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A defect in ATP-citrate lyase links acetyl-CoA production, virulence factor elaboration and virulence in *Cryptococcus neoformans*

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Summary

The interaction of *Cryptococcus neoformans* with phagocytic cells of the innate immune system is a key step in disseminated disease leading to meningoencephalitis in immunocompromised individuals. Transcriptional profiling of cryptococcal cells harvested from cell culture medium or from macrophages found differential expression of metabolic and other functions during fungal adaptation to the intracellular environment. We focused on the *ACL1* gene for ATP-citrate lyase, which converts citrate to acetyl-CoA, because this gene showed elevated transcript levels in macrophages and because of the importance of acetyl-CoA as a central metabolite. Mutants lacking *ACL1* showed delayed growth on medium containing glucose, reduced cellular levels of acetyl-CoA, defective production of virulence factors, increased susceptibility to the antifungal drug fluconazole and decreased survival within macrophages. Importantly, *acl1* mutants were unable to cause disease in a murine inhalation model, a phenotype that was more extreme than other mutants with defects in acetyl-CoA production (e.g., an acetyl-CoA synthetase mutant). Loss of virulence is likely due to perturbation of critical physiological interconnections between virulence factor expression and metabolism in *C. neoformans*. Phylogenetic analysis and structural modeling of cryptococcal Acl1 identified three indels unique to fungal protein sequences; these differences may provide opportunities for the development of pathogen-specific inhibitors.

Keywords

acetyl-CoA; pathogenesis; capsule; metabolic remodeling; ATP-citrate lyase

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Introduction

The basidiomycete yeast *Cryptococcus neoformans* causes life-threatening pulmonary infections and meningoencephalitis in immunocompromised individuals, killing an estimated 600,000 people each year (Park *et al.*, 2009). In fact, cryptococcosis is one of the most important HIV-related opportunistic infections, particularly in developing regions such as sub-Saharan Africa (Park *et al.*, 2009). Infectious propagules (spores or desiccated yeast cells) initiate infection in the lung and the fungus must adapt to different nutritional and physical conditions in the host during dissemination to the brain. These conditions include the intracellular environment of phagocytic cells, and extracellular locations in the lung, bloodstream and central nervous system (Kronstad *et al.*, 2011b). There is increasing evidence for an adaptive program in *C. neoformans* that coordinates nutrient acquisition and the elaboration of virulence factors, as recently reviewed (Kronstad *et al.*, 2011a). In particular, specific transcription factors coordinately regulate the expression of anti-phagocytic functions, the remodeling of central carbon metabolism, the expression of specific nutrient acquisition systems, the response to hypoxia, and elaboration of the three main virulence factors. These factors include the pigment melanin in the cell wall, a polysaccharide capsule and the ability to grow at body temperature (Kronstad *et al.*, 2011a).

In the context of metabolic adaptation to the host, transcriptional profiling of cryptococcal cells during murine lung infection revealed elevated expression of genes involved in the glyoxylate pathway, gluconeogenesis, β -oxidation, amino acid biosynthesis and acetyl-CoA utilization and production. The expression pattern suggested that the fungus must metabolically adapt to a glucose-limited environment in the host (Hu *et al.*, 2008). Factors that influence acetyl-CoA are of particular interest because of the central position of this metabolite in pathways for the utilization of glucose and other carbon sources (Fig. 1). In addition, the importance of acetate to the acetyl-CoA pathway during cryptococcosis is suggested by the moderate virulence defect of an *acs1* deletion mutant lacking acetyl-CoA synthase in *C. neoformans* (Hu *et al.*, 2008). Interestingly, metabolite profiling revealed that acetate is the most abundant metabolite found in cryptococcomas and cryptococcal cell supernatants; this substrate can quickly be metabolized to acetyl-CoA in glucose-depleted conditions (Himmelreich *et al.*, 2003).

Very little is known about carbon source utilization during mammalian infection by *C. neoformans*. Differential expression of metabolic functions has been observed by transcriptional profiling of cryptococcal cells obtained from the cerebrospinal fluid (CSF) of rabbits (Steen *et al.*, 2003). In a recent study, Price *et al.* (2011) found that mutants with defects in glycolytic functions such as pyruvate kinase and hexose kinase, exhibited severe attenuation of virulence in a murine inhalation model as well as decreased persistence in rabbit CSF. In addition to serving as a carbon source, glucose is also known to repress melanin production, while capsule production is influenced by a wide variety of factors including carbon source availability (Kronstad *et al.*, 2011a). Interestingly, *C. neoformans* mutants with defects in the glyoxylate pathway are not attenuated for virulence, although a *pck1* mutant with a defect in gluconeogenesis has a virulence defect (Idnurm *et al.*, 2007; Panepinto *et al.*, 2005; Rude *et al.*, 2002). Lipids may also be important carbon sources during infection because defects in peroxisomal and mitochondrial β -oxidation attenuate virulence (Kretschmer *et al.*, 2012).

The pool of acetyl-CoA is important for a number of different cellular processes including histone and protein lysine acetylation, as well as a wide variety of biosynthetic reactions (Wang *et al.*, 2010; Wellen *et al.*, 2009). The three main pathways for generating cytosolic acetyl-CoA in eukaryotic cells are through β -oxidation of fatty acids, the conversion of acetate by acetyl-coA synthetase (*Acs1*), and the oxidation of mitochondria-derived citrate

by ATP-citrate lyase (Acl1) (Hynes and Murray, 2010; Vorapreeeda *et al.*, 2012). ATP-citrate lyase (Acl1) catalyzes the reaction, citrate + CoA + ATP $\rightarrow$ acetyl-CoA + oxaloacetate + ADP + P_i in the presence of magnesium ions (Sun *et al.*, 2011). The recent work of Wellen *et al.* (2009) showed that Acl1 is required for histone acetylation in response to growth factor stimulation and during differentiation of mammalian cells, and that glucose availability can affect histone acetylation in an Acl-dependent manner. In light of the upregulation of transcripts for functions involved in the production and use of acetyl-CoA during cryptococcal infection, and the potential role of Acl1 to coordinate the transcriptional response to changes in metabolism with cellular differentiation, our goal was to understand the role of Acl1 in the metabolic adaptation of *C. neoformans* to the host environment, particularly in the context of the integration of metabolism and virulence factor expression. Here we report that loss of Acl1 results in a carbon-source-dependent defect in elaboration of the polysaccharide capsule and the inability of the fungus to cause disease.

Results

Serial analysis of gene expression during macrophage interaction

To investigate cryptococcal gene expression during an interaction with host immune cells, we generated SAGE libraries from fungal cells harvested after six hours of incubation in either tissue culture medium (38,544 tags) or in co-culture with macrophages (23,904 tags). A comparison of the two libraries identified differential expression of tags representing transcripts for a variety of cellular processes including heat shock and stress response, protein biosynthesis, transport, vesicle trafficking, respiration, growth, nucleotide metabolism and processing, chromatin structure, RNA interference, cell cycle and cytokinesis, as well as carbohydrate and lipid metabolism (Table S1 in the supplemental material). The wide variety of affected pathways suggests extensive transcriptional adaptation of the pathogen in response to the intracellular environment of macrophages. A similar pattern of cryptococcal gene expression in response to phagocytosis was observed by Fan *et al.*, (2005). Seventeen genes that were found to be upregulated in the SAGE data were validated by qRT-PCR and nine of these were found to be upregulated by at least 2 fold, indicating a > 50% true positive hit rate from the screen (Fig. S1 in the supplemental material). Notably, transcripts were elevated for functions in central carbon and lipid metabolism, and amino acid synthesis, as previously observed in SAGE studies of expression changes during pulmonary challenge (Table 1, Hu *et al.*, 2008). In particular, tags for genes encoding functions for acetyl-CoA production and use were upregulated and these included ATP-citrate lyase (13x), alcohol dehydrogenase (6x), long chain fatty acid CoA ligase (4x), homocitrate synthase (7x) and the multifunctional enzyme Mfe2 for peroxisomal β -oxidation (3x); the *ACL1* and *MFE2* genes were among those validated by qRT-PCR. The SAGE data for the subset of genes related to metabolism are presented in Table 1, and the position of acetyl-CoA in central carbon metabolism is summarized in Fig. 1. In light of the importance of acetyl-CoA in carbon metabolism and biosynthesis, the 13-fold higher number of tags for the gene encoding ATP-citrate lyase (*ACL1*) during the interaction with macrophages prompted a more detailed analysis of the contribution of this change to virulence.

Growth on different carbon sources is affected by loss of Acl1

Initially, three independent *acl1* deletion mutants were constructed by overlap PCR and biolistic transformation in the α mating type background to study the phenotypic consequences of perturbation of acetyl-CoA production via glycolysis and citrate formation. All genotypes were confirmed by PCR screening and genomic hybridization (Fig. S2 in the supplemental material). We first examined the wild-type and mutant strains for growth on different carbon sources and found that *acl1* cells had a growth defect on 2% and 0.2%

glucose (Fig. 2A). In contrast, the mutants grew as well as wild type on acetate or ethanol/glycerol, thus indicating that the glucose to acetyl-CoA pathway in this mutant was perturbed while the acetate to acetyl-CoA pathway (through Acs1) remained functional (Fig. 2A). A more detailed examination of the growth kinetics of the *acI1* mutants in glucose revealed an increased lag phase prior to exponential growth compared to wild-type cells; however the *acI1* cells eventually reached cell densities that were comparable to wild type (Fig. 2B). The growth kinetics of the mutants in acetate mirrored those of the wild-type strain (Fig. 2B). Overall, these results indicated that Acl1 is important for growth on glucose as a carbon source and that low levels of glucose support growth, perhaps due to some production of acetyl-CoA via pyruvate, fatty acids, acetaldehyde and acetate. To more closely represent the low nutrient intracellular environment of the host, further experiments were carried out with 0.2% glucose.

We next performed spot assays in the presence of 0.2% glucose and 0.2% acetate to compare the influence of agents that provoke nitrosative, oxidative or cell-wall stress on the growth of wild type *versus* mutant cells. Cells spotted on glucose-containing media were grown until the mutants reached wild-type levels of growth on YNB plates so that carbon source growth defects could be distinguished from stress responses. No differences between wild-type and mutant cells were observed in response to nitrosative or oxidative stress on either carbon source (data not shown); however, a pronounced growth defect in *acI1* mutants was observed on congo red medium in the presence of glucose (Fig. 2C). Wild type levels of growth were restored for the mutants on congo red medium with acetate as the carbon source (Fig. 2C). A subtle growth defect was also observed on caffeine, a general cell wall stress agent, in the presence of glucose; this defect was also repaired in the presence of acetate. Growth in the presence of calcofluor white to challenge cell wall integrity did not reveal differences between wild-type and mutant cells regardless of carbon source. The growth defect on congo red in the presence of glucose but not acetate suggests that loss of Acl1 affects glucan moieties in the cell wall, whereas chitin, the cellular target of calcofluor white, appears to be unaffected.

Loss of ACL1 influences virulence factor production

The loss of *ACL1* resulted in a pronounced capsule defect in that no cell-associated capsule could be visualized by India ink staining and DIC microscopy after 24 hours growth in low-iron medium (LIM, which contains 0.5% glucose) (Fig. 3A). Capsule was not detected in any of the three deletion mutants. In mammalian cells, the consequences of an Acl1 defect on histone and protein lysine acetylation under glucose-replete conditions can be rescued by the addition of acetate (Wellen *et al.*, 2009). We therefore replaced glucose with 0.5% acetate in LIM and found that capsule formation was restored in the mutants. This result suggests that there is a critical threshold of acetyl-CoA required for normal capsule production, which cannot be met in the absence of Acl1 when glucose is the primary carbon source. The production of capsule was also examined after prolonged incubation of stationary phase cells and the *acI1* mutants were never observed to produce a cell-associated capsule.

O-acetylated residues on the *C. neoformans* capsule polysaccharide (GXM) are immunodominant epitopes recognized by mono- and polyclonal antibodies. Previously, Alexa-555 conjugated antibodies were used to demonstrate that the sites of O-acetylated capsule overlapped with C3 complement deposition, and that the antibody produced a bright, punctuate or negative binding pattern depending on the capsule induction conditions and the age of the cells (Gates-Hollingsworth and Kozel, 2009). We therefore examined the O-acetylation of capsule on mutant and wild-type cells by fluorescence microscopy. Specifically, the binding patterns of Alexa-555 conjugated O-acetyl-specific capsule antibody 1326 (Gates-Hollingsworth and Kozel, 2009) were characterized in the wild-type

strain and the *acII* mutants. We also constructed a *casI* knockout mutant to use as a control because Cas1 functions in capsule acetylation in *C. neoformans* (Janbon *et al.*, 2001; Kozel *et al.*, 2003). Cells were grown in LIM with either 0.5% glucose or 0.5% acetate as a carbon source, formaldehyde-killed and labeled before examination by fluorescence microscopy. Mutant *casI* cells showed a negative binding pattern regardless of carbon source as expected (data not shown). Wild-type cells showed bright exterior labeling of parental and daughter cells, regardless of the carbon source used for induction. Cells with the *acII* deletion grown under glucose conditions showed a thin layer of capsule binding, as expected for overall reduced capsule production, but generally did not show the punctate pattern. This fluorescence indicates that there is sufficient acetyl-CoA still produced in the cell for the acetylation of capsule, even in the absence of Acl1 (data not shown).

The loss of Acl1 also resulted in a defect in melanin formation that was detectable after 48 hours of growth on L-DOPA medium containing glucose, compared to wild-type cells (Fig. 3B). Mutant cells began to accumulate pigment after 48hrs but never achieved wild-type levels of melanin, nor did they achieve wild-type levels of growth on L-DOPA medium. Such a significant delay in melanin production with glucose as the primary carbon source might cause the mutants to be more susceptible to host defense mechanisms such as oxidative killing, although we did not observe this phenotype in our assays. Melanin formation and growth were fully restored in the mutants when acetate was used as the carbon source in the L-DOPA media (Fig. 3B).

Loss of Acl1 attenuates virulence

The defects in virulence factor production and growth on glucose for the *acII* mutants suggested that Acl1 would be important for virulence in a mouse inhalation model of cryptococcosis. We tested this prediction by inoculating ten female BALB/c mice intranasally with cells of the wild-type strain or the *acII* mutants. All mice infected with the wild-type cells died between days 18 and 21, while mice infected with the mutants survived for the duration of the experiment (asymptomatic and sacrificed on day 50) (Fig. 4A). These results indicated that loss of Acl1 in *C. neoformans* renders the cells avirulent. Fungal burden analysis revealed substantially higher levels of wild-type cells in both the lungs (8 Log₁₀ CFU/g) and brain (4 Log₁₀ CFU/g), compared to *acII* cells, which could only be isolated at very low levels from the lungs of infected mice (<1 Log₁₀ CFU/g) (Fig. 4B). In particular, it was notable that no cells were cultured from brain tissue of mice infected with the *acII* mutants. The complete absence of *acII* mutants in the brain may implicate *ACL1* in dissemination to other organs after infection. However, it is likely that this result mainly reflects poor proliferation or survival in the lung, as well as the influence of the observed defects in other virulence factors.

Cryptococcal uptake and survival in macrophages is decreased in the absence of Acl1

Stimulated J774.1 macrophage-like cells were incubated with cryptococcal cells at an MOI of 1:1 for 1 and 24hr periods to determine intracellular fungal uptake and survival. Survival and replication within macrophages was calculated as the ratio of CFU mL⁻¹ obtained from macrophage lysis after 24hr incubation compared to CFUs obtained after 1hr incubation. All three independent mutants exhibited impaired intracellular survival rates compared to wild type, and CFUs obtained after 24hr were between 30–60% of cellular uptake values (Fig. 5A). Wild-type cells, however, exhibited efficient replication and survival (180% of uptake values). These results are consistent with the inability of the *acII* mutants to persist in lung tissue and to cause disease. Mutant cells, while differing in survival ability, were taken up into macrophages at a similar rate compared to the wild-type strain (Fig. 5B).

The metabolic source of acetyl-CoA alters drug susceptibility

With the exception of growth on glucose, the phenotypes examined above do not result in an obvious direct way from a defect in acetyl-CoA production. We therefore examined growth in the presence of an azole antifungal drug based on the reasoning that an *acI* mutant may exhibit increased susceptibility because acetyl-CoA is critical for ergosterol biosynthesis. Fluconazole is a widely prescribed antifungal drug that acts by inhibiting lanosterol 14-demethylase (the product of the *ERG11* gene), an essential cytochrome P450 enzyme in the ergosterol pathway (Revankar *et al.*, 2004). The minimum inhibitory concentration (MIC) of fluconazole was determined for the *acI* mutants and the wild-type strain using the broth dilution method. The *acI* mutants were found to be 4-fold more susceptible to fluconazole than the wild-type strain (Table 2). In addition to the *acI* mutants, we also tested two other metabolic mutants predicted to have defects in acetyl-CoA production. These included a mutant in the *MFE2* gene that encodes the multifunctional enzyme for the second and third steps in the oxidation of fatty acids in the peroxisome. *MFE2* was also found as a differentially expressed tag in the macrophage SAGE dataset (Table 1) and a defect in this gene attenuates virulence (Kretschmer *et al.*, 2012). We also tested the *acsI* mutant with a defect in the enzyme acetyl-CoA synthetase that converts acetate to acetyl-CoA in the cytosol (Hu *et al.*, 2008). While the *acsI* deletion mutant shared the same MIC as the wild-type strain, the *mfe2* mutant also exhibited increased susceptibility to fluconazole (Table 2). This difference in drug susceptibility suggests that perturbation of the acetate to acetyl-CoA pathway does not affect the cell's ability to overcome sterol inhibition by fluconazole, while disruption of the glucose to acetyl-CoA or β -oxidation pathways increases fluconazole susceptibility through altered sterol biosynthesis. It is possible that there is redundancy due to other acetyl-CoA synthetase paralogs that function in the ACS pathway and this may buffer the effects of fluconazole stress on the cell; however, the growth defect observed for the *acsI* mutant on acetate would argue against this possibility (Hu *et al.*, 2008).

Time-kill experiments were also performed with a standard concentration of mutant cells and increasing concentrations of fluconazole to determine whether the fungistatic growth inhibition by fluconazole was converted to a lethal effect by the *acI* deletion, thus causing the reduced MICs. A $>3\log_{10}$ (99.9% killing) reduction in CFU mL⁻¹ after a 48hr exposure to a given concentration of fluconazole was defined as a fungicidal chemical genetic interaction. Time-kill experiments require a higher inoculum than that used in standard MIC determinations; thus time-kill MIC values were determined to account for this increase in cell density (Table 2). Inocula of each strain were incubated with time-kill MIC and ¼ MIC (growth control) concentrations of fluconazole for 48 hrs and dilutions of each preparation were spotted on YPD plates and grown for a further 48 hrs at which time cells were counted. Time-kill MIC levels of fluconazole failed to reduce mutant growth to fungicidal levels and we therefore conclude that loss of *ACL1* did not convert fluconazole growth inhibition to lethality in *acI* mutants.

Because azole drug susceptibility was affected by the loss of *ACL1*, we reasoned that the *acI* mutants likely produced lower levels of acetyl-CoA. To test this hypothesis, mutant and wild-type cells were grown to mid-exponential phase in 0.2% glucose-supplemented YNB, and the acetyl-CoA levels in cell lysates were quantified. We found that *acI* mutants contained 20–50% of the acetyl-CoA levels found in wild-type cells (Fig. 6). The *acIΔ* mutants therefore have less available acetyl-CoA for biosynthetic processes, and other pathways were not able to fully compensate for loss of *AcI1*.

Hydroxycitrate is a known inhibitor of mammalian *AcI1* (Szutowicz *et al.*, 1976) and we therefore attempted to mimic the gene deletion effects observed in the *acI* mutant by chemical inhibition with hydroxycitrate. Even at the highest concentrations tested ($>500 \mu\text{g mL}^{-1}$), no reduction in capsule production or growth inhibition was observed. The failure of

this small molecule to inhibit *Cryptococcus* could be due to cellular impermeability, perhaps due to the polysaccharide capsule or cell wall (which are not present in mammalian cells) or the lack of peripheral citrate transporters.

Loss of *Acl1* influences the expression of *LAC1*, but not capsule genes

In light of the phenotypes of the *acl1* mutants, we examined the expression levels of different acetyl-CoA metabolism genes (*ACS1*, *MFE2*, *ACL1*), as well as virulence genes involved in capsule formation (*CAP60*, *CAP10*, *CAS1*) and melanin production (*LAC1*). For this analysis, gene expression was examined by qRT-PCR for cells of the three *acl1* mutants and the wild-type strain grown to mid-exponential phase in YNB with 0.2% glucose as carbon source. *ACL1* transcripts were reduced 60-fold in the *acl1* mutants compared to wild type, as expected in the knockout control (data not shown), but expression levels for *ACS1* and *MFE2* remained constant in all strains regardless of deletion of the *ACL1* gene (Fig. 7A). Genes involved in capsule formation all exhibited wild-type levels of expression (Fig. 7A) but the striking finding was that *LAC1* encoding the predominant laccase for melanin production was down-regulated 5–25 fold in each of the three independent mutants (Fig. 7B). This down regulation of *LAC1* expression is consistent with the observed decrease in melanin production in the *acl1* mutants, and it may reflect the impact of perturbed central carbon metabolism on the regulatory circuit that controls melanin formation. In particular, glucose is known to suppress melanin formation (Zhu and Williamson, 2004).

Although the *acl1* mutants exhibit a capsule defect, genes involved in capsule formation were not differentially expressed suggesting that capsule components, while still synthesized at wild-type levels, may be altered in assembly, secretion or attachment to the cell surface. It is possible that the culture conditions used for RNA preparation were not optimal for capsule gene expression resulting in similar transcript levels between mutants and wild type. However the increased mutant sensitivity to congo red suggested that the loss of *ACL1* may impair the glucan component of the cell wall, which is known to anchor the capsule. Preliminary transcriptome analysis of the *acl1* mutant suggested that genes for certain glucan synthases are downregulated in the absence of *ACL1* (E.G. manuscript in preparation). qPCR of different glucan synthases showed that while α -glucan synthase (*AGS1*) is slightly upregulated in the mutants, the genes for β -glucan synthesis-associated proteins (*SKN1*, CNAG_06832; *KRE62*, CNAG_06031) are downregulated 2–4x (Fig. 7B). Previously, deletion of glucan synthases in *C. neoformans* has been shown to result in mutants that were avirulent, slow growing and defective in capsule attachment although they continue to shed capsule into the environment (Reese *et al.*, 2007). Therefore, a capsule blot was performed to determine the quantity of polysaccharide shed into the extracellular medium by the *acl1* mutants and wild-type strain. While a small quantity of capsule is shed by wild type, *acl1* mutants shed much more capsule, consistent with a capsule attachment defect (Fig. 7C).

Phylogenetic analysis, identification of fungal-specific indels and structural modeling of *Acl1*

While *Acl1* orthologs in basidiomycete fungi and animals are encoded by a single gene, those of the ascomycetes, plants and a small subset of bacteria are encoded by two genes. It has been suggested that the single *ACL1* genes in basidiomycetes and animals originally arose from two genes as the result of ancient gene fusion event (Hynes and Murray, 2010). Despite sharing 54% sequence identity to the human *Acl1* protein ortholog, and the similarity in genomic structure of cryptococcal *ACL1* to animal sequences, the basidiomycete ortholog appears to be most closely related to *Acl1* sequences in other fungi.

While the prevalence of *ACL1* appears to be widespread (Fig. 8), it is notably absent from the *Saccharomyces* and *Candida* genomes (Vorapreeda *et al.*, 2012).

The X-ray crystal structure is available for the human ortholog of Acl1 (Sun *et al.*, 2010, 2011), and we therefore aligned the *C. neoformans* H99 sequence with that of the human enzyme to compare the positions of key residues. This analysis indicated that the catalytic His760 for autophosphorylation during intermediate formation and the residues of the ATP- and citrate-binding pockets are conserved in the *C. neoformans* enzyme. In mammalian cells, Acl1 is known to be hierarchically regulated through phosphorylation by cAMP-dependent protein kinase (PKA) (Pierce *et al.*, 1981) and GSK-3, as well as transcriptionally controlled by the sterol regulatory element-binding protein (SREBP) (Kim *et al.*, 2010; Sato *et al.*, 2000). The regulatory Ser455 for PKA phosphorylation was also conserved, however, the Thr445 and Ser451 required for GSK-3 activation were not present, suggesting regulatory differences for the *Cryptococcus* enzyme.

Three fungal-specific indels were identified in the global alignments of Acl1 proteins, and these consisted of insertions of 6, 15 and 21 amino acids found in almost all fungal Acl1 sequences, but absent in organisms from all other phyla (Fig. 9–11). While the crystal structure of Acl1 is available for the human enzyme, no structural information is known for Acl1 in *C. neoformans*. A 3D model of the *C. neoformans* Acl1 protein was estimated using the online structure assembly tool I-TASSER and the structural restraints from the human structure PDB file (3pff) (Fig. 12). Indel alignments were overlaid with secondary structure motifs determined from the *C. neoformans* Acl1 model. All indels were composed of random coil structures positioned between helices and β strands. The 6 amino acid insertion was of particular interest because it was found between residues Asn203/Pro204 and Asp216 in the corresponding region of the human enzyme; these residues are thought to bind phosphate or coordinate Mg^{2+} in the ATP binding pocket. The 21 amino acid insertion is also of interest because it is possible that this region may be able to enter the catalytic cleft (Fig. 12). Molecular dynamics simulations would be required to investigate these properties further. Overall, these sequence changes appear to produce structural alterations between the human and fungal orthologs that may result in functional differences for potential inhibitor development.

Discussion

In this study, we examined the prediction that ATP-citrate lyase (Acl1) plays an important role in metabolic remodeling during growth in mammalian hosts because elevated *ACL1* transcript levels were identified in a SAGE analysis of *C. neoformans* cells after their uptake by macrophages. The SAGE analysis revealed many commonalities between the transcripts regulated by interactions of *C. neoformans* with macrophages *in vitro*, and our previous SAGE data obtained from cells during murine pulmonary challenge (Hu *et al.*, 2008). In both studies, tags for genes involved in central carbon metabolism, lipid and amino acid synthesis transcripts were elevated, although there were some differences between the studies with regard to specific genes. Some similarities in datasets were also observed between the SAGE analysis and a microarray transcriptome analysis of cryptococcal cells interacting with macrophages (Fan *et al.*, 2005). For example, genes encoding functions for amino acid and phosphate transport, fatty acid and lipid metabolism, translational machinery, and histone and chromatin remodeling were observed in both studies, although the gene sets were not identical, most likely due to differences in experimental methods. Notably, the altered levels of the *ACL1* transcript observed in our current SAGE were not detected in either of the previous *in vivo* expression studies (Hu *et al.*, 2008).

We observed a complete attenuation of virulence for the *ac11* mutants and this likely due to multiple defects including slower growth in nutrient-limited environments, reduced uptake and survival within macrophages, reduced production of melanin, and limited elaboration of the antiphagocytic capsule at the cell surface. Studies of acapsular mutants reveal that capsule is one of the most important virulence factors for infection and dissemination of *C. neoformans* to the brain (Griffiths, 2012; Moyrand *et al.*, 2002; Moyrand *et al.*, 2007; Moyrand *et al.*, 2008; Wilder *et al.*, 2002). Our studies indicate that the loss of capsule in *ac11* mutants may not be due to altered gene expression (based on our analysis of a limited number of *CAP* genes), nor to altered O-acetylation due to reduced cellular acetyl-CoA levels. Increased sensitivity to congo red in the presence of glucose suggests that insufficient levels of acetyl-CoA caused by deletion of *ACL1* alters glucan levels or the composition in the cell wall. This in turn would result in defective capsule attachment and capsule shedding, as we observed. It is possible that the loss of Acl1, which uses citrate from the TCA cycle as a substrate, may result in the accumulation of intermediates that interfere with the biosynthesis of glucan from glucose. Reese and Doering (2003) identified α -1,3-glucan as a component of the cell wall required for capsule anchoring, and silencing of α -1,3-glucan synthase expression by RNAi produced slow growing, acapsular cells that were unable to assemble a capsule although they generated polysaccharide components. Furthermore, in a study by Rachini *et al.* (2007) acapsular fungal cells, but not encapsulated cryptococcal cells, were opsonized by anti- β -glucan monoclonal antibodies and more efficiently phagocytosed and killed by human monocytes and murine peritoneal macrophages. Changes in capsule deposition alter cell ultrastructure and availability of cell surface molecules in ways which may modify interactions with host cells and tissues during infection; this may help explain the reduced survival of *ac11* mutants within macrophages observed in our work.

For *C. neoformans*, the increased susceptibility to fluconazole for an *ac11* mutant is likely due to perturbed sterol and lipid synthesis arising from reduced levels of acetyl-CoA, as demonstrated by our quantification of acetyl-CoA. Altered lipid composition might also affect vesicular transport as well as the synthesis of other molecules that are dependent on acetyl-CoA. In *Pseudomonas aeruginosa*, deletion of *aceA*, one of the structural subunits of pyruvate dehydrogenase which produces acetyl-CoA from pyruvate, results in downregulation of type III secretion genes suggesting that acetyl-CoA plays a role in secretion of pathogenic effectors (Rietsch and Mekalanos, 2006). In yeast, sterols and sphingolipids are enriched at the trans-Golgi network from where they are sorted via Golgi-derived vesicles to the plasma membrane (Schrack *et al.*, 2012). Sterols and sphingolipid-enriched membrane rafts are associated with specific classes of proteins which play roles in many biological processes such as cell polarity, protein trafficking and signal transduction (Klemm *et al.*, 2009; Mousley *et al.*, 2012; Schrack *et al.*, 2012; Surma *et al.*, 2011). A trafficking defect could also potentially account for the reduced size of the polysaccharide capsule in the *ac11* mutant and we note that depleted acetyl-CoA stores might also affect the acetylation of the capsule. Fluorescence microscopy with antibodies specific for O-acetylated GXM epitopes indicated that while the smaller *ac11* mutant capsules did not appear to be acetylated differently compared to wild type, there was less antibody binding overall raising the possibility of altered antigenicity. The importance of capsule acetylation has been clearly demonstrated in de-O-acetylation studies, achieved either by deletion of the capsule O-transacetylase (*CAS1*) or by chemical depletion, which resulted in cells with reduced capsules as well as altered antibody binding, complement activation and tissue accumulation (Janbon *et al.*, 2001; Kozel *et al.*, 2003). It seems likely therefore that defective capsule and cellular structures would contribute to the growth defects observed in *ac11* mutants of *C. neoformans*, as well as complete avirulence in a mouse model.

In addition to insufficient acetyl-CoA levels for metabolic processes, the pleiotropic phenotypes of an *ac11* mutant may also be due to alterations in both protein lysine activation

and histone modification. The latter defects are known consequences of altering cellular acetyl-CoA concentrations, and acetylation alters gene expression by directly influencing chromatin structure, and by acting as a molecular tag for the recruitment of chromatin-modifying complexes. For example, Wellen *et al.* (2009) showed that Acl1-derived acetyl-CoA is used by histone acetyltransferases (HATS) for histone acetylation in mammalian cells, thus linking metabolism to the regulation of gene expression. Son *et al.* (2011) also recently demonstrated that histone H4 in the ascomycete *Gibberella zeae* is differentially acetylated in *acl1(aclA) acl2(aclB)* mutants compared to wild-type cells. Also, loss of some HATS (such as Gcn5 which partially acetylates the amino-terminal tails of histone H3 at repressed promoters), results in a number of phenotypes similar to the *acl1* mutant in *C. neoformans* (O'Meara *et al.*, 2010a). That is, the *gcn5* mutant exhibited capsule defects and avirulence in a murine model of cryptococcosis (O'Meara *et al.*, 2010a). Furthermore, deletion of *ADA2*, which encodes a component of transcriptional regulator SAGA (Spt-Ada-GCN5) complex, was found to be a novel regulator of capsule and virulence in *C. neoformans* (Haynes *et al.*, 2011). In addition to the phenotypes observed in our work, Nowrousian *et al.* (1999) demonstrated the importance of ACL activity to the life cycle of *Sordaria macrospora* in which ACL was expressed at the beginning of the sexual cycle and was required for the initial stages of sexual development and the maturation of fruiting bodies. Defects in sexual development have also been reported for ATP-citrate lyase mutants in *Gibberella zeae* and *Aspergillus nidulans* by Son *et al.* (2011) and (Hynes and Murray, 2010), respectively. A lower acetyl-CoA concentration may also affect the activation of a number of metabolic processes as it has been reported that up to 90% of central metabolism enzymes are regulated by acetylation (Wang *et al.*, 2010). In light of the central importance of cytosolic acetyl-CoA for so many different pathways, it is likely that *ACL1* participates in the elaboration of virulence factors through complex metabolic and gene regulation networks. Our ongoing work includes transcriptome changes in the absence of *ACL1*.

Our study also indicates that alternative pathways to produce acetyl-CoA make different contributions to virulence. The three main pathways for producing cytosolic acetyl-CoA in eukaryotic cells are through β -oxidation of fatty acids (e.g., involving the multifunctional enzyme Mfe2), from acetate through the action of acetate synthetase (Acs1), and from citrate through the action of ATP-citrate lyase (Acl1). Genes from these three pathways were upregulated in SAGE studies of *C. neoformans* cells interacting with macrophages or during infection indicating that the production of acetyl-CoA is important for adaptation of *C. neoformans* to the host environment. Deletion of *ACL1* completely attenuated virulence, while the *mfe2* and *acs1* mutants exhibited reduced virulence to differing degrees (Kretschmer *et al.*, 2012) (Hu *et al.*, 2008). The similarity of phenotypes obtained for the *acl1* and *mfe2* mutant strains suggest that the citrate to acetyl-CoA and β -oxidation pathways play key roles in metabolism during infection and to a greater extent than Acs1. As previously noted, *ACL1* homologs are absent in species such as *Saccharomyces cerevisiae* and *Candida albicans*. Comparative genomic analysis has identified a number of different cytosolic enzymes in these organisms through which the biosynthesis of acetyl-CoA could be achieved such as pyruvate kinase, pyruvate dehydrogenase, malic enzyme, lactate dehydrogenase and acetyl-CoA synthetase, which are also found in *C. neoformans* (Vorapreeda *et al.*, 2012).

Our transcriptional analysis identified Acl1 as a potentially important factor in the interaction of *C. neoformans* with macrophages. The loss of this enzyme results in a number of phenotypes, including increased anti-fungal drug susceptibility and, importantly, complete loss of virulence. These results therefore suggest that Acl1 may be an important target for novel anti-fungal strategies. In spite of the high level of homology between human and *C. neoformans* protein orthologs, which enabled structural modeling of *C. neoformans* Acl1,

we were able to identify three fungal-specific indels in the cryptococcal Acl1 sequence. These insertions in fungal Acl1 sequences form additional random coils between more complex regions of secondary structure in which the amino acids are buried within the protein, whereas the indel regions were often exposed and may form surface protein loops for protein-protein interactions. Moreover, the 6 aa insertion we identified is positioned between residues known to form the ATP binding pocket required for catalysis. Although it is not clear at present how these indels are positioned compared to the catalytic histidine residue of the active site, other such insertions have been reported to cause changes in protein structure resulting in differences in enzyme kinetics, specificity, and/or regulation (Artsimovitch *et al.*, 2003; Cherkasov *et al.*, 2006; Diner and Hayes, 2009; Takahata *et al.*, 2010; Wood *et al.*, 2009). It is possible that such an insertion in fungal Acl1 sequences may lead to phyla-specific biochemical/physiological changes. Leveraging structure-function differences between human and fungal enzymes may lead to the discovery of small molecules capable of specifically inhibiting fungal Acl1.

Experimental Procedures

Strains, plasmids and media

The serotype A strain H99 (*C. neoformans* var. *grubii*) was used along with the *C. neoformans* H99 mutant strains *mfe2Δ* and *acs1Δ* (Hu *et al.*, 2008; Kretschmer *et al.*, 2012). The strains were maintained on YPD medium (1% yeast extract, 2% peptone, 2% dextrose and 2% agar). Selectable markers for the transformation of *C. neoformans* were from plasmids pCH233 (nourseothricin resistance, 100 μg mL⁻¹) and pJAF1 (neomycin resistance, 200 μg mL⁻¹). Low iron medium (LIM) was composed of 0.5% glucose or acetate, 38 mM L-asparagine, 2.3mM K₂HPO₄, 1.7 mM CaCl₂·2H₂O, 0.3 mM MgSO₄·7H₂O, 20 mM HEPES, 22 mM NaHCO₃ plus 1 mL L⁻¹ 1000X salt solution (0.005g L⁻¹ CuSO₄·5H₂O, 2g L⁻¹ ZnSO₄·7H₂O, 0.01g L⁻¹ MnCl₃·4H₂O, 0.46g L⁻¹ sodium molybdate, 0.057g L⁻¹ g L⁻¹ boric acid in 1L of iron-chelated H₂O). The solution was iron-chelated (BIORAD chelex-100), the pH was adjusted to 7.4, and autoclaved. Finally, 0.4 mg L⁻¹ thiamine was added. L-DOPA medium contained 7.6 mM L-asparagine, 5.6 mM glucose, 22 mM KH₂PO₄, 0.5 mM L-DOPA and 20 g L⁻¹ agar. The solution was autoclaved after the pH was adjusted to 5.6, and 1 mg L⁻¹ thiamine and 5 μg L⁻¹ biotin-HCl were added.

SAGE analysis of *C. neoformans* cells isolated from macrophages

The murine macrophage-like cell line J774A.1 was maintained at 37°C in 10% CO₂ in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FBS), 1% nonessential amino acids, 100 μg mL⁻¹ penicillin-streptomycin, and 4 mM L-glutamine (Invitrogen). *Cryptococcus* cells were opsonized with monoclonal antibody 18B7 against capsule (1 μg mL⁻¹), and macrophages were treated with recombinant mouse gamma interferon (IFN-gamma) (50 U mL⁻¹) and lipopolysaccharide (LPS) (0.3 μg mL⁻¹) prior to co-incubation at a multiplicity of infection (MOI) of 1:1. Macrophages were inoculated with H99 cells and washed after 1 h of inoculation to remove unattached, extracellular fungal cells. After 6 h of incubation, sterile, ice-cold distilled H₂O was applied to each well to lyse the macrophages, and the fungal cells (4 × 10⁷) were harvested by centrifugation. H99 control cells were prepared by growth under the same condition but without macrophages, and 10⁸ cells were harvested.

SAGE library construction, sequencing and analysis

SAGE library construction, sequencing and analysis were as previously described (Hu *et al.*, 2007; Hu *et al.*, 2008; Steen *et al.*, 2002; Steen *et al.*, 2003). Briefly, RNA was isolated from lyophilized cells by vortexing with glass beads (3.0 mm, acid-washed and RNase-free) for

15 min in 15 mL of TRIZOL extraction buffer (Invitrogen, Carlsbad, CA, USA). The mixture was incubated for 15 min at room temperature and total RNA was isolated according to the manufacturer's instructions. Total RNA was used directly for SAGE library construction as described by Velculescu *et al.* (1995) using the I-Long SAGE kit (Invitrogen). The tagging enzyme for cDNA digestion was *Nla*III and 29 PCR cycles were performed to amplify the ditags during library construction. Colonies were screened by PCR (M13F and M13R primers) to assess the average clone insert size and the percentage of recombinants. Clones from the libraries were sequenced by BigDye primer cycle sequencing on an ABI PRISM 3700 DNA analyzer. Sequence chromatograms were processed using PHRED (Ewing *et al.*, 1998), and vector sequence was detected using Cross_match (Gordon *et al.*, 1998). Fourteen-base-pair tags were extracted from the vector-clipped sequence, and an overall quality score for each tag was derived based on the cumulative PHRED score. Duplicate ditags and linker sequences were removed as described previously (Steen *et al.*, 2002). Only tags with a predicted accuracy of 99% were used, and statistical differences between tag abundance in different libraries were determined as described (Audic and Claverie, 1997). The libraries yielded 38,544 tags from cells grown in media without macrophages for 6hr and 23,904 tags from cells grown in media with macrophages for 6hr. An overview of the abundance classes for both SAGE libraries is presented in Table S1 in the supplementary material, with both the number of different tag sequences and the total number of tags present in each class for the cells from the 6h infection and the control. All libraries were normalized to 23,904 to allow direct comparisons, and the tags that appeared less than once in any given library were removed. The EST database available for strain H99 at the University of Oklahoma's Advanced Center for Genome Technology (<http://www.genome.ou.edu/cneo.html>) was used for the preliminary assignment of tags to genes. When an EST sequence could not be identified for a particular tag, the genomic sequence for H99 at the Duke University Center for Genome Technology (<http://cgt.genetics.duke.edu/data/index.html>) and the Broad Institute (<http://cneo.genetics.duke.edu/>) was used to identify contigs with unambiguous tag assignments. Note that a limitation of the SAGE approach is that some transcripts are not detected because of low abundance and/or the absence of an *Nla*III site for transcript processing. The control (fungal cells alone) and cryptococcal-macrophage interaction SAGE datasets were deposited to the NCBI under accession number GSM986206 and GSM986207 respectively.

Deletion of the *ACL1* and *CAS1* genes

An *acl1::NAT* deletion allele was constructed using a modified overlap PCR procedure (Davidson *et al.*, 2002; Yu *et al.*, 2004). The primers ACL1-1/ACL1-3 and ACL1-4/ACL1-6 were used with genomic DNA to obtain the left and right arms for the deletion construct (Table S2 in the supplemental materials). The *NAT* selectable marker was amplified using primers ACL1-2/ACL1-5 and the plasmid pCH233. The *ACL1::NAT* allele results in the deletion of the complete open reading frame of *ACL1* (4253 bp), CNAG_04640. The resulting PCR product (3.5kb) was used to transform strain H99 by biolistic transformation (Davidson *et al.*, 2000). Transformants were grown on YPD plates containing nourseothricin (100 $\mu\text{g mL}^{-1}$) and screened by colony PCR with Extaq polymerase (Takara) using primer pairs ACL1-7/ACL1-8 and ACL1-9/ACL1-10. Primer ACL1-9 was designed from the region upstream of *ACL1* and ACL1-10 was designed for the *NAT* cassette. Transformants in which the wild-type allele was replaced were confirmed by genomic hybridization as described (Hu and Kronstad, 2006). Three mutants designated *ACL1-15*, *ACL1-16* and *ACL1-3* contained the deletion allele and were selected for further study. Primer sequences are listed in Tables S2 and S3 in the supplemental materials.

Cell stress and growth assays

To examine the response of *C. neoformans* wild-type, *ac11-15*, *ac11-16* and *ac11-3* strains to various nutrient sources, exponentially growing cultures were washed, re-suspended in H₂O and adjusted to a concentration of 10⁶ cells mL⁻¹. The cell suspensions were diluted 10-fold serially in water, and 5 µL of each dilution was spotted onto YPD and YNB agar plates (yeast nitrogen base without amino acids and ammonium sulfate) supplemented with 5g L⁻¹ ammonium sulfate and a carbon source (2% or 0.2% glucose, 2% or 0.2% sodium acetate trihydrate, or 2% glycerol and 2% ethanol). Plates were incubated for 2–5 days at 30°C and photographed.

To compare the effects of nitrosative, oxidative and cell-wall stress on wild-type and mutant cells, different stress-inducing chemicals were added to YNB agar plates containing either 0.2% glucose or acetate. Nitrosative, oxidative and cell-wall stresses were induced with 4 mg mL⁻¹ NaNO₂, 100µM tert-butyl alcohol, 0.5 mg mL⁻¹ caffeine, 0.5% congo red or 30 µg mL⁻¹ calcofluor white. Inocula were prepared and spotted as stated above and glucose-containing plates were incubated until growth of mutant colonies reached levels comparable to that of wild-type cells (4–5 days).

Assays of growth in liquid media were performed by diluting rinsed, exponentially growing mutant and wild-type cells in YNB (no carbon source) supplemented with either 0.2% acetate or glucose to a final concentration of 10⁵ cells mL⁻¹. Cultures were grown at 30°C with agitation for up to 5 days. Optical density (OD₆₀₀) for each culture was determined every 12h. Experiments were performed in triplicate.

Macrophage infection assay

To investigate the uptake and survival rates of wild type and *ac11* mutant strains within macrophages, J774.1 macrophage-like cells were grown to 80% confluence in DMEM supplemented with 10% fetal bovine serum and 4mM L-glutamine at 37°C with 5% CO₂. Single colonies of wild type and mutant strains were inoculated in YPD and grown overnight at 30°C. Cultures were rinsed 3 times in PBS. Confluent macrophages grown in DMEM were stimulated 2h prior infection with 150 ng mL⁻¹ phorbol myristate acetate (PMA). Inocula containing 2×10⁶ wild type or mutant fungal cells were then opsonized in fresh DMEM with 0.5 µg mL⁻¹ of the monoclonal antibody 18B7 for 30 mins at 37°C. The opsonized cells (100 µL) were incubated with the stimulated macrophages for 1 hr and 24 hr at 37°C with 5% CO₂. Macrophages containing internalized cryptococci were washed thoroughly 4 times with PBS to remove free extracellular fungal cells, and then lysed with sterile water for 30 min at room temperature. Lysate dilutions were plated on YPD +chloramphenicol (25 µg mL⁻¹) agar and incubated at 30°C for 48 hrs, at which time the resulting CFUs were counted. To determine cryptococcal uptake into macrophages, macrophage and cryptococcal cells were prepared as described above with the following exceptions. After stimulation, the opsonized cells were incubated with the stimulated macrophages for 2 hr at 37°C with 5% CO₂. Cells were rinsed 4 times with PBS, fixed with 4% paraformaldehyde, rinsed a further 2 times with PBS and mounted. Macrophages containing fungal cells were quantified by DIC microscopy (100X magnification). A minimum of 100 cells were counted per replicate. Experiments were carried out in triplicate.

Capsule formation and melanin production

Low iron medium (LIM) was used to induce capsule formation. A single colony from a YPD plate for each strain was cultured overnight at 30°C in LIM. After incubation, the capsule was stained with India ink and examined by differential interference microscopy (DIC, Zeiss Axioplan 2 imaging system, 100X magnification). To examine melanin production, a single colony of each strain was incubated overnight at 30°C in liquid YNB

medium, the cells were washed and diluted to 10^6 cells mL^{-1} . Five microlitres of serial dilutions from this stock were spotted onto L-DOPA plates containing 0.1% glucose or acetate. The plates were incubated for 3 days at 30°C , and melanin production was monitored and photographed daily.

Capsule blotting

To determine whether capsule was synthesized and shed in the *acII* mutants, the quantity of shed polysaccharide was assessed by a blotting technique of culture medium filtrate, using an anti-GXM antibody to probe for capsule, as previously described by Yoneda *et al* (O'Meara *et al.*, 2010b; Yoneda and Doering, 2008). Briefly, capsule production was induced by inoculating each mutant, wild type and complement strain in 5 mL of LIM. Cultures were grown for 1 week at 30°C , and 50 μL aliquots were diluted to a cell density (OD_{600}) of 0.2. The aliquots were boiled for 15 mins. to denature enzymes, the cells were pelleted and supernatants removed. Supernatants were electrophoresed on an agarose gel and transferred to nylon membrane using the Southern blot technique. The membrane was blocked using Tris-Buffered Saline-Tween-20 with 5% milk and the polysaccharide was detected using the primary monoclonal antibody 18b7 (1/10 000 dilution) and anti-mouse peroxidase-conjugated secondary antibody (1/5 000 dilution, Jackson Labs). Antibody binding was visualized using Amersham ECL Western blotting detection reagents (GE Healthcare) and a BioRad Chemidoc MP imaging system.

Fluconazole and hydroxycitrate susceptibility

Minimal inhibitory concentrations (MIC) values were determined by standard CLSI protocols (CLSI, 2008). In brief, a series of two-fold fluconazole dilutions were made in DMSO (final concentrations ranged from 0 to $500 \mu\text{g mL}^{-1}$), across the rows of a 96-well flat plate (Sarstedt, Newton, NC, USA), with the exception of wells containing DMSO for growth and sterility controls. Overnight cultures in YNB were diluted in water to an OD_{600} of 0.11, followed by a 1:100 dilution in water, and a final 1:20 dilution in either YPD or YNB media. A standard volume of inoculum (195 μL) was added to each well. Cultures were grown for 48 hr at 30°C , visually inspected for growth, and then incubated further and monitored daily for up to 2 weeks. The MIC as determined by visual inspection was the first drug concentration that yielded no growth. Experiments were performed in duplicate. Similarly, the MIC for hydroxycitrate (Sigma, USA) diluted in water ($1200\text{-}0 \mu\text{g mL}^{-1}$) was performed for wild-type *C. neoformans* strain H99.

Time-kill experiments were performed to determine whether the inhibitory growth effects of fluconazole and the *ACL1* gene deletion were fungicidal. Time-kill MIC values were determined as above with the exception that overnight cultures diluted to OD_{600} of 0.11 were further diluted 1:25 in YNB + 0.2% glucose, as a higher inoculum was required for time-kill spotting. The same diluted cultures were prepared for the time-kill assay and 95 μL of culture was added to 5 μL of diluted fluconazole in triplicate (final concentrations for each strain were at time-kill MIC and $\frac{1}{4}$ MIC levels). At 0, 24 and 48 hr, dilutions from each well were spotted on YPD agar plates, incubated for 48 h at 30°C and colony counts determined. A fungicidal effect was defined as $>3\log_{10}$ (99.9% killing) reduction in CFU mL^{-1} at time-kill concentrations after 48 h incubation (Spitzer *et al.*, 2011). Experiments were carried out in triplicate.

qRT-PCR and gene expression

Overnight cultures in YNB were diluted to 10^5 cells mL^{-1} in 50mL of fresh YNB (without amino acids or ammonium sulfate) supplemented with 5 g L^{-1} ammonium sulfate and 0.2% glucose. Cultures were incubated at 30°C until they reached the mid-exponential phase of growth, the cells were then collected, flash frozen in liquid N_2 and stored at -80°C . Total

RNA was extracted from frozen cells using an Qiagen RNeasy minikit according to the manufacturer's protocol. Subsequently, cDNA was synthesized using a Verso cDNA kit (Thermo Scientific). The resulting cDNA was used for real-time PCR with primers targeted to the 3' regions of transcripts. Primers were designed using the PrimerSelect program from the DNASTar program package. The Power SYBR Green PCR mix (Applied Biosystems) was used according to the manufacturer's recommendations. An Applied Biosystems 7500 Fast Real-time PCR system was used to detect and quantify the PCR products with the following conditions: incubation at 95°C for 10 min followed by 40 cycles of 95°C for 15 s, and 60°C for 1 min. The cDNA of the 18s gene was used to normalize the data. The sequences of the primers for the qRT-PCR analysis are listed in Table S4 in the supplementary material.

Virulence assay

For virulence assays, female BALB/c mice (4–6 weeks old) were obtained from Charles River Laboratories (Ontario, Canada). *C. neoformans* cells were grown in YNB supplemented with 2% acetate overnight at 30°C, washed in PBS and re-suspended at 1.0×10^6 cells mL⁻¹ in PBS. Inoculation was by intranasal instillation with 50 µL of cell suspension (inoculum of 2.0×10^5 cells per BALB/c mouse). Groups of ten mice were inoculated for each strain. The status of the mice was monitored twice daily post inoculation. For the determination of fungal burdens in organs, infected mice were euthanized by CO₂ inhalation and organs were excised, weighed and homogenized in 1 mL of PBS using a MixerMill (Retsch). Serial dilutions of the homogenates were plated on YPD agar plates containing 35 µg mL⁻¹ chloramphenicol and colony-forming units were counted after incubation for 48 h at 30°C. All experiments with mice were conducted in full compliance with the guidelines of the Canadian Council on Animal Care and approved by the University of British Columbia's Committee on Animal Care (protocol A08-0586).

Quantification of cellular acetyl-CoA

Overnight cultures in YNB were diluted to 10⁵ cells mL⁻¹ in YNB media supplemented with 0.2% glucose and grown to midlog at 30°C. The cells were then rinsed twice with PBS, counted using a hemocytometer and once harvested, the cells were flash frozen in liquid N₂ and stored at -80°C. The cells were resuspended in 500 µL of water followed by bead beating for 5 minutes. Two hundred microlitres of cell lysate were centrifuged to remove cell debris and 100 microlitres of clarified lysate was used for acetyl-CoA quantification with a PicoProbe™ Acetyl-CoA quantification Kit (BioVision, San Francisco, USA) as per the manufacturer's instructions, with the exception that the samples were incubated for 1 hr at 37°C. Background sources of CoA such as free CoASH and succinyl-CoA were quenched as part of the quantification protocol, and acetyl-CoA was then converted to CoA through the enzyme-linked assay. Acetyl-CoA levels were determined using fluorescence measurements at 589nm for extrapolation from an acetyl-CoA standard curve, after sample measurements were corrected for background. Concentrations were normalized for the number of cells in the sample and the assays were performed in triplicate.

Phylogenetic analysis and structure modeling

The *C. neoformans* H99 Acl1 protein sequence (CNAG_04640) was obtained from the Broad Institute database and used as a probe to retrieve the protein sequences of available animal and basidiomycete homologs from the NCBI database. The *Aspergillus niger* AclA and AclB sequences were used to retrieve the protein sequences of all other bacterial, ascomycete and plant homologs, which were then concatenated for each species. Sequences were aligned with Align Plus 4 from the Clone Manager program package (Sci Ed Central) using the BLOSUM62 scoring matrix and default parameters. Pairwise sequence identity between the *Homo sapiens* and *C. neoformans* Acl1 sequences were also determined using

this program. Genetic distances for bootstrapped data sets (100 replicates) were calculated by Kimura's (1983) method (Kimura, 1983) and neighbor-joining consensus trees based on the calculated distances were constructed and a consensus tree was obtained. The trees were rooted using *Thermovibrio ammonificans* (a member of the *Aquificales* group of bacteria) sequence. All of the phylogenetic programs that were used to construct the tree are part of the TREECON for Windows software package (Van de Peer and De Wachter, 1997).

Indels were identified through visual inspection of global alignments of Acl1 orthologs from available representative species of different phyla. Local sequence information for various conserved indels and their flanking conserved regions from different species were compiled into signature files.

The *C. neoformans* Acl1 structure model was predicted by the structure assembly program I-TASSER (Roy *et al.*, 2010) (University of Michigan, Ann Arbor, Michigan, USA). The 3D model was constructed using the human crystal structure folding restraints specified in the PDB file 3pff (Sun *et al.*, 2011). Secondary structure information was mapped onto alignments to correlate indel positions with structural motifs.

Statistical Analysis

Significant differences in intracellular survival and uptake rates, fungal load and cellular acetyl-CoA differences levels ($p < 0.05$) were determined by unpaired t-tests using GraphPad Prism 5 for Windows (Graph-Pad Software, San Diego, CA). Analysis of survival differences was performed by the logrank test using GraphPad Prism 5 ($p < 0.0001$).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Sources of cytoplasmic acetyl-CoA and the role of acetyl-CoA in central carbon metabolism and other processes.

Figure 2.

Growth of *acI1* mutants and wild-type strains on different carbon sources. A) Cells were spotted at decreasing concentrations on solid media. The plates were incubated for 2–5 days at 30°C. Spot assays on solid substrates show that mutant *acI1* cells and wild-type cells have the same patterns of growth on YNB (no carbon) supplemented with acetate or ethanol and glycerol as a carbon source. B) Growth of mutant and wild type cells inoculated in liquid YNB culture supplemented with either 0.2% acetate or glucose. C) Wild type and *acI1* mutants spotted on YNB with agents that provoke cell wall stress (calcoflour white, caffeine and congo red), supplemented with either 0.2% glucose or 0.2% acetate, were grown for 4 days at 30°C.

Figure 3. Deletion of *ACL1* influences the major virulence factors. A) Capsule formation was examined by overnight growth in LIM containing either 0.5% glucose or acetate, followed by staining with India ink and visualization by DIC microscopy. B) Melanin production was tested by spotting a dilution series of cells on L-DOPA medium containing either 0.1% acetate or glucose for 3 days at 30°C.

Figure 4.

ACL1 is required for virulence in a mouse inhalation. A) BALB/c mice were infected intranasally with wild-type or mutant cells and monitored daily for the onset of disease symptoms. Mice succumbed to infection with the wild-type strain at 18–21 days post inoculation. All three independent mutants were avirulent (logrank analysis $p < 0.0001$). Asterisks indicate a high level of confidence in significance for the virulence curves. B) Fungal burdens in the brains and lungs of infected mice were determined by plating homogenized tissue on YPD plates followed by incubation for 48 hours at 30°C. The burdens of three different mice per strain were determined and averaged ($p < 0.05$). Experiments were carried out in triplicate. Values are reported as the mean $\pm$ SD.

Figure 5.

Uptake and survival of wild-type and *acI1* mutant strains co-cultured with J774.1 macrophage-like cells. A) Intracellular survival of cells within macrophages (asterisks indicate $p < 0.05$). B) Phagocytosis of wild-type and mutant cryptococcal strains in J774.1 cells ($p > 0.05$). Wild-type and mutant strains were incubated at for 1 hr and 24 hrs in DMEM with macrophages at an MOI of 1:1 at 37°C. CFUs from lysed macrophages were obtained at the different time points and survival was calculated as the ratio of CFUs at 24 hrs vs 1 hr of incubation. Experiments were carried out in triplicate. Values are reported as the mean $\pm$ SD.

Figure 6.

Quantification of cellular acetyl-CoA levels. Cells were grown to midlog in 0.2% glucose supplemented YNB and acetyl-CoA levels in cell lysates were quantified by the PicoProbeTM acetyl-CoA quantification assay (asterisks indicate significance, $p < 0.05$). Acetyl-CoA was enzymatically converted to NADH, which was bound by the PicoProbeTM, fluorescing in a dose-dependent manner at 585nm. Acetyl-CoA levels were normalized for cell number. Experiments were carried out in triplicate. Values are reported as the mean $\pm$ SD.

Figure 7. qRT-PCR analysis of virulence gene transcription. A) and B) qRT-PCR analysis of differential gene expression shows that the metabolic shift to a low glucose environment results in the downregulation of *LAC1* and β -glucan synthase genes in the absence of *ACL1*. Capsule gene expression, however, is not affected in *acI1* mutants. Mutant and wild-type strains were grown to midlog in 0.2% glucose supplemented YNB in triplicate and compared for transcription levels of several metabolic and virulence genes by qRT-PCR. Experiments were carried out in triplicate. Values are reported as the mean $\pm$ SD. The analyzed genes were as follows: *ACS1*, acetyl-CoA synthetase; *MFE2*, fatty acid oxidase; *CAS1*, capsule acetylase; *CAP60*, putative mannosyltransferase; *CAP10*, putative xylosyltransferase; *LAC1*, laccase; *AGS1*, α glucan synthase; *KRE62* and *SKN1*, β -glucan synthesis associated proteins. C) Capsule blot showing increased shed capsule by *acI1* mutants compared to wild type. Cells were grown for 1 week in LIM media. Aliquots of cell-free culture media were boiled and separated on a 1% agarose gel. After transfer, the membrane was processed using standard Western Blotting techniques.

Figure 8.

Phylogeny, distribution and genomic structure of ATP-citrate lyase. ATP-citrate lyase is encoded by a single gene in basidiomycete fungi and animals, while it is encoded by two separate genes in ascomycete fungi as denoted in the top left-hand image comparing the genomic structure of *ACL1* homologs. The two AclA and AclB protein sequences from the ascomycetes and other organisms were concatenated to form a single sequence per species. These sequences were then compared with single polypeptide sequences from basidiomycete and animal phyla in the phylogenetic analysis. Despite these genetic differences, all fungi branch together as a monophyletic clade. While *ACL* genes are widely distributed, they are found in only a few bacterial phyla and are also not present in *Saccharomyces* or *Candida* species.

Figure 9.

A fungal-specific indel composed of 15 aa insertion in Acl1 distinguishes cryptococcal orthologs from the human form. Based on structure modeling, the indel was found in regions of random coil secondary structure, flanked by more organized α -helices and β -strands. Amino acid numbering for indel positioning is denoted according to the *C. neoformans* sequence on the top line.

21 aa insertion in *Cryptococcus*

Figure 10.

A 21–27 aa insertion found only in fungal Ac11 orthologs was identified by global alignments of available Ac11 protein sequences. The indel is comprised of a random coil element that may be able to enter the active site, based on structure modeling data. Amino acid numbering for indel positioning is denoted according to the *C. neoformans* sequence on the top line.

Figure 11.

The 6 aa insertion in fungal Acl1 sequences is not found in any other species, including humans. This fungal-specific indel lies between important residues of the ATP binding site (boxed in green). Amino acid numbering for indel positioning is denoted according to the *C. neoformans* sequence on the top line.

Figure 12.

Model of cryptococcal Acl1 configured according to similarity and structural restraints of the human ortholog (3pff.pdb) using the I-TASSER structure modeling platform. The positions of the indel insertions with respect to the active site histidine (blue) are shown. Indels at amino acid positions 23–37, 228–233 and 260–280 (relative to the *C. neoformans* sequence) are shown in cyan, magenta and red respectively.

Table 1

Upregulated SAGE tags encoding predicted functions in carbohydrate, lipid and amino acid metabolism.

Tag	DMEM alone	Macrophage Co-culture	<i>C. neoformans</i> H99 gene number	Predicted Function and gene name
Carbohydrate Metabolism				
ATTGAATGTA	7	45	CNAG_00984	Alcohol dehydrogenase, ADH1
TAGTGCCCG	3	16	CNAG_07561	6-phosphogluconate dehydrogenase
AAGGTTGATG	1	14	CNAG_03146	alpha amylase
GCACGGAGAT	5	13	CNAG_02133	6-phosphogluconolactonase
CATTACTGCA	3	13	CNAG_01102	Glucose 1-dehydrogenase
ATTCAAAAA	1	13	CNAG_04640	ATP-citrate lyase, ACL1
ATAAGCTTTC	0	12	CNAG_07745	Mannitol-1-phosphate dehydrogenase
GTCGTAGAGT	2	9	CNAG_03072	Enolase 3
TGTCAAAAAA	0	7		Transaldolase
CCATATGTTT	0	4	CNAG_06666	Glycogen phosphorylase-like protein
CATTTCTTCA	0	4	CNAG_02552	Transketolase
Lipid Metabolism				
CATTTATGAA	1	11	CNAG_03936	1,4-benzoquinone reductase
TAACCATATA	1	10	CNAG_04869	Butyrylcholinesterase
AATGTATTAA	1	8	CNAG_05229	Stomatin-like protein
CAAATTCATT	1	7	CNAG_04443	Dienelactone hydrolase family
TCATTTCACT	2	6	CNAG_04392	Sterol-binding protein, MFE2
AAGCAGAGGA	0	4	CNAG_04484	Long chain fatty acid CoA ligase
Amino Acid Metabolism				
CATAGTTTTA	4	22	CNAG_00879	NAD-specific glutamate dehydrogenase
CATACAGGTC	3	13	CNAG_00457	Glutamine synthetase
CATCTGTTGA	1	7	CNAG_00993	Homocitrate synthase

Tags are listed by abundance based on the macrophage co-culture library and show higher levels than the DMEM growth control.

Table 2

Minimal inhibitory concentration of fluconazole of wild type and different metabolic mutants in *C. neoformans* H99, as determined by the broth dilution method.

Strain	YPD ($\mu\text{g mL}^{-1}$)	YNB ($\mu\text{g mL}^{-1}$)	Time-kill MIC
Wild type H99	15	15	15
<i>acs1</i>	15	15	N/D
<i>mfe2</i>	3.75	3.75	N/D
<i>ac11-15</i>	3.75	3.75	7.5
<i>ac11-16</i>	3.75	3.75	7.5
<i>ac11-3</i>	3.75	3.75	7.5

Determinations were made after 48 hrs at 30°C and confirmed after 5 days growth under the same conditions. Time kill MIC determinations were made using the higher inoculums required for the assays.