD- <i>YAT1_{Sc}</i>	This study
pHZ111	pRS426-GPD- <i>YAT2_{Sc}</i>	This study
pHZ112	pRS426-GPD- <i>CAT2_{Sc}</i>	This study
pHZ113	pRS426-GPD- <i>CTN1_{Ca}</i>	This study
pHZ114	pRS426-GPD- <i>CTN3_{Ca}</i>	This study
pHZ115	pRS426-GPD- <i>CTN2_{Ca}</i>	This study

Fig. 1. Schematic representation of the compartmentalization of alternative carbon sources, metabolic pathways and the carnitine shuttle. The *C. albicans* CATs Ctn1p, Ctn2p and Ctn3p are shown with their predicted localization and function. The figure is adapted from Swiegers *et al.* (2001).

intermediates, is transported by three CATs. Fig. 1 shows the organization of these pathways inside the cell and the ‘carnitine shuttle’ that serves to transport the key intermediate acetyl-CoA between the compartments.

Mutant strain construction

There are three genes coding for predicted CATs in *C. albicans*: *CTN1* (*YAT1*, *CAT1*, *orf19.4551*), *CTN2* (*CAT2*, *orf19.4591*) and *CTN3* (*YAT2*, *CAT3*, *orf19.2809*). All three are upregulated significantly following macrophage phagocytosis (by 20.4-, 7.2- and 3.9-fold, respectively; Lorenz *et al.*, 2004). Ctn1p is predicted to be associated with the outer mitochondrial membrane and is 58 % similar to *S. cerevisiae* *YAT1*. Ctn3p has two peroxisome-targeting signal motifs; otherwise it is 53 % similar to the cytosolic ScYat2p. *CTN2* has two predicted translation initiation sites, with a mitochondrial targeting signal after the first methionine, and a peroxisome-targeting signal after the second methionine, suggesting a dual localization. This is a similar architecture to the yeast *CAT2* gene, whose product is both peroxisomal and mitochondrial (Elgersma *et al.*, 1995). These proteins are 63 % similar to each other (Prigneau *et al.*, 2004).

To disrupt these three genes, we used the *SAT1* flipper approach (Reuß *et al.*, 2004) to precisely remove the ORFs of *CTN1*, *CTN2* and *CTN3* (see Methods). The *SAT1* cassette (encoding resistance to nourseothricin) was excised and the second allele was disrupted in the same way. All three mutants were complemented by inserting one copy of the ORF back at its original locus as described (Reuß *et al.*, 2004; see Methods). We also constructed a Δ ctn1 Δ ctn3 double mutant as part of the process of making a strain lacking all three *CTN* genes (we have not tested the Δ ctn1 Δ ctn2 or Δ ctn2 Δ ctn3 combinations). To date we have been unable to construct this triple mutant strain, which may indicate that this is an essential gene family. All mutants described here are homozygous deletions unless stated otherwise (see Table 1).

CTN mutants do not grow on certain non-fermentable carbon sources

We used a spot dilution assay to test the ability of the CAT mutant strains to assimilate different carbon sources. Strains were grown to exponential phase, washed and serially diluted, then spotted using a pin replicator to minimal YNB medium containing 2 % glucose, potassium acetate, ethanol, sodium citrate, lactate or the monounsaturated fatty acid oleate as the sole carbon source (Fig. 2). We included a Δ fox2 mutant, lacking the β -oxidation gene, as a control; this mutant has pleiotropic defects on several carbon sources (Ramírez & Lorenz, 2007; Piekarska *et al.*, 2006).

As shown in Fig. 2, the Δ ctn1 mutant was unable to grow on ethanol, potassium acetate and sodium citrate, although its growth on oleate and lactate was indistinguishable from the wild-type strain in this plate assay. The Δ ctn3 mutant did not show defects in growth on any of the carbon sources tested; this mutant was previously reported to have no phenotype in the presence of glucose, acetate or methanol (Prigneau *et al.*, 2004). The double Δ ctn1 Δ ctn3 mutant had the same phenotype as the single Δ ctn1 mutant. The Δ ctn2 mutant failed to grow on media containing acetate, ethanol, or oleate and was significantly compromised on citrate. Growth was restored in the complemented strains. We also tested a second, independent mutant for each gene and observed identical results (data not shown).

These findings are in contrast to *S. cerevisiae*, in which the Δ yat1, Δ yat2 and Δ cat2 mutants do not have any growth defect on non-fermentable carbon sources unless the peroxisomal citrate synthase *CIT2* is also mutated (van Roermund *et al.*, 1995; Swiegers *et al.*, 2001). Though these proteins and metabolic pathways are very highly conserved, several similar phenotypic discrepancies have been recently described (Piekarska *et al.*, 2006; Ramírez & Lorenz 2007; Sexton *et al.*, 2007; M. A. Ramírez & M. C. Lorenz, unpublished observations), suggesting that there has been

Fig. 2. Growth assay on different carbon sources. *C. albicans* wild-type (SC5314), $\Delta fox2$ (MRC6), $\Delta ctn1$ (MLC48), $\Delta ctn3$ (MLC50), $\Delta ctn1 \Delta ctn3$ (MLC53), $\Delta ctn1::CTN1$ (MLC58), $\Delta ctn3::CTN3$ (MLC60), $\Delta ctn2$ (H3C30) and $\Delta ctn2::CTN2$ (H3C32) strains were grown to mid-exponential phase and serially diluted 1:5 in a 96-well plate. The dilutions were applied using a multi-prong replicator onto minimal YNB medium containing the indicated carbon sources at 2%.

significant adaptation of the function or regulation of carbon metabolism in these species.

CTN genes are upregulated in macrophages and on non-fermentable carbon sources

To address the regulation of these genes in *C. albicans*, we assayed gene expression following phagocytosis and in the presence of various carbon sources. All three *CTN* genes have been shown to be induced following macrophage phagocytosis by microarray (Lorenz *et al.*, 2004) and Northern analysis (Prigneau *et al.*, 2004). *CTN3*, additionally, was repressed by glucose and induced by multiple carbon sources including glycerol, oleate, serum, acetate, ethanol and methanol (Prigneau *et al.*, 2004). Given the homology between these genes, there is some concern that cross-hybridization could complicate this analysis, so we re-examined gene expression, testing for specificity using our knockout strains.

We collected wild-type *C. albicans* (SC5314) 1 h after macrophage phagocytosis and assayed gene expression by RT-PCR (Fig. 3a). *CTN1* and *CTN3* were strongly upregulated, as shown previously (Lorenz *et al.*, 2004; Prigneau *et al.*, 2004), though we were unable to demonstrate induction of *CTN2* (which was the least induced CAT by microarray). The RT-PCR primers did not amplify a band from the corresponding mutant strains, demonstrating specificity (data not shown).

We next examined the expression pattern of these genes in different carbon sources by Northern blot analysis (Fig. 3b). None of the CAT-encoding genes were detectable in glucose- or lactate-grown cells; *CTN1* and *CTN3* were induced in acetate-grown cells and further induced moderately (*CTN3*) or dramatically (*CTN1*) in oleate. *CTN2* was detectable only after growth on the fatty acid oleate (Fig. 3b). The probes are specific to each gene and there was no cross-hybridization (data not shown). In summary, all three CAT genes were upregulated on non-fermentable carbon sources, most strongly in cells exposed to oleate.

The induction of the CAT genes by non-fermentable carbon sources is similar to that of the *S. cerevisiae* homologues. In *S. cerevisiae* *YAT1* was repressed by glucose and induced by

ethanol (Schmalix & Bandlow, 1993). *CAT2* was induced tenfold in oleate as compared to glucose (Atomi *et al.*, 1993).

CTN1 and CTN3 complement S. cerevisiae mutants

The *C. albicans* *CTN* genes are annotated based on their sequence homology to *S. cerevisiae* *YAT1*, *YAT2* and *CAT2*. To determine the validity of these assignments, we tested whether the *C. albicans* *CTN* genes can complement the *S. cerevisiae* mutant phenotypes. Since in *S. cerevisiae*, mutation in CAT genes alone does not yield a growth defect on non-fermentable carbon sources, we introduced the $\Delta cat2$ mutation into $\Delta yat1$, $\Delta yat2$ and $\Delta cat2$ strains (see Methods). Indeed, as previously described (Swiegers *et al.*, 2001), the double mutants were unable to grow on ethanol as sole carbon source (Fig. 4 and data not shown). The double mutant strains were then transformed with high-copy plasmids containing the CAT genes from *S. cerevisiae* or *C. albicans* under the control of a constitutive *GPD1* promoter.

As shown in Fig. 4, the $\Delta cat2 \Delta cat$ double mutant strains transformed with empty vector were unable to grow on ethanol as the sole carbon source, and overexpression of the *S. cerevisiae* genes complements these phenotypes. *CaCTN1* restores growth on ethanol in both the $\Delta yat1$ and $\Delta yat2$ mutants, while *CaCTN3* complements only $\Delta yat2$. We were unable to complement $\Delta cat2$ with any of the *C. albicans* genes, and the *CaCTN2* gene did not function in *S. cerevisiae*. This can occasionally be the result of the altered codon usage in *C. albicans*, but we found no CTG codons in *CTN1* or *CTN2*. *CTN3*, which is functional in the *S. cerevisiae* $\Delta yat2$ mutant, has five CTG codons. Thus, this analysis confirms that there is some conservation of function in this gene family but again suggests that there are some significant differences in carbon metabolism between these related species.

CTN mutants undergo hyphal morphogenesis on solid media

In a previous study, deletion of *CTN3* was found to disrupt filamentation on solid media (Prigneau *et al.*, 2004). We

Fig. 3. Expression pattern of *CTN1*, *CTN3* and *CTN2*. (a) Wild-type (SC5314) cells were added to flasks containing macrophages (10^8 cells, giving a 2 : 1 *C. albicans* : macrophage ratio) in RPMI plus 10 % serum and incubated for 1 h. Control cells were incubated in the same medium without macrophages. Cells were harvested and RNA extracted. The expression of these genes was determined by RT-PCR. For each gene/condition, three 1 : 10 serial dilutions of the RNA (indicated by the gradient sign) were amplified (see Methods). 18S rRNA was amplified as a control. (b) SC5314 was grown to mid-exponential phase in minimal YNB medium with glucose, collected by centrifugation, washed with water, and shifted to medium containing 2 % glucose (Glu), potassium acetate (Ace), oleate (Ole) and lactate (Lac) for 1 h. RNA was extracted from these cells and gene expression was determined by Northern analysis.

tested whether the deletion of the other two genes also affects colony morphology under the same conditions. The wild-type and Δ *ctn1*, Δ *ctn2* and Δ *ctn3* mutant strains were incubated on solid YP-glycerol, YP-serum or medium 199. After 4 days of growth at 37 °C, all four strains formed long peripheral hyphae (Fig. 5 and data not shown), with no difference between the wild-type and the mutant strains under the conditions tested. In contrast to the earlier report for Δ *Actn3*, we found that none of these mutants were

affected in their filamentation ability. This difference in phenotype may be due to the *URA3* deletion marker used in the earlier study; chromosomal position effects of this gene have been found to alter several phenotypes, including hyphal differentiation (Bain *et al.*, 2001; Lay *et al.*, 1998).

***CTN* mutants are not attenuated in virulence in the mouse model**

Previous work has demonstrated that perturbations in alternative carbon source utilization pathways led to mild to significant attenuation in virulence in a mouse model of haematogenously disseminated candidiasis. These include β -oxidation of fatty acids via the Δ *fox2* mutation (Ramírez & Lorenz, 2007; Piekarska *et al.*, 2006), the glyoxylate cycle via the Δ *icl1* mutation (Ramírez & Lorenz, 2007; Lorenz & Fink, 2001), and gluconeogenesis via the Δ *fbp1* (Ramírez & Lorenz, 2007) or Δ *pck1* (Barelle *et al.*, 2006) mutations. To determine if CATs play any role in survival and pathogenesis *in vivo*, we tested the Δ *Actn* mutants in a standard mouse tail vein injection model. Briefly, 10^6 *C. albicans* cells (yeast form) were injected into outbred adult female mice. Infected mice were monitored for signs of infection and euthanized when moribund according to approved protocols.

Mice infected with wild-type strains had a mean time to death (MTD) of 3.9 days (Fig. 6). Mice infected with the mutants had a comparable death rate, with the MTD for Δ *ctn1*, Δ *ctn2* and Δ *ctn3* being 4.8, 4.0 and 4.2 days respectively (Fig. 6a, b, c). The double mutant Δ *ctn1* Δ *ctn3* behaved similarly (Fig. 6d). We also tested an independent strain for each mutant and found the same results. Thus, deletions in the *CTN* genes have no obvious effect on *in vivo* survival and virulence even though they caused defects in carbon utilization *in vitro* that were similar to other avirulent mutants.

DISCUSSION

In this study, we have examined the function and regulation of the three predicted *C. albicans* CAT genes, *CTN1*, *CTN2* and *CTN3*. Mutations in two of the three genes (*CTN1*, *CTN2*) confer growth defects in the presence of non-fermentable carbon sources and all three genes are induced under these conditions. However, strains lacking one or two of these genes retain full virulence, marking a discrepancy between *in vitro* carbon utilization and *in vivo* virulence phenotypes, in contrast to other recent reports for metabolic genes (Piekarska *et al.*, 2006; Ramírez & Lorenz, 2007).

Both the *C. albicans* Δ *ctn1* and Δ *ctn2* mutant strains were unable to grow on media containing ethanol or acetate. Both of these compounds are converted to acetyl-CoA and transported into the mitochondria via the carnitine shuttle, where they can be assimilated via the TCA and/or glyoxylate cycles. Ctn2p is predicted to be in both the

Fig. 4. Complementation of the *S. cerevisiae* mutants. *S. cerevisiae* strains $\Delta yat1 \Delta cit2$, $\Delta yat2 \Delta cit2$ and $\Delta cat2 \Delta cit2$ were transformed with pRS426-GPD-ScYAT1, ScYAT2, ScCAT2, CaCTN1, CaCTN3 and CaCTN2 respectively. Transformants were patched to minimal YNB medium containing 2% glucose and incubated at 30 °C for 3 days before replica plating to minimal YNB medium containing 2% glucose, or 2% ethanol plus carnitine (10 mg l⁻¹), and incubated at 30 °C for 3 (glucose) or 10 (ethanol) days.

peroxisome and the mitochondrial matrix while Ctn1p is associated with the mitochondrial outer membrane (see Fig. 1). Combined with these localizations, our data suggest that acetyl-CoA is converted to acetylcarnitine on the mitochondrial surface by Ctn1p, transported into the matrix, and converted back to acetyl-CoA by Ctn2p before incorporation into the TCA cycle. During growth on fatty acids, the acetyl-CoA produced by peroxisomal β -oxidation is converted to acetylcarnitine by Ctn2p, and could move from the peroxisome to the mitochondria (via the cytosol) with no need for Ctn1p and, indeed, $\Delta ctn1$ mutants can use oleate as a carbon source. The weak growth of $\Delta ctn2$ mutants on citrate is potentially explained by the presence of a mitochondrial citrate transport protein; *C. albicans* has an uncharacterized homologue of the well-studied *S. cerevisiae* Ctn1p protein (Kaplan, *et al.*, 1996), thus citrate can probably enter the mitochondria in the absence of CATs. The phenotypes we observed clearly indicate a need for CAT activity during citrate metabolism, however.

As previously reported, $\Delta ctn3$ strains do not have carbon utilization phenotypes that we could detect (Prigneau *et al.*, 2004). CTN1 complemented deletions in both *S. cerevisiae* YAT1 and YAT2, while CTN3 complemented only the $\Delta yat2$ mutation. This demonstrates a basic conservation of function of these proteins, though this is not absolute, since none of these could restore growth to a *S. cerevisiae* $\Delta cat2$ mutant.

In an earlier study, CTN3 was found to affect filamentous growth, as $\Delta ctn3$ strains were unable to form hyphae in several inducing conditions (Prigneau *et al.*, 2004). In contrast, we report here that deletion of the CTN genes did not disrupt formation of hyphae. While there may be some differences in strain background, both studies used SC5314-derived strains. In the study of Prigneau *et al.* (2004), the CTN3 mutant strains were constructed using the 'URA blaster' method (Fonzi & Irwin, 1993). This discrepancy may be related to URA3 'position effects', which have been subsequently recognized to cause

Fig. 5. Peripheral filaments of *C. albicans*. wild-type (SC5314), $\Delta ctn1$ (MLC48), $\Delta ctn3$ (MLC50) and $\Delta ctn2$ (HZC30) strains from fresh YPD plates were struck to single colonies on YP medium containing 3% glycerol or 10% serum. Plates were incubated at 37 °C for 4 days before photographing under a Zeiss dissecting scope.

Fig. 6. Virulence test in the mouse model of disseminated candidiasis. A total of 10^6 cells of each strain were injected via the tail vein. All assays used the wild-type strain SC5314 as a control. (a) $\Delta actn1$ (MLC48) vs $\Delta actn1::CTN1$ (MLC58); (b) $\Delta actn2$ (H3C30) vs $\Delta actn2::CTN2$ (H3C32); (c) $\Delta actn3$ (MLC50) vs $\Delta actn3::CTN3$ (MLC60); (d) $\Delta actn1 \Delta actn3$ double mutant (MLC53).

numerous phenotypes, including morphogenetic, that are unrelated to the deleted gene (Bain *et al.*, 2001; Brand *et al.*, 2004; Lay *et al.*, 1998). The current study did not use *ura3* auxotrophic strains; instead the mutations were constructed in a prototrophic strain using a dominant drug-resistance marker, nourseothricin (Reuß *et al.*, 2004).

Although the *CTN* mutant strains had dramatic growth defect on non-fermentable carbon sources *in vitro*, there was no attenuation of virulence in the systemic infection model. We found this result surprising, given that the *in vitro* phenotypes of the $\Delta actn$ strains are similar to those of published mutants with known virulence defects, such as $\Delta icl1$, $\Delta fbp1$, $\Delta pck1$ and $\Delta fox2$ (Lorenz & Fink, 2001; Piekarska *et al.*, 2006; Ramírez & Lorenz, 2007; Barelle *et al.*, 2006). This discrepancy may result from greater expression of the *CTN* genes in the host environment, mitigating effects of single gene deletions on overall CAT activity. Alternatively, *C. albicans* could import simple carbon molecules, such as acetate or lactate, from the host that do not require intracellular transport for utilization or that make use of other transport systems, such as the mitochondrial succinate–fumarate shuttle or the citrate transporter. In a co-culture assay with murine macrophage

cell line J774A, all the deletion strains were able to form hyphae and escape from macrophage engulfment at the same rates as the wild-type control (data not shown). This has been observed previously, even in strains with *in vivo* virulence defects (Lorenz & Fink, 2001; Ramírez & Lorenz, 2007) and we believe that the short duration of the macrophage assay does not allow sufficient time to reveal starvation-related defects.

In contrast to the $\Delta actn1$ and $\Delta actn2$ mutations, there is no phenotype associated with the deletion of the *S. cerevisiae* CAT genes (*YAT1*, *YAT2*, *CAT2*) (Kispal *et al.*, 1993; Schmalix & Bandlow, 1993; Swiegers *et al.*, 2001). Indeed, any of these single mutants will grow on non-fermentable carbon sources unless the *CIT2* gene, encoding a peroxisomal isoform of citrate synthase, is also deleted. This indicates a flexibility not present in *C. albicans*, an idea reinforced by the presence of three *CIT* isoforms in *S. cerevisiae*, and only a single gene in *C. albicans* (*CIT1*, highly homologous to all three *S. cerevisiae* proteins). This redundancy likely allows *S. cerevisiae* to run much of the glyoxylate cycle in either the peroxisome or mitochondria and increases the spectrum of compounds that could be shuttled between compartments, thereby reducing the

importance of acetyl-CoA transport under most circumstances. Why this is the case is not clear, but it is tempting to speculate that it improves the efficiency of ethanol utilization, a process more important in the highly fermentative *S. cerevisiae* than in other yeasts.

Though these carbon metabolic pathways and enzymes are highly conserved, there have been several previous examples in which gene deletions do not have the same phenotype in these related species. Snf1p, a kinase involved in glucose repression, is essential in *C. albicans*, but not in *S. cerevisiae* (Petter *et al.*, 1997). In addition, mutations of the *C. albicans* *fox2* gene, encoding an enzyme of β -oxidation, has several pleiotropic phenotypes not seen in the *S. cerevisiae* mutant (Piekarska *et al.*, 2006; Ramírez & Lorenz, 2007). It is worth considering whether the more restricted localization of carbon metabolic pathways in *C. albicans* that we propose here underlies this pleiotropy. The adaptation of metabolic networks to suit the *in vivo* niche of *C. albicans* is likely to be an important aspect of the biology of this pathogen.

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