Role of Acetyl Coenzyme A Synthesis and Breakdown in Alternative Carbon Source Utilization in *Candida albicans*

Aaron J. Carman, Slavena Vylkova, and Michael C. Lorenz*

Department of Microbiology and Molecular Genetics, The University of Texas Health Science Center at Houston, Houston, Texas 77030

Received 28 July 2008/Accepted 4 August 2008

Acetyl coenzyme A (acetyl-CoA) is the central intermediate of the pathways required to metabolize nonfermentable carbon sources. Three such pathways, i.e., gluconeogenesis, the glyoxylate cycle, and β-oxidation, are required for full virulence in the fungal pathogen *Candida albicans*. These processes are compartmentalized in the cytosol, mitochondria, and peroxosomes, necessitating transport of intermediates across intracellular membranes. Acetyl-CoA is trafficked in the form of acetate by the carnitine shuttle, and we hypothesized that the enzymes that convert acetyl-CoA to/from acetate, i.e., acetyl-CoA hydrolase (*ACH1*) and acetyl-CoA synthetase (*ACS1* and *ACS2*), would regulate alternative carbon utilization and virulence. We show that *C. albicans* strains depleted for *ACS2* are uviable in the presence of most carbon sources, including glucose, acetate, and ethanol; these strains metabolize only fatty acids and glycerol, a substantially more severe phenotype than that of *Saccharomyces cerevisiae* *acs2* mutants. In contrast, deletion of *ACS1* confers no phenotype, though it is highly induced in the presence of fatty acids, perhaps explaining why *acs2* mutants can utilize fatty acids. Strains lacking *ACH1* have a mild growth defect on some carbon sources but are fully virulent in a mouse model of disseminated candidiasis. Both *ACH1* and *ACS2* complement mutations in their *S. cerevisiae* homolog. Together, these results show that acetyl-CoA metabolism and transport are critical for growth of *C. albicans* on a wide variety of nutrients. Furthermore, the phenotypic differences between mutations in these highly conserved genes in *S. cerevisiae* and *C. albicans* support recent findings that significant functional divergence exists even in fundamental metabolic pathways between these related yeasts.

*Corresponding author. Mailing address: Department of Microbiology and Molecular Genetics, The University of Texas Health Science Center at Houston, 6431 Fannin St., Houston, TX 77030. Phone: (713) 500-7422. Fax: (713) 500-5499. E-mail: Michael.Lorenz@uth.tmc.edu.

*Published ahead of print on 8 August 2008.*
in a mouse model of disseminated candidiasis. The phenotypes of the *C. albicans* *acs2*Δ mutant are more pronounced than those of the *S. cerevisiae* homolog, indicating some divergence of function, as has been seen previously (27, 29, 42), though CaACS2 fully complements an *S. cerevisiae* mutation. This work continues our efforts to define critical carbon metabolic processes in the important pathogen *C. albicans*.

**MATERIALS AND METHODS**

**Strains and media.** The *C. albicans* strains used in this study are listed in Table 1. Standard yeast media were used (34), including YNB (0.17% yeast nitrogen base, 0.5% ammonium sulfate) and YPD (1% yeast extract, 2% peptone, 2% dextrose). YNB was supplemented with carbon sources as indicated at a final concentration of 2%, 5′-Fluoroorotic acid medium (YNB plus 2% glucose, 0.2 mM uracil, 0.2 mM uridine, and 0.1% 5′-fluoroorotic acid) (3) was also used. *Candida albicans* strains were transformed either by electroporation (30) or by use of a modified lithium acetate method (5).

The *Saccharomyces cerevisiae* strains used in this study are listed in Table 1 and are derived from strain BY4741 (4). Strains were transformed using a modified lithium acetate method (33).

**Candida albicans mutant construction.** All *C. albicans* mutants were constructed in RM1000 (HIS3 Ura3). For the *ach1Δ* and *acs1Δ* mutants, we constructed two disruption constructs, one using the hisG-URA3-hisG cassette (9) and the other using the *Candida dubliniensis* *HIS1* gene (26). Briefly, 300 bp of the 5′ and 3′ untranslated regions flanking the gene were amplified by overlapping PCR into one fragment, with a BamHI site in the middle. This was cloned as a HindIII/XbaI fragment into pBSKI-I. The *hisG-URA3-hisG* cassette was removed from pCUB-6 (9) by a BamHI/BglII/PvuII digest and then ligated into the fusion plasmids cut with BamHI. In parallel, the *CdHIS1* gene was amplified as a BamHI cassette and inserted into the BamHI site in the fusion plasmids. The resulting disruption plasmids are listed in Table 2. Complementing constructs were made by cloning PCR-amplified genes into plasmid pClp10 (22) (Table 2).

The first allele of *ACS2* was knocked out using a *HIS1* disruption construct as described above (pAC26). Correct heterozygous mutants were confirmed by PCR. The second allele of *ACS2* was put under the control of the *MET3* repressible promoter. The first 971 bp of *ACS2* were cloned into pCaDis (7). An overlapping PCR strategy was used to introduce a unique EcoRV cut site into the middle of the fragment. The resulting plasmid (pAC48) was linearized with EcoRV and transformed into the *ACS2* heterozygote mutant. Correct recombinants were confirmed by PCR.

**TABLE 1. Fungal strains**

| Species and strain | Genotype | Source or reference |
|--------------------|----------|---------------------|
| **C. albicans**    |          |                     |
| SC5314 (wild type) | Prototroph|                     |
| RM1000 (ura3 his1) | ura3::Xin443/ura3::Xin443 his1::hisG his1::hisG rps10::CIp10-URA3/RPS10 | This study |
| ACC16 (ach1Δ [no. 1]) | ura3::Xin443/ura3::Xin443 his1::hisG his1::hisG ach1Δ::hisG/ach1Δ::HIS1 | This study |
| ACC17 (ach1Δ+ACH1) | ura3::Xin443/ura3::Xin443 his1::hisG his1::hisG ach1Δ::hisG/ach1Δ::HIS1 | This study |
| ACC20 (ach1Δ [no. 2]) | ura3::Xin443/ura3::Xin443 his1::hisG his1::hisG ach1Δ::hisG/ach1Δ::HIS1 | This study |
| ACC21 (ach1Δ+ACH1) | ura3::Xin443/ura3::Xin443 his1::hisG his1::hisG ach1Δ::hisG/ach1Δ::HIS1 | This study |
| ACC9 (acs1Δ+ACH1) | ura3::Xin443/ura3::Xin443 his1::hisG his1::hisG acs1Δ::hisG/acs1Δ::HIS1 | This study |
| ACC10 (acs1Δ+ACS1) | ura3::Xin443/ura3::Xin443 his1::hisG his1::hisG acs1Δ::hisG/acs1Δ::HIS1 | This study |
| ACC13 (acs1Δ [no. 2]) | ura3::Xin443/ura3::Xin443 his1::hisG his1::hisG acs1Δ::hisG/acs1Δ::HIS1 | This study |
| ACC14 (acs1Δ+ACS1) | ura3::Xin443/ura3::Xin443 his1::hisG his1::hisG acs1Δ::hisG/acs1Δ::HIS1 | This study |
| ACC24 (acs2Δ/ACS2-MET3p [no. 1]) | ura3::Xin443/ura3::Xin443 his1::hisG his1::hisG acs2Δ::HIS1/URS3-MET3p-ACS2 | This study |
| ACC25 (acs2Δ/ACS2-MET3p [no. 2]) | ura3::Xin443/ura3::Xin443 his1::hisG his1::hisG acs2Δ::HIS1/URS3-MET3p-ACS2 | This study |
| MRC10 (icl1Δ) | ura3::Xin443/ura3::Xin443 icl1Δ::hisG/icl1Δ::HIS1 RPS10::URA3 | This study |
| MRC11 (icl1Δ+ICL1) | ura3::Xin443/ura3::Xin443 icl1Δ::hisG/icl1Δ::HIS1 RPS10::ICL1-URA3 | This study |
| **S. cerevisiae** |          |                     |
| BY4741 | his3 leu2 met1 ura3 MATa | 4 |
| BY4741 ach1Δ (library) | his3 leu2 met1 ura3 MATa ach1Δ::G418 | 40 |
| BY4741 acs1Δ (library) | his3 leuc2 met1 ura3 MATa acs1Δ::G418 | 40 |
| BY4741 acs2Δ (ACY1) | his3 leu2 met1 ura3 MATa acs2Δ::G418 | This study |

**TABLE 2. Plasmids**

| Plasmid | Description | Source or reference |
|---------|-------------|---------------------|
| pFA6KanMX | Contains G418 resistance marker | 38 |
| pCaDis | MET3 promoter | 7 |
| pClp10 | RPS10 integrating plasmid | 22 |
| pCUB6 | hisG-URA3-hisG | 9 |
| pAC1 | pClp10-CaACS1 | This study |
| pAC3 | pClp10-CaACH1 | This study |
| pAC7 | pBSK-acs1Δ::hisG-URA3-hisG | This study |
| pAC12 | pBSK-ach1Δ::hisG-URA3-hisG | This study |
| pAC24 | pBSK-ach1Δ::HIS1 | This study |
| pAC25 | pBSK-acs1Δ::HIS1 | This study |
| pAC26 | pBSK-acs1Δ::HIS1 | This study |
| pAC48 | pCaDis-ACS2-MET3p | This study |
| p415-GPD | pRS415-GPD1p LEU2 2μ | 20 |
| pAC60 | pRS415-GPD1p-CaACH1 | This study |
| pAC61 | pRS415-GPD1p-CaACS1 | This study |
| pAC62 | pRS415-GPD1p-CaACS1 | This study |
| pAC63 | pRS415-GPD1p-CaACS1 | This study |
| pAC64 | pRS415-GPD1p-CaACS1 | This study |
| pAC65 | pRS415-GPD1p-CaACS1 | This study |
A. **ACH1 locus**

| ACH1p | histG | HIST1 |

**RPS10 locus**

| RPS10 | or |

| RPS10 | or |

| RPS10 | or |

**Genotype**

| ach1Δ/Δ + ACH1 ACC16, ACC20 |

**Strain name**

| ach1Δ/Δ + ACH1 ACC17, ACC21 |

| ach1Δ/Δ + ACH1 ACC17, ACC21 |

**FIG. 1.** Construction of *C. albicans* mutant strains. (A and B) Both alleles of *ACH1* (A) and *ACS1* (B) were disrupted in RM1000 sequentially by replacing one allele with *C. dubliniensis* *HIS1* and the other with *hisG-URA3-hisG*. After selection on 5-fluoroorotic acid medium, *URA3* was reintroduced at the *RPS10* locus using plasmid Ctp10 either unlinked (mutant strains) or linked (complemented strains) to a wild-type copy of the gene. (C) The first allele of *ACS2* was replaced with the *HIS1* marker, and the *MET3* promoter linked to *URA4* was integrated upstream of the second allele, replacing the native *ACS2* promoter.

**Saccharomyces cerevisiae plasmid construction.** *S. cerevisiae* overexpression constructs are listed in Table 2. *S. cerevisiae* *ACH1*, *ACS1*, and *ACS2* were amplified by PCR and cloned into plasmid p415-GPD (20) between SpeI and XhoI sites to produce plasmids pAC64 (p415-GPD-CaACH1), pAC65 (p415-GPD-CaACS1), and pAC66 (p415-GPD-CaACS2), respectively. For the heterologous complementation experiments, *C. albicans* *ACH1*, *ACS1*, and *ACS2* were cloned into the p415-GPD vector. CaACS1 and CaACS2 were inserted between SpeI and XhoI sites to produce plasmids pAC64 (p415-GPD-CaACS1) and pAC65 (p415-GPD-CaACS2), while CaACH1 was inserted between SmaI and PstI sites to produce plasmid pAC63 (p415-GPD-CaACH1).

**In vitro growth assays.** For spot dilution assays, strains were grown in liquid YNB-glucose at 30°C to mid-log phase, washed twice with water, and transferred to a 96-well plate at an optical density of 600 nm (OD600) of 1.0. Cells were then serially diluted fivefold and spotted using a multipipette to solid YNB medium containing 2% glucose, potassium acetate, ethanol, lactate, glycine, or oleate and incubated at 30°C for 3 to 7 days as indicated in the figure legends.

For liquid growth assays, strains were grown in YNB-glucose at 30°C overnight. The next day the cells were collected by centrifugation, washed twice with water, and diluted into fresh YNB medium containing the appropriate carbon source at an OD600 of 0.05.

**Northern analysis.** *Candida albicans* SC5314 cells and *acs2Δ/MET3p-ACS2* cells were grown overnight in YNB-glucose, collected by centrifugation, and washed twice with water. Cells were then added to fresh YNB medium containing glucose alone or glucose plus 5 mM methionine and cysteine.

The cells were grown for 1, 2, or 5 h and then collected by centrifugation and quickly frozen on dry ice-ethanol. RNA was extracted using hot acid-phenol (1) and cDNA was then made using reverse transcriptase (Invitrogen) and then purified with Roche Quick Spin columns. The blots were incubated in prehybridization buffer (5 × SSC [1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 50% formamide, 5% Denhardt’s solution, 0.1% sodium dodecyl sulfate [SDS] [1]), and 100 μg/ml single-stranded DNA) for 2 h at 42°C. Blots were then transferred to fresh prehybridization buffer containing the appropriate labeled probe and incubated at 42°C overnight. The next morning the blots were washed and exposed to film for autoradiography. Images were processed using a Storm PhosphorImager. Blots were then stripped by incubation in stripping solution (0.2% SDS in Tris-EDTA) for 30 min at 65°C. Blots were rehybridized with a probe specific for 18S rRNA as a control.

**Reverse transcription-PCR (RT-PCR).** *C. albicans* SC5314 cells and *acs2Δ/MET3p-ACS2* cells were grown overnight in YNB-glucose, collected by centrifugation, and washed twice with water. Cells were then added to fresh YNB medium containing glucose alone or glucose plus 5 mM methionine and cysteine. The cells were grown for 1, 2, or 5 h and then collected by centrifugation and quickly frozen on dry ice-ethanol. RNA was extracted using hot acid-phenol (1). cDNA was then made using reverse transcriptase (Invitrogen), and 300-bp internal fragments of either *ACS2* or *ACT1* were PCR amplified from 10-fold serial dilutions of cDNA.

**Immunoblot analysis of histone H3 and histone H4 acetylation.** SC5314, *acs1Δ*, and *acs2Δ/MET3p-ACS2* cells were grown in 25 ml YNB with 2% glucose overnight, collected by centrifugation, washed twice with phosphate-buffered saline (PBS) and diluted 1:10 in fresh YNB with 2% glucose to an OD of 1.0; then 50 mg/ml cysteine-methionine was added to the cells and 10-ml aliquots were taken at 0, 10, 20, 30, 60, and 120 min and frozen immediately at −80°C. Cells were resuspended in histone extraction buffer (12) supplemented with protease inhibitors, followed by glass bead lysis. Extracts were centrifuged at 7,000 × g for 7 min to isolate the cytosolic fraction. Equal amounts of total protein (10 μg) were separated using 10% SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes, and probed with either anti-acetyl-histone H3 polyclonal antibody (AR-0143; LP Bio) or anti-acetyl-Lys5 histone H4 polyclonal antibody (AR-0119; LP Bio). The blots were developed according to the manufacturer’s recommendations using the ECL kit (Pierce Biotechnology). After the anti-acetyl-H3 and -H4 antibodies were stripped, the membranes were reprobed with anti-histone H3 (AR-0144; LP Bio) or anti-histone H4 (ab10158; Abcam Inc.) polyclonal antibodies, respectively, and developed as described earlier.

**In vivo virulence assays.** Mouse virulence assays were performed as previously described (29) using adult (21- to 25-g) female outbred ICR mice (Harlan). C.
albicans} strains were grown in YPD to mid-log phase and collected by centrifugation. Cells were washed twice with PBS and resuspended in PBS, and 10^6 yeast form cells were injected via the tail vein in 0.1 ml PBS. Ten mice were infected per group. Mice were monitored and were euthanized when moribund. Survival data were analyzed using Prism5 (Graphpad Software) and the log rank test. Statistical significance was defined as a P value of less than 0.05. All animal assays were conducted in accordance with protocols approved by the University of Texas Health Science Center Animal Welfare Committee.

RESULTS

Mutant strain construction. Candida albicans has close homologs of the S. cerevisiae ACH1/ACS genes. ACH1 (orf19.3171) encodes a putative acetyl-CoA hydrolase which catalyzes the hydrolysis of acetyl-CoA to free acetate and CoA and is required for acetate utilization in S. cerevisiae (6, 14, 16). ACS1 encodes a 675-residue acetyl-CoA synthetase, one of two C. albicans enzymes responsible for catalyzing the formation of acetyl-CoA from free acetate and CoA. ACS1 (orf19.1743) is 64% identical to the S. cerevisiae ACS1 gene product at the amino acid level. The C. albicans ACS2 gene (orf19.1064) encodes the second putative acetyl-CoA synthetase, a 671-amino-acid protein with 68% identity to the S. cerevisiae Acs2 enzyme. Each of these genes is induced in macrophage-phagocytosed cells (ACS1, 3.3-fold; ACS2, 2.8-fold; ACH1, 6.1-fold) (17), and we were thus interested in their in vitro and in vivo functions.

We constructed mutants with mutations in each gene, as shown in Fig. 1. Homozygous deletions of ACH1 and ACS1 were constructed, along with complemented strains in which the wild-type gene was reintegrated at the RPS10 locus using plasmid CIP10 (Fig. 1) (see Materials and Methods). Attempts to construct a homozygous aces2Δ/Δ strain were unsuccessful; this was expected since the S. cerevisiae gene is essential for growth in glucose (37). Instead, we constructed a strain in which one allele was deleted and the other was under the control of the methionine-repressible MET3 promoter (7) (see Materials and Methods). For each gene, multiple independent deletion strains were generated and analyzed.

ACS2 is essential for growth in most carbon sources. The addition of methionine and cysteine (5 mM each) strongly represses transcription from the MET3 promoter (7). RT-PCR analysis of our aces2Δ/MET3p-ACS2 strain indicated that ACS2 message was undetectable 1 hour after addition of Met-Cys to a logarithmically growing culture (Fig. 2A). ACT1, a control message, was unaffected. This treatment also resulted in the rapid cessation of growth: the aces2Δ/MET3p-ACS2 strain stopped growing within 1 hour of addition of Met-Cys to cultures in YPD, while the wild-type strain continued exponential growth (Fig. 2B). These data indicate that repression of the aces2Δ/MET3p-ACS2 construct effectively depletes ACS2 mRNA and, as a result, inhibits growth.

S. cerevisiae aces2Δ mutants cannot grow on media containing glucose (37); because of this, ACS2 was reported to be an essential gene by the genome-wide functional profiling project (11, 40). However, earlier work showed that Sacces2Δ strains could grow in the presence of ethanol or acetate (37). We tested the C. albicans aces2Δ/MET3p-ACS2 strain by plating serial dilutions on several different carbon compounds in the presence and absence of 5 mM methionine and cysteine (Fig. 3). As expected from Fig. 2B and previous work with S. cerevisiae, this strain failed to grow when glucose was the sole carbon source. Surprisingly, however, depletion of aces2 also blocked utilization of acetate, ethanol, lactate, and citrate. In each case, growth was similar to wild type when methionine and cysteine were omitted from the medium (Fig. 3). Repression of the aces2Δ/MET3p-ACS2 construct did not affect growth when either glycerol or oleate was the sole carbon source, indicating that ACS2 is not absolutely essential in C. albicans but is required for growth on a wider range of carbon sources than is the S. cerevisiae homolog.

ACH1 and ACS1 mutants have limited in vitro phenotypes. We sought to test the homozygous achi1ΔΔ and acs1ΔΔ mutants for carbon utilization phenotypes using a spot dilution assay. Growth of the achi1Δ mutant on glucose, potassium acetate, or ethanol (Fig. 4) or on oleate (data not shown) was unchanged compared to that of the wild type. In contrast, loss of ACH1 conferred a mild retardation of growth in the presence of ethanol and acetate, phenotypes that were complemented by the restoration of a single copy of ACH1 (Fig. 4). A mutant lacking isocitrate lyase (ICL1), a strain with well-documented growth defects on ethanol and acetate (27, 29), was used as a control.

In vitro expression analysis of ACH1, ACS1, and ACS2 on different carbon sources. We examined the expression of ACH1,
ACS1, and ACS2 during growth in different carbon sources. RNA was obtained from wild-type and mutant strains incubated for 1 h at 30°C in fresh YNB containing different carbon sources (see Materials and Methods). As shown in Fig. 5A, ACH1 was present at low levels in glucose-containing medium and was highly induced when acetate, ethanol, or glycerol was the carbon source. This agrees with previous findings that S. cerevisiae ACH1 is glucose repressed (15). Interestingly, ACH1 expression was almost undetectable when lactate was the sole carbon source. ACS1 and ACS2 had largely opposite expression patterns (Fig. 5B and C). In glucose, acetate, ethanol, glycerol, and citrate, ACS2 is the more highly expressed of the two. ACS1 was most highly expressed in oleate, while ACS2 expression appeared lower, perhaps explaining why the acs2ΔΔ mutant is able to grow in the presence of oleate. Probe specificity was confirmed using the acs1/H9004 and acs2/H9004 strain.

Functional conservation and complementation between C. albicans and S. cerevisiae. The ACH and ACS genes of C. albicans were classified as such based on sequence homology. We addressed functional conservation by heterologously expressing the C. albicans genes in S. cerevisiae strains lacking ACH1 or ACS2 (acs1Δ mutants have no phenotype in S. cerevisiae [see Materials and Methods]). The deletion strains were transformed with plasmids containing the S. cerevisiae or C. albicans genes under the control of the strong, constitutive GPD1 promoter.

As shown in Fig. 6, the C. albicans genes complement the S. cerevisiae deletions, indicating conservation of function. Deletion strains transformed with these plasmids were serially diluted onto minimal medium containing either 2% glucose or potassium acetate as the sole carbon source. Expression of CaACH1 or ScACH1 restored growth to a comparable level in the Scach1Δ strain (Fig. 6A). Similarly, CaACS2 complemented the Scacs2Δ strain (Fig. 6B). Overexpression of either gene in BY4741 did not affect growth on glucose or potassium acetate.

Additionally, we show that heterologous expression of C. albicans ACS1 does not restore growth of the Scacs2Δ strain.

FIG. 3. ACS2 is required for growth on diverse carbon sources. Serial 1:5 dilutions of the wild-type (SC5314; upper strain in each pair) or acs2ΔΔMET3p-ACS2 (ACC24; lower strain) strain were spotted to YNB plates containing the indicated carbon sources without (left) and with (right) 5 mM methionine-cysteine. Growth was observed after 3 days, except for citrate plates (5 days), at 30°C.
Overexpression of ScACS1 also does not compensate for the absence of ACS2. Because these genes were expressed from the constitutive GPD1 promoter, this indicates that carbon source-based expression differences do not explain the radical difference in phenotypes between acs1 and acs2 mutants in the two species. The S. cerevisiae Acs1 and Acs2 enzymes are known to have distinct kinetic properties (36), and these, or possibly localization differences, likely explain the observed phenotypes.

acs mutations do not affect global histone acetylation. It was recently reported that ACS2 is a primary source of acetyl-CoA used for nuclear histone acetylation and, thus, global transcriptional regulation in S. cerevisiae (35). We tested whether the C. albicans Acs2 enzyme performs a similar function by assaying N-terminal acetylation of histones H3 and H4 using antibodies specific for the acetylated proteins (see Materials and Methods). When we repressed transcription of the acs2/H9004/MET3p-ACS2 allele by adding Met-Cys, there was no change in acetylation of H3 or H4 over the course of 3 hours, far longer than necessary to see the cessation of growth (data not shown). We also tested the acs1/H9004/strain and, likewise, found no difference in acetylation (data not shown). We conclude that acetyl-CoA synthesis via the Acs enzymes is not required for histone acetylation in C. albicans, in contrast to published data for S. cerevisiae.

ACH1 and ACS1 are not required for virulence in a mouse model of disseminated candidiasis. Previous work had shown that C. albicans makes use of nonglucose carbon sources during systemic infection in animal models of disseminated infection (2, 18, 27, 29). However, not all mutations that impair growth on alternative carbon sources are necessary in vivo; we have shown that deletion of carnitine acetyltransferases does not attenuate virulence (34a, 42). To determine the role of acetyl-CoA metabolism in vivo, we tested the acs1Δ/Δ and ach1Δ/Δ strains in the standard tail vein injection mouse model of disseminated hematogenous candidiasis (Materials and Methods). We found that neither mutation significantly reduces virulence compared to complemented or wild-type strains (Fig. 7). The ach1Δ/Δ strain was somewhat variable in this assay, so the assay was repeated several times; we concluded that if there was an effect, it was minor and not statistically significant. This is perhaps not surprising, since the in vivo phenotypes are mild.

We did not evaluate loss of ACS2 in vivo. This experiment would have required reconstructing the conditional ACS2 al-
lele using a tetracycline-regulated promoter (23), as the MET3 promoter is not appropriate for in vivo studies. However, the growth defects of ACS2-depleted strains are so severe that it is extremely unlikely that they would retain virulence; it has been shown that other mutants that cannot grow on glucose are avirulent in vivo (2, 31).

**DISCUSSION**

In this work, we have examined the in vitro and in vivo roles of acetyl-CoA/acetate interconversion in *C. albicans* by focusing on acetyl-CoA hydrolase (ACH) and acetyl-CoA synthetase (ACS). *C. albicans* acs2Δ mutants, while not completely inviable, are unable to assimilate a wide variety of carbon compounds, including glucose, ethanol, and acetate. In contrast, deletion of *C. albicans* ACS1 produced no observable phenotype but could support growth as the sole Acs enzyme in the presence of fatty acids or glycerol. Expression analysis is consistent with these findings, with ACS2 as the dominant isoform in most conditions. Deletion of ACH1 conferred a mild reduction in growth on some nonfermentable carbon sources, notably acetate and ethanol. Neither acs1Δ nor ach1Δ mutants, however, were attenuated in a mouse model of candidiasis.

Interest in carbon metabolism in pathogens has increased recently due to observations that suggest that at least some environments within the host are deficient in glucose, the preferred nutrient for fungi and many bacteria. Many genes encoding key steps of alternate carbon metabolism are strongly induced, including those of the glyoxylate cycle (e.g., ICL1), β-oxidation (e.g., FOX2), and gluconeogenesis (e.g., FBP1).

---

**FIG. 6.** Cross-species complementation of ACH1 and ACS2. *S. cerevisiae* strains transformed with the indicated plasmids were grown overnight in SD-Ura, serially diluted 1:5, and spotted to YNB with the indicated carbon source (2%, wt/vol). (A) The wild-type strain (BY4741) and an ach1Δ strain were transformed with ACH1 from either *S. cerevisiae* or *C. albicans* under the control of the GPD1 promoter (see Materials and Methods). (B) The wild-type strain and an acs2Δ strain were transformed with ACS2 from either species. (C) The acs2Δ strain was transformed with plasmids expressing ACS1 or ACS2 from either species.
and these pathways are required for full virulence in mouse models of disseminated infection (2, 18, 27, 29). Similarly, the glyoxylate cycle is required for virulence in M. tuberculosis (19, 21). Because these pathways are compartmentalized in eukaryotic cells, intermediates (acytetyl-CoA being the most important) must be transported across organelar membranes. For acetyl-CoA this occurs by converting it to acetate via acetyl-CoA hydrolase (Ach1), conjugating it to carnitine via carnitine acetyltransferases, causing it to cross the membrane by an unknown mechanism, then reversing the process on the other side, using acetyl-CoA synthetase (ACS1, ACS2) to regenerate this molecule. Similar to the findings here, we and others have shown that the carnitine acetyltransferases are induced in phagocytosed cells and are required for the assimilation of nonfermentable carbon sources (17, 28, 34a, 42).

Mutations that disable β-oxidation of fatty acids, the glyoxylate cycle, or gluconeogenesis impair growth on alternative carbon sources and attenuate virulence, moderately to severely depending on the mutation (2, 18, 27, 29). In contrast, mutations in carnitine acetyltransferases and in ACH1 do not compromise virulence despite causing in vitro phenotypes (34a, 42). What is the reason for the difference? For ACH1, the mutant has very mild in vitro phenotypes, and one might not expect this to reduce virulence. In contrast, the importance of acetyl-CoA metabolism can be seen with ACS2, a gene that is essential for viability under most conditions. Similarly, to date we have been unable to construct a strain lacking all three carnitine acetyltransferases (CTN1, CTN2, and CTN3), suggesting that this gene family may also be essential (42).

In the case of both ACS2 and CTN, the phenotypes of C. albicans differ from those of the model yeast S. cerevisiae. Mutation of acs2 in C. albicans confers a much more extensive phenotype than in S. cerevisiae, in which ACS2 is required only for growth in sugars. Similarly, C. albicans single ctn mutants have carbon utilization defects that are more extensive than those of the cognate S. cerevisiae mutants (28, 34a, 42). Finally, C. albicans fox2 deletion strains are deficient in β-oxidation, as expected from S. cerevisiae precedents, but also do not grow in the presence of ethanol or acetate (27, 29). Taken together, these findings suggest that the regulation and function of carbon metabolic pathways have diverged between these two yeasts with vastly different natural environments. Given the importance of carbon metabolism in vivo, we continue efforts to understand these differences.

Finally, nutrient acquisition is a fundamental challenge for all organisms, pathogen or otherwise. While it has been appreciated for many years that mammalian hosts effectively sequester some nutrients, such as iron, from microbes, it is becoming increasingly clear that nutrient deprivation in the host is a general condition of pathogenic species, one that must be overcome as a prerequisite to disease progression. We note that many of these alternative carbon pathways are highly conserved among microorganisms but are absent from mammals, and thus they make attractive drug targets. Further research will be required to determine whether inhibitors of these processes have value as antimicrobial agents.

ACKNOWLEDGMENTS

We thank P. Sudbery and P. Magee for strains and plasmids and D. Garsin and K. Morano for comments on the manuscript. We also thank M. Ramirez for assistance with the mouse virulence assays. This work was supported by NIH award AI075091 to M.C.L.

REFERENCES

1. Ausubel, F. M., B. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl. 2000. Current protocols in molecular biology. John Wiley & Sons, Edison, NJ.
2. Barett, C. J., C. L. Priest, D. M. Maccallum, N. A. Gow, F. C. Odds, and A. J. Brown. 2006. Niche-specific regulation of central metabolic pathways in a fungal pathogen. Cell Microbiol. 8:961–971.
3. Boeke, J. D., J. Trueheart, G. Natsoulis, and G. R. Fink. 1987. 5-Fluoroorotic acid as a selective agent in yeast molecular genetics. Methods Enzymol. 154:164–175.
4. Brachmann, C. B., A. Davies, G. J. Cost, E. Caputo, J. Li, P. Hieter, and J. D. Boeke. 1998. Designer deletion strains derived from Saccharomyces cerevisiae S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. Yeast 14:115–132.
5. Braun, B. R., and A. D. Johnson. 1997. Control of filamentation formation in Candida albicans by the transcriptional repressor TUP1. Science 277:105–109.
6. Buu, L. M., Y. C. Chen, and F. J. Lee. 2003. Functional characterization and localization of acetyl-CoA hydrolase, Acihp, in Saccharomyces cerevisiae. J. Biol. Chem. 278:17203–17209.
7. Care, R. S., J. Trevethick, K. M. Binley, and P. E. Sudbery. 1999. The MET3 promoter: a new tool for Candida albicans molecular genetics. Mol. Microbiol. 34:792–798.
8. De Virgilio, C., N. Burckert, G. Barth, J. M. Neuhaus, T. Boller, and A. Wiemken. 1992. Cloning and disruption of a gene required for growth on acetate but not on ethanol: the acetyl-coenzyme A synthetase gene of Saccharomyces cerevisiae. Yeast 8:1043–1051.
9. Fonzi, W. A., and M. Y. Irwin. 1993. Isogenic strain construction and gene mapping in Candida albicans. Genetics 134:717–728.
10. Fradin, C., P. De Groot, D. MacCallum, M. Schaller, F. Klis, F. C. Odds, and B. Hube. 2005. Granulocytes govern the transcriptional response, morphology and proliferation of Candida albicans in human blood. Mol. Microbiol. 58:597–615.
11. Giaver, G., A. M. Chu, L. N. Connelly, L. Riles, S. Versonneau, S. Dow, A. Lucanu-Danila, K. Anderson, B. Andre, A. P. Arkin, A. Astromoff, M. El Bakoury, R. Bangham, R. Benito, S. Brachet, S. Campanaro, M. Curtis, K. Davis, A. Deutschbauer, K. D. Entian, P. Flaherty, F. Fourny, D. J.
Garfinkel, M. Gerstein, D. Gotte, U. Guldener, J. H. Hegemann, S. Hempel, Z. Herman, D. F. Jaramillo, D. E. Kelly, S. L. Kelly, P. Kotter, D. LaBonte, D. C. Lamb, N. Lan, H. Liang, H. Liao, L. Liu, C. Luo, M. Lussier, R. Mao, P. Menard, S. L. Ooi, J. L. Revuelta, C. J. Roberts, M. Rose, P. Ross-Macdonald, B. Scherens, G. Schimmack, B. Shaffer, D. D. Shoemaker, S. Sookhai-Mahadeo, R. K. Storms, J. N. Stratmann, G. Valle, M. Voet, G. Volckaert, C. Y. Wang, T. R. Ward, J. Wilhelm, E. A. Winzeler, Y. Yang, G. Yen, E. Youngman, K. Yu, H. Bussey, J. D. Boeke, M. Snyder, P. Philippson, R. W. Davis, and M. Johnston. 2002. Functional profiling of the Saccharomyces cerevisiae genome. Nature 418:387–391.

12. Kizer, K. O., T. Xiao, and B. D. Strahl. 2006. Accelerated nuclei preparation and methods for analysis of histone modifications in yeast. Methods 40:296–302.

13. Kujau, M., H. Weber, and G. Barth. 1992. Characterization of mutants of the yeast Varroia lipolytica defective in acetyl-coenzyme A synthetase. Yeast 8:193–203.

14. Lee, F. J., L. W. Lin, and J. A. Smith. 1989. Purification and characterization of an acetyl-CoA hydrolase from Saccharomyces cerevisiae. Eur. J. Biochem. 184:21–28.

15. Lee, F. J., L. W. Lin, and J. A. Smith. 1990. A glucose-repressible gene encodes acetyl-CoA hydrolase from Saccharomyces cerevisiae. J. Biol. Chem. 265:7413–7418.

16. Lee, F. J., L. W. Lin, and J. A. Smith. 1996. Acetyl-CoA hydrolase involved in acetal utilization in Saccharomyces cerevisiae. Biochim. Biophys. Acta 1297:105–109.

17. Lorenz, M. C., J. A. Bender, and G. R. Fink. 2004. Transcriptional response of Candida albicans upon internalization by macrophages. Eukaryot. Cell 3:1076–1087.

18. Lorenz, M. C., and G. R. Fink. 2001. The glyoxylate cycle is required for fungal virulence. Nature 412:83–86.

19. McKinney, J. D., K. Homer zu Bentrup, E. J. Munoz-Elias, A. Miczak, B. Chen, W. T. Chan, D. Swenson, J. C. Sacchettini, W. R. Jacobs, Jr., and D. G. Russell. 2000. Persistence of Mycobacterium tuberculosis in macrophages and mice requires the glyoxylate shunt enzyme isocitrate lyase. Nature 406:735–738.

20. Mumberg, D., R. Muller, and M. Funk. 1995. Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. Gene 156:119–122.

21. Munoz-Elias, E. J., and J. D. McKinney. 2005. Mycobacterium tuberculosis isocitrate lyases 1 and 2 are jointly required for in vivo growth and virulence. Nat. Med. 11:638–644.

22. Murad, A. M., P. R. Lee, I. D. Broadbent, C. J. Barelle, and A. J. Brown. 2000. Clp10, an efficient and convenient integrating vector for Candida albicans. Yeast 16:325–327.

23. Nakayama, H., T. Mio, S. Nagahashi, M. Kokado, M. Arisawa, and Y. Aoki. 2000. Tetracycline-regulatable system to tightly control gene expression in the plant pathogenic fungus Magnaporthe grisea. Mol. Microbiol. 33:610–618.

24. Wach, A., A. Brachat, R. Pohlmann, and P. Philippson. 1995. New heterologous modules for classical or PCR-based gene disruptions in Saccharomyces cerevisiae. Yeast 11:1793–1808.

25. Wang, Z. Y., C. R. Thornton, M. J. Kershaw, L. Dehao, and N. J. Talbot. 2003. The glyoxylate cycle is required for temporal regulation of virulence by the plant pathogenic fungus Magnaporthe grisea. Mol. Microbiol. 47:1601–1612.

26. Winzeler, E. A., D. D. Shoemaker, A. Astromoff, H. Liang, K. Anderson, B. Andre, R. Bangham, R. Benito, J. D. Boeke, H. Bussey, A. M. Chu, C. Connelly, K. Davis, F. Dietrich, S. W. Dow, E. Paramonova, F. C. Odds, P. Krom, and B. Pohlmann. 2004. New heterologous modules for classical or PCR-based gene disruptions in Saccharomyces cerevisiae. Nature 429:527–531.