MINIREVIEW

Intersection of fungal fitness and virulence in Cryptococcus neoformans

John C. Panepinto1 & Peter R. Williamson1,2

1Section of Infectious Diseases, Department of Medicine, University of Illinois at Chicago and 2Jesse Brown VA Medical Center, Chicago, IL, USA

Correspondence: John C. Panepinto, Section of Infectious Diseases, University of Illinois at Chicago, 808 S. Wood Street, Bld 910, RM 881, MC 735, Chicago, IL 60612, USA. Tel.: +1 312 9968068; fax: +1 312 4131657; e-mail: panepijc@uic.edu

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Introduction

The ability of Cryptococcus neoformans to inflict damage on the host, thereby causing disease, has increasingly been the focus of research since the beginning of the AIDS epidemic in the US. Though HIV therapy has reduced infections in industrialized nations, C. neoformans still manages to manifest itself in emerging HIV-infected populations in underdeveloped countries as an opportunistic infection in these immunocompromised patients, often resulting in death (Bicanic & Harrison, 2005).

In the majority of intact hosts, innate and adaptive immunity overcomes the ability of C. neoformans to cause damage to the host and instead inflicts damage on the infecting organism, resulting in suppression of infection or clearance. In cases where the immune system is incapable of overcoming the infection, and host damage reaches a threshold of clinically definable disease, antifungal therapy is required to reduce the ability of the infecting organism to cause further damage. Reduction of damage to the host by antifungals occurs either by direct injury to the infecting organism or by impairing the ability of the infecting organism to grow, rendering it susceptible to even compromised host defenses.

The mechanism by which the majority of antifungal agents reduce the ability of susceptible fungal pathogens to inflict damage to the host is by targeting essential cellular processes. For example, polynye and azole antifungals target membrane integrity and synthesis, respectively, and 5-fluorocytosine, after bioconversion to a nucleoside analog, targets nucleic acid synthesis (Block et al., 1973; Haynes et al., 1996). Echinocandins, the newest class of antifungals, target fungal cell wall synthesis, but are ineffective against C. neoformans (Kartsonis et al., 2003). Genes involved in the synthesis of sterols, nucleic acids and cell wall components are not considered classical virulence factors because they do not directly inflict damage on the host (Casadevall & Pirofski, 2001). While complete inhibition of these essential pathways should theoretically result in fungal death, the incomplete inhibition often encountered during clinical therapy may result only in reduced capacity to propagate within the host, thus requiring participation by the host immune system to eliminate the pathogen. This capacity to propagate within a unique environment, in this case within host tissue, suggests the use of the word fitness. Fitness allows understanding of the behavior of complex populations within an ecological niche and was first quantitated by British biologist J. B. S. Haldane (1924). Pathogenic fitness, then, may provide a quantitative attribute within the specific environment of the host that requires sufficient tolerance to stress for the successful pathogen. Such a tenuous balance...
between the fitness of a damaged pathogen and the reduced defenses of the immunocompromised patient may therefore be one factor in the often unpredictable nature of clinical outcome despite antifungal therapy. It follows that an understanding of how a fungal pathogen resists the stressful events encountered in the host may have implications in understanding the ‘clinical conundrum’ of a poor clinical response in the face of effective therapy.

**Laccase as a virulence factor and a marker of fitness**

The ability to produce melanin pigments was strongly correlated with virulence in early studies of cryptococcal infection (Rhodes et al., 1982; Kwon-Chung & Rhodes, 1986). The first genetic evidence that laccase was important for virulence came from studies in which the gene encoding the laccase enzyme, CNLAC1, was deleted in a strain of *C. neoformans* var. *grubii* (Salas et al., 1996). Laccase has been associated with the production of immunomodulatory catecholamines from brain, as well as protection from antifungals and oxidative products of macrophages (Huffnagle et al., 1995; Liu et al., 1999a, b; Duin et al., 2002). In addition, laccase activity has been found to be a marker of stress, as induction of laccase correlated with substrate starvation and the presence of potentially toxic metals (Zhu & Williamson, 2004). As molecular genetic studies of *C. neoformans* increased, the use of *C. neoformans* var. *grubii* strain H99 became more prevalent, as serotype A strains appear more genetically tractable and the ability to complement by transformation put less emphasis on classical genetics. The selection of laccase-negative mutants by insertion mutagenesis experiments performed in the H99 genetic background has identified numerous genes important for the expression of laccase, all of which have demonstrated a remarkable degree of virulence attenuation, reinforcing the correlation between laccase activity and pathogenic potential (Erickson et al., 2001; Zhu et al., 2003; Zhu & Williamson, 2003; Panepinto et al., 2005). However, the use of molecular genetics to delete the LAC1 and LAC2 genes from *C. neoformans* strain H99 demonstrated that the loss of LAC1 alone, or of both LAC1 and LAC2, results in a less impressive but significant virulence attenuation in a complement-deficient mouse strain (Pukkila-Worley et al., 2005). A better understanding of the contribution of laccase to virulence will require deletion of the laccase genes in other cryptococcal genetic backgrounds using additional animal models. Though any conclusion regarding the contribution of laccase activity to the overall virulence of *C. neoformans* var. *grubii* in a single strain must be made with great caution, the rather small contribution to virulence in one model and the strong association of laccase activity within numerous clinical strains observed over the last three decades may be partly due to an additional ability of laccase to act as a genetic marker for cellular processes that are important for virulence. The following sections will highlight how laccase-deficient mutants of *C. neoformans* var. *grubii* strain H99 have been used to probe basic cellular processes genetically associated with laccase, resulting in the identification of important and novel stress-related and virulence-associated pathways.

**The Δvph1 mutant: laccase as a marker for vacuolar function**

Evidence for the importance of vesicular acidification to laccase expression in *C. neoformans* came when an insertion mutant with a deficiency in melanization was found to be mutated in *VPH1*, the gene encoding the 95-kDa regulatory subunit of the vacuolar H^+^-ATPase (Erickson et al., 2001). The VPH1 mutant was found to be avirulent in a mouse model of cryptococcosis and exhibited deficits in the production of several virulence-associated factors. In addition to a defect in melanization, the Δvph1 mutant exhibited impaired growth at 37 °C, and reduced production of capsule. Complementation with intact *VPH1* restored all phenotypes to wild type. The Δvph1 mutant produced normal amounts of the LAC1 transcript and laccase protein, but the protein was found to be non-functional. The defect in laccase activity could be restored by incubation of laccase-induced Δvph1 cells in weakly acidic buffer supplemented with copper (Zhu et al., 2003). Bafilomycin A, a drug that inhibits vacuolar acidification, was also able to reduce laccase activity in wild-type *C. neoformans* (Fig. 1) (Erickson et al., 2001). This suggests that vesicular acidification is necessary for the appropriate post-translational modification of the copper-dependent laccase protein. The effect of VPH1 loss on capsule is less clear, but bafilomycin A treatment significantly reduced capsule production in wild-type *C. neoformans*, suggesting that production of capsule is also dependent on vesicular acidification (Fig. 1) (Erickson et al., 2001).

The acidification of various membrane-bound subcellular compartments is known to be of importance in all eukaryotes. Secretion of proteins and proper posttranslational modifications require acidification of Golgi and endoplasmic reticulum lumens (Stevens & Forgac, 1997). Proper acidification is important for the regulation of ion homeostasis and pH gradients such that cell integrity can be maintained. The vacuole is the site of deposition for carbon and nitrogen stores for use in times of nutrient deprivation, as well as the site of autophagic digestion, required for the regulated recycling of cellular components (Reggiori & Klionsky, 2002; Wilson et al., 2002).

Vesicular acidification was first demonstrated to be important for the survival of *C. neoformans* by the
investigation of weak bases as potential therapeutic agents (Weber et al., 2000; Harrison et al., 2002). Weak bases, such as quinacrine, have been used to successfully treat malaria and are believed to function by accumulating in the parasite’s food vacuole, preventing acidification (Loria et al., 1999). Consequences of the failure to acidify the food vacuole are believed to include buildup of heme from the digestion of hemoglobin to toxic levels and the inability to detoxify peroxide accumulations. Quinacrine accumulates in the C. neoformans vacuole and causes fungal cell death at an optimal temperature of 37°C and optimal pH of 7.4 (Harrison et al., 2000). Other compounds that counteract vesicular acidification, ammonium chloride and bafilomycin A, a specific inhibitor of V-type H⁺-ATPases, also reduce the growth of C. neoformans (Reggiori & Klionsky, 2002).

The phenotype Δvph1 mutant illustrates the importance of vesicular acidification in relation to the fitness of C. neoformans by growth defects that result in impaired propagation and to pathogenesis by deficiencies in laccase activity and capsule production. Therapeutic targeting of the vacuolar acidification machinery would be expected to impair the ability of the organism to grow within the host, and would dampen the expression of its two most characterized virulence factors, thus reducing pathogenic fitness (Harrison et al., 2000).

**The Δlc1 mutant: laccase as a marker for copper homeostasis**

Another insertion mutant was found to exhibit a transcriptional defect in laccase activity (Fig. 2a). The insertion was found to disrupt a gene with homology to the CLC – type voltage-gated chloride channels encoded by GEFL of Saccharomyces cerevisiae and CLC-A of Schizosaccharomyces pombe (Zhu & Williamson, 2003). The S. cerevisiae Δgefl1 mutant is defective in high-affinity iron transport mediated by the multi-copper oxidase Fet3 that is found on the cell surface (Davis-Kaplan et al., 1998). It was discovered that chloride ion was essential for the conversion of the Fet3 apoenzyme into its copper-containing active form. Because laccase shares multi-copper oxidase activity with Fet3, it was correctly hypothesized that copper would restore laccase activity to the Δlc1 mutant (Fig. 2b) (Zhu & Williamson, 2003). Further studies have demonstrated that the presence of exogenous copper is capable of inducing transcription of laccase, most likely through the action of a copper-dependent transcription factor. One potential candidate for the mediation of LAC1 induction in response to copper is the OXY2 locus, predicted to encode a putative transcriptional activator because of phenotypic homology to S. cerevisiae Δoxy2 mutants that lack the transcriptional regulator of high-affinity copper transport genes (Nyhus & Jacobson, 2004). Mutation of the OXY2 locus in C. neoformans decreased laccase activity and resulted in decreased uptake of copper, conferring resistance to high exogenous copper but sensitivity to copper chelation. Addition of the copper chelator bathocuproinedisulfonic acid to wild-type cells decreases laccase activity, suggesting further that laccase activity is sensitive to copper availability (Zhu &

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**Fig. 1.** Laccase and capsule inhibition with bafilomycin A₁. Top: Wild-type (a), vph1 mutant (c) and VPH1-reconstituted (b) Cryptococcus neoformans were grown in YPD for 2 days and then incubated in the presence of the indicated amounts of bafilomycin A₁ on asparaginoneoformans were grown in YPD for 2 days and then incubated in the presence of the indicated amounts of bafilomycin A₁ on asparaginenorepinephrine agar without glucose for 16 h. Bottom: Wild-type cells in the absence (left panel) or in the presence (right panel) of 100 µM bafilomycin A₁ were incubated on malt agar for 7 days and examined in the presence of India ink. From Ericson et al. (2001).

**Fig. 2.** Copper restoration of melanin production in the Δlc1 mutant. Fresh cells were inoculated on asparaginone-containing plates in the presence of 100 µM bathocuproinedisulfonic acid (a), 10 µM (b) or 100 µM CuSO₄ (c). Strains shown are wild type (H99), Δlc1 (mLac3), and Δcnlac1. From Zhu & Williamson (2003).
Williamson, 2003). Loss of CLC1 significantly attenuated virulence and caused reduction of capsule production, suggesting that regulation of ion homeostasis is essential for pathogenic growth. Other genes involved in copper homeostasis have been demonstrated to be important for laccase activity, including the copper transporter encoded by CCC2, and the copper chaperone encoded by ATX1, but their effect on virulence has yet to be determined (Walton et al., 2005).

In addition to the effect of the Δccl1 mutation on the copper loading of laccase and a potential copper-dependent transcriptional activator, other effects of copper loading defects will significantly impair the fitness of C. neoformans. Oxygen consumption of the S. cerevisiae Δgef1 mutant was significantly reduced, indicating mitochondrial insufficiency, most likely due to defects in metal-loading of the cytochrome oxidase subunits (Greene et al., 1993). Mitochondrial insufficiency would be expected to severely impair the ability of C. neoformans to survive under glucose starvation conditions, as it relies heavily on respiration for ATP production. Disregulation of copper homeostasis would potentially decrease pathogenic fitness by impairing survival and reducing laccase expression in the mammalian host.

The Δvad1 mutant and NOT1 overexpression: laccase as a marker of stress-sensitive gene expression?

The C. neoformans Δvad1 mutant was first identified as an inserional mutant with reduced laccase activity (Fig. 3). VAD1 encodes a virulence-associated DEAD-box protein homologous to Dhh1 of S. cerevisiae, a component of the mRNA deadenylase/decapping complex (Panepinto et al., 2005). Assessment of the LAC1 transcript by Northern blot revealed decreased expression of LAC1 mRNA, suggesting that the laccase deficiency of the Δvad1 mutant was the result of reduced transcription. The severe virulence attenuation that results from the Δvad1 mutation in C. neoformans suggests that the regulation of mRNA degradation is crucial for the ability of C. neoformans to be a pathogen. The Δvad1 mutant was assayed for the ability to express virulence traits other than laccase, and was found to produce wild-type amounts of capsule, urease and phospholipase (Panepinto et al., 2005). Since the LAC1 knockout of C. neoformans strain H99 retains some measure of virulence, the severe attenuation seen in the Δvad1 mutant could not be explained by laccase deficiency alone (Salas et al., 1996; Puikkila-Worley et al., 2005). Additionally, it would be unlikely that defects in mRNA degradation that normally increase mRNA abundance would directly modulate laccase expression. Therefore, a forward genetic approach was undertaken to identify genes exhibiting Vad1-dependent regulation of transcript abundance that might shed light on the mechanism of the Δvad1 laccase deficit. Differential display reverse transcription PCR was used to identify genes that are differentially regulated in the Δvad1 mutant compared to the wild type under laccase-inducing conditions. Several genes with either increased or decreased mRNA abundance in the Δvad1 mutant were identified and further characterized. Two genes that were found to exhibit decreased mRNA abundance in the Δvad1 mutant relative to the wild type were PCK1 and TUF1 (Panepinto et al., 2005).

PCK1 encodes phosphoenolpyruvate carboxykinase (PEPCK), a key regulatory enzyme in gluconeogenesis (Valdés-Hevia et al., 1989; Yin et al., 1996). PEPCK can also act in anaerobic reactions that fill the Krebs cycle with intermediates by converting PEP to oxaloacetate (Lea et al., 2001). This most often occurs under conditions of elevated CO2 concentration, as would be encountered during growth in human tissue (Aguilera et al., 2005). In S. cerevisiae, PCK1 was found to be highly up-regulated in response to elevated CO2 concentration during carbon limitation (Aguilera et al., 2005). PCK1 was verified to be down-regulated in the Δvad1 mutant by Northern blot analysis (Panepinto et al., 2005). Deletion of PCK1 in wild-type C. neoformans results in a
significant attenuation of virulence when 10^6 cells of the
mutant were injected into the lateral tail vein of NIH Swiss
albino mice. The ΔpkcI mutant grew normally on medium
containing glucose, but the mutant’s utilization of alterna-
tive carbon sources was severely impaired. This suggests that
alternative carbon metabolism is important for the ability of
C. neoformans to cause disease, and is dependent on intact
Vad1 function. Although the ΔpkcI mutant exhibited no
defect in laccase activity, PCK1 transcription is co-regulated
with LAC1 in response to glucose starvation, suggesting that
the basic biological functions that are required for the
induction of laccase transcription are also required for the
transcription of PCK1. The failure to appropriately commu-
nicate glucose starvation to the transcriptional machinery
appears to impact pathogenic fitness, as the necessary
metabolic alterations to allow replication are not induced.
Under conditions of pathogenic growth, especially growth
within a phagosome, the ability to cope with nutrient
starvation is probably the key to survival.

Much like LAC1, PCK1 transcription is induced in C.
neoformans in response to glucose starvation, suggesting that
carbon repression governs its expression (Panepinto
et al., 2005). The regulation of LAC1 expression by carbon
source supports the proposed environmental role of laccase
in the degradation of lignin found in the bark of trees for use
as a source of carbon (Zhu & Williamson, 2004). The failure of
the Δvad1 mutant to induce either LAC1 or PCK1
expression in response to glucose deprivation suggests that
VAD1 is a regulator of carbon repression in C. neoformans.
The S. cerevisiae homologue of VAD1, DHH1, exhibits a
defect in utilization of alternative carbon sources, suggesting
that it, too, is defective in release of carbon repression (Hata
et al., 1998). Given that two genes, both of which influence
the virulence of C. neoformans, are regulated by carbon
repression, constitutive engagement of the carbon repres-
sion machinery is a potential therapeutic target for
C. neoformans infection. Laccase is also repressed by the
glucose analogue 2-deoxyglucose, which can be phosphory-
lated at the 6-position but not metabolized further (Fig. 4).
This suggests that glucose, or a phosphorylated derivative, is
a potential second messenger signaling glucose repression of
LAC1 transcription in C. neoformans. Although the use of
antimetabolite compounds has not yet been explored as
adjunctive therapy with antifungals, 2-deoxyglucose has
been demonstrated to enhance the anti-tumor effect of
etoposide in ascites tumor-bearing mice, acting as an
antimetabolite inhibitor of glycolysis (Gupta et al., 2005).
The use of antimetabolite therapy as adjunctive treatment for
C. neoformans infection warrants exploration, as 2-deoxyglucose
would not only inhibit glycolysis, but would also serve as a repressor of laccase expression, impairing both replication and virulence factor production, reducing
pathogenic fitness.

How might Vad1 modulate transcription of PCK1 and
LAC1? The laccase promoter is unusually large for a fungal
promoter, stretching approximately 2 kb upstream of the
start codon. Electrophoretic mobility shift assay studies
demonstrate that distinct regions within the LAC1 promoter
form a complex with yet unidentified regulatory proteins
and the composition of the complexes varies under different
growth conditions (Zhang et al., 1999). The region encoding
the TATA box is sufficient to promote full induction of
laccase transcription in response to glucose starvation, but is
still glucose-repressible. Close inspection of the TATA region
reveals a consensus binding site for the CRE1 repressor, 5′-
SYGGAG-3′ (Cubero & Scaccoccio, 1994). The mechanism of
carbon catabolite repression in C. neoformans most likely
differs from that of S. cerevisiae, more closely resembling
that seen in the higher ascomycetes such as Aspergillus
nidulans. Sequence searches of the C. neoformans genome
reveal the presence of homologues of the A. nidulans CRE
system, including the creA repressor and regulators creB-D
(Table 1). The CRE system elicits glucose repression by the
regulation of the ubiquitination status of the CreA repressor
(Lockington & Kelly, 2001). The creB and creC genes encode
proteins with deubiquitinating activity that appears to
counteract the ubiquitin ligase-recruiting function of the
creD gene product (Lockington & Kelly, 2001; Boase & Kelly,
2004). Further work is necessary to determine whether the
C. neoformans CRE system is responsible for the glucose
sensitivity of the laccase promoter in the region adjacent to
the TATA box. Additional evidence for disparity in the
regulation of carbon repression between S. cerevisiae and
C. neoformans comes from study of the cAMP-PKA signaling
pathway. In S. cerevisiae, mutations that activate the cAMP-
PKA pathway constitutively engage carbon repression, pre-
venting expression of carbon-sensitive genes (Monteiro &
Table 1. Cryptococcus neoformans homologues of the Aspergillus nidulans CRE carbon catabolite repression system. Protein sequence of Aspergillus nidulans CreA (AAO2858), CreB (AAO4454), CreC (AAO63188) and CreD (AAO10351) were used to search the C. neoformans annotated genome database at The Institute for Genome Research (TIGR) (http://www.tigr.org/tdb/e2k1/cna1) using the Blastp algorithm.

<table>
<thead>
<tr>
<th>Aspergillus nidulans gene</th>
<th>Gene function</th>
<th>Cryptococcus neoformans annotation</th>
<th>P-value</th>
</tr>
</thead>
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<tr>
<td>creA</td>
<td>DNA binding transcriptional repressor</td>
<td>183.m01849</td>
<td>1.0e-32</td>
</tr>
<tr>
<td>creB</td>
<td>Deubiquitinating enzyme</td>
<td>167.m03314</td>
<td>3.7e-64</td>
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<tr>
<td>creC</td>
<td>WD40 protein in complex with CreB</td>
<td>163.m06508</td>
<td>1.4e-42</td>
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<tr>
<td>creD</td>
<td>Ubiquitin ligase recruitment</td>
<td>179.m00715</td>
<td>1.5e-24</td>
</tr>
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Fig. 5. Cartoon depiction of the regulatory functions carried out by the Saccharomyces cerevisiae Ccr4-NOT complex. Adapted from Collart (2003).

Netto, 2004). In C. neoformans, mutations that disable the cAMP-PKA pathway repress laccase expression. The downstream target of PKA that modulates laccase expression has not been identified (Hicks et al., 2004). It is possible, however, that repression of LAC1 in cAMP-PKA mutants results from defects in transcription independent of carbon repression, as PKA is also an important regulator of stress-sensitive transcription.

A transcript encoding the C. neoformans homologue of NOT1, the largest component of the Ccr4-NOT complex, was found to be up-regulated in the Δvad1 mutant by differential display (Panepinto et al., 2005). In S. cerevisiae, the VAD1 homologue is a component of the Ccr4-NOT complex, a large multi-protein complex that serves to regulate mRNA metabolism through modulation of both transcription and mRNA stability (Fig. 5) (Collart, 2003). In addition to interaction with the mRNA degradation machinery, the Ccr4-NOT complex has been demonstrated to co-purify with components of two distinct transcriptional activation complexes, the SAGA complex, which regulates transcription from consensus TATA elements, and TFIID and associated TAFs, which regulate POLII dependent transcription from TATA-less promoters found upstream of ‘housekeeping’ genes (Bai et al., 1999; Badarinarayana et al., 2000). NOT1 encodes the largest component of the CCR4-NOT complex, and serves as a scaffold on which the complex is assembled (Collart & Struhl, 1994). The N-terminal domain of Not1 interacts with the deadenylase/decapping machinery, whereas the C-terminal domain interacts with the transcriptional regulatory machinery and the other NOT proteins (Maillet et al., 2000). Not1 is an essential gene in S. cerevisiae, but mutational analysis has demonstrated that Not1 is a regulator of promoter occupation, altering the balance of expression between TATA-containing and TATA-less promoters (Collart & Struhl, 1994; Liu et al., 1998; Oberholzer & Collart, 1999). Not1 has also been suggested to directly repress transcription, but localization of Not1 to the nucleus has not been demonstrated (Oberholzer & Collart, 1999).

When the NOT1 mRNA levels were reduced in the Δvad1 mutant by expression of NOT1-interfering RNA, a partial restoration of laccase activity was observed (Fig. 6) (Panepinto et al., 2005). This is consistent with the hypothesis that, through up-regulation of NOT1, Vad1 modulates LAC1 through the TATA box. When NOT1 was overexpressed in the wild type, laccase activity was found to be reduced, suggesting that NOT1 alone is able to repress laccase transcription. These data suggest that NOT1 is an epistatic regulator of pathogenic fitness downstream of VAD1, both identified by their associations with the virulence factor, laccase.

Another transcript found to be down-regulated in the Δvad1 mutant under laccase-inducing conditions was TUF1, encoding a mitochondrial elongation factor required for the translation of mitochondrion-encoded transcripts (Winzler et al., 1999). This down-regulation of TUF1 was postulated to result in a defect in growth in glycerol-containing media observed in the Δvad1 mutant. Deletion of TUF1 was attempted in the wild type, but no homologous recombinants were obtained after screening over 1500 colonies. RNA interference (RNAi) was used as an alternative approach to examine the effects of TUF1 down-regulation in the wild type. RNAi directed against TUF1 resulted in severely attenuated growth even in glucose-grown cultures, and absence of growth on medium containing the respiration-dependent carbon source glycerol (Panepinto et al., 2005). The importance of TUF1 to the growth of C. neoformans demonstrates the dependence of this organism on mitochondrial function for normal
growth. The \textit{S. cerevisiae} homologue of \textit{TUF1} is dispensable for normal growth on glucose-containing medium but, unlike \textit{S. cerevisiae}, \textit{C. neoformans} is unable to ferment. \textit{TUF1} is also an essential gene in the non-fermentative yeast \textit{Schizosaccharomyces pombe} (Chiron \textit{et al.}, 2005). These results suggest that \textit{Vad1} regulates mitochondrial function in \textit{C. neoformans}.

The reduction of \textit{TUF1} expression in the \textit{\Delta vad1} mutant has the potential to have a severe impact on pathogenic fitness independent of laccase production. Genes encoded by the mitochondrion of \textit{C. neoformans} would be expected to exhibit attenuated translation in the \textit{\Delta vad1} mutant due to down-regulation of \textit{TUF1} expression. Indeed, the mitochondrial cytochrome \textit{c} oxidase subunit 1 gene, \textit{COX1}, has been demonstrated to be up-regulated in \textit{C. neoformans} during neuropathogenesis, suggesting a role in pathogenic fitness (Toffaletti \textit{et al.}, 2003). In addition to components of the mitochondrial respiratory chain, the mitochondrial genome also encodes the multi-subunit NADH dehydrogenase as well as mitochondrial-specific ribosomal subunits, tRNAs and ATP production machinery (Xu, 2002; Toffaletti \textit{et al.}, 2004). Given the importance of these components in respiration, and the dependence of \textit{C. neoformans} on the respiratory chain, a severe impact of attenuated mitochondrial translation on pathogenic fitness would be expected. The genetic association of mitochondrial functions and laccase is most likely due to the role of both in nutrient utilization evolved in the ecological niche of \textit{C. neoformans}, where degradation of lignin by laccase is important (Zhu \& Williamson, 2004), an association presumably important to pathogenic fitness in the host as well.

\textbf{Outlook}

The work of many investigators has demonstrated that the expression of virulence traits is intimately linked to basic biological processes that determine fitness of the fungal pathogen during infection. The study of processes that maintain pathogenic fitness will identify features that are required for the pathogenic process, some of which may be unique to \textit{C. neoformans}, helping to understand the important mechanisms of pathogenesis and increasing the number of potential therapeutic targets. The work presented in this review also offers several potential therapeutic targets for fungal-specific pharmacological interventions.

It is difficult to predict, when studying the basic biology of \textit{C. neoformans}, whether the chosen pathway will affect virulence factor production. Much of the recent work to identify regulators of virulence factor expression has been performed using forward genetic screens. Insertional mutagenesis has been performed by a number of methods and has identified key regulators of laccase expression and virulence, including \textit{VAD1}, \textit{VPH1}, \textit{CLC1}, \textit{SNF5} and \textit{CCC2} (Erickson \textit{et al.}, 2001; Zhu \& Williamson, 2003; Idnurm \textit{et al.}, 2004; Panepinto \textit{et al.}, 2005; Walton \textit{et al.}, 2005). In screening for defects in virulence factor production, the investigator is allowing the organism to ‘speak’ about what it requires for the expression of a given trait. This method has limitations, however, because the effects due to loss of essential genes cannot be seen. The use of genome-wide RNAi screens will aid in the discovery of important regulators of virulence factor expression, especially those which are essential (Liu \textit{et al.}, 2002).

While the true measure of ‘virulence’ remains the ability of an organism to inflict damage on its host, we must also consider the converse (Casadevall \& Pirofski, 2001). The function of host immunity is to inflict damage on the invading organism, and this is effective against \textit{C. neoformans} in most immunocompetent patients. In those patients who are immunocompromised, or who are infected with an organism that overpowers the host defense, antifungal agents are employed to aid the host in damaging and eradicating the infecting organism. In cases where antifungal therapy fails, the balance of the immune status...
of the patient and the pathogenic fitness of the infecting organism become the defining factors of clinical outcome. This review highlights some of the many levels on which fitness and virulence intersect in *C. neoformans*, identified through the study of laccase regulation (Fig. 7). The hope of identifying new antifungal targets requires continued efforts to understand the basic biology of this important pathogen and how it responds to the hostile environment of the host.

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**References**


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