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Nitrogen Metabolite Repression of Metabolism and Virulence in the Human Fungal Pathogen *Cryptococcus neoformans*

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ABSTRACT

Proper regulation of metabolism is essential to maximizing fitness of organisms in their chosen environmental niche. Nitrogen metabolite repression is an example of a regulatory mechanism in fungi that enables preferential utilization of easily assimilated nitrogen sources, such as ammonium, to conserve resources. Here we provide genetic, transcriptional, and phenotypic evidence of nitrogen metabolite repression in the human pathogen *Cryptococcus neoformans*. In addition to loss of transcriptional activation of catabolic enzyme-encoding genes of the uric acid and proline assimilation pathways in the presence of ammonium, nitrogen metabolite repression also regulates the production of the virulence determinants capsule and melanin. Since GATA transcription factors are known to play a key role in nitrogen metabolite repression, bioinformatic analyses of the *C. neoformans* genome were undertaken and seven predicted GATA-type genes were identified. A screen of these deletion mutants revealed *GAT1*, encoding the only global transcription factor essential for utilization of a wide range of nitrogen sources, including uric acid, urea, and creatinine—three predominant nitrogen constituents found in the *C. neoformans* ecological niche. In addition to its evolutionarily conserved role in mediating nitrogen metabolite repression and controlling the expression of catabolic enzyme and permease-encoding genes, Gat1 also negatively regulates virulence traits, including infectious basidiospore production, melanin formation, and growth at high body temperature (39°–40°). Conversely, Gat1 positively regulates capsule production. A murine inhalation model of cryptococcosis revealed that the *gat1*Δ mutant is slightly more virulent than wild type, indicating that Gat1 plays a complex regulatory role during infection.

NITROGEN is a major component of macromolecules ranging from proteins to nucleic acids that are essential to all living organisms (Carroll and Salt 2004). Yet while almost 80% of the earth's atmosphere is made up of diatomic nitrogen gas, very few species are able to break the strong triple bond that exists between the two atoms to reduce gaseous nitrogen into a state that is biologically available (Postgate 1998). In organisms unable to utilize nitrogen directly from the atmosphere, the ability to scavenge reduced forms of nitrogen from alternative sources is crucial and elaborate control mechanisms are needed to ensure its constant supply.

As major decomposers of organic material in the biosphere, fungi are well known for their ability to utilize numerous compounds as nitrogen sources by expressing catabolic enzymes and permeases in a range of pathways. Readily assimilated compounds such as ammonium and glutamine are usually the preferred nitrogen sources of fungi, although in the absence of these sources, less easily assimilated nitrogen sources such as amines, amides, purines, and pyrimidines may also be utilized (Marzluf 1997). In members of the phylum Ascomycota, the selective utilization of these secondary nitrogen sources is typically tightly controlled by transcriptionally regulating the synthesis of the enzymes and permeases required for their scavenging and degradation (Mitchell and Magasanik 1984; Marzluf 1997; Fraser *et al.* 2001; Magasanik and Kaiser 2002). Achieved through a global regulatory circuit known as either nitrogen metabolite repression or nitrogen catabolite repression, this process ensures secondary nitrogen source degrading pathways are not expressed when more easily assimilated nitrogen sources are available, thereby maximizing the fitness of the organism in its ever-changing local environment (Wiame *et al.* 1985; Marzluf 1997; Fraser *et al.* 2001). By combining nitrogen metabolite repression with pathway-specific induction mediated by an array of dedicated transcription factors, resources are even more tightly conserved until preferred nitrogen sources are depleted (Davis *et al.*

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1993; Feng and Marzluf 1998; Berger *et al.* 2006, 2008). In this way, the fungus activates the transcription of catabolic genes only when their substrates are immediately available.

In ascomycete species where it has been observed, nitrogen metabolite repression is mediated by transcription factors belonging to the GATA family. These proteins are distinguished by their highly conserved DNA-binding domain consisting of a Cys_2/Cys_2-type zinc finger motif followed by an adjacent basic region and are named after the DNA sequence 5′-GATA-3′ that they recognize (Ko and Engel 1993; Merika and Orkin 1993; Ravagnani *et al.* 1997). GATA transcription factors are widely found in eukaryotes, including metazoans and plants, and have diverse biological functions. For example, in the nematode worm *Caenorhabditis elegans*, the GATA factor ELT-2 is required for immunity to bacterial and fungal pathogens such as *Salmonella enterica* and *C. neoformans* (Kerry *et al.* 2006). In the plant *Arabidopsis thaliana*, the GATA factor HAN regulates the development of flower and shoot apical meristems (Zhao *et al.* 2004).

The first GATA factor mutant, however, was identified in *Neurospora crassa* on the basis of its central role in nitrate metabolism (Fincham 1950). Subsequent analyses showed that these positively acting nitrogen regulatory GATA factors often act synergistically with pathway-specific transcription factors to activate the expression of catabolic enzyme and permease-encoding genes required to assimilate secondary nitrogen sources when preferred sources are lacking (Davis *et al.* 1993; Feng and Marzluf 1998; Berger *et al.* 2006, 2008). As a consequence, loss of such global *trans*-acting GATA regulatory genes including *areA* in *Aspergillus nidulans* and *nit-2* in *N. crassa* render these species incapable of utilizing nitrogen sources other than ammonium or glutamine (Arst and Cove 1973; Stewart and Vollmer 1986; Fu and Marzluf 1987; Kudla *et al.* 1990; Caddick 1992; Marzluf 1997). These GATA factors have also been shown to affect virulence in a number of pathogenic fungi. *Aspergillus fumigatus areA*Δ, clinical *Saccharomyces cerevisiae gln3*Δ, and *Candida albicans gat1*Δ or *gln3*Δ mutants all exhibit reduced virulence compared to wild type in murine virulence assays (Hensel *et al.* 1998; Limjindaporn *et al.* 2003; Kingsbury *et al.* 2006; Liao *et al.* 2008).

The importance of nitrogen metabolism in the study of *C. neoformans*, an opportunistic pathogen of the phylum Basidiomycota that causes life-threatening meningoencephalitis predominantly in immunocompromised individuals, is also evident (Casadevall and Perfect 1998). The primary ecological niche of *C. neoformans* is nutrient-rich pigeon guano; 70% of the nitrogen present is in the form of uric acid with the rest consisting primarily of xanthine, urea, and creatinine (Staib *et al.* 1976; Staib *et al.* 1978; Casadevall and Perfect 1998). In contrast, nitrogen availability becomes scarce upon infection of humans. Beyond these obvious differences in nitrogen availability that *C. neoformans* often encounters as part of its infection cycle, there are more subtle indications that nitrogen metabolism may also play a role in the regulation of virulence. The antiphagocytic polysaccharide capsule is a well-known virulence factor that is highly induced in the presence of uric acid (Staib *et al.* 1976, 1978). Assimilation of uric acid as a nitrogen source requires a number of catabolic enzymes including the virulence factor urease, a nitrogen-scavenging enzyme shown to play a role in central nervous system invasion (Cox *et al.* 2000; Olszewski *et al.* 2004; Shi *et al.* 2010). While evidence that nitrogen availability may play a role in pathogenicity of *C. neoformans* is mounting, little is known about this process.

In this study we provide the first proof of canonical ascomycete-like nitrogen metabolite repression in the basidiomycete *C. neoformans*. We also reveal that beyond the traditional role in nitrogen scavenging, this regulatory mechanism controls the coordinated production of the virulence factors capsule and melanin. Study of deletion mutants of every predicted GATA-type gene in the *C. neoformans* genome revealed the existence of only one positively acting nitrogen regulatory GATA factor: Gat1. Gat1 mediates nitrogen metabolite repression and is required for utilization of most nitrogen sources including the preferred ammonium; this represents an anomaly from archetypical nitrogen metabolite repression. Gat1 also functions to negatively regulate mating, melanin production at human body temperature (37°), and growth at high body temperature (39°–40°) and is required for capsule synthesis. Importantly, this complex series of changes in the regulation of *C. neoformans* virulence composite combines to create an unexpected phenotype during murine infection: the *gat1*Δ mutant is slightly more virulent than wild type. Together, these studies indicate that in addition to controlling the regulon of genes for nitrogen acquisition, Gat1 is a key coordinator of multiple virulence-associated phenotypes.

MATERIALS AND METHODS

Strains and media: Cryptococcus strains (supporting information, Table S1) were grown in YPD (1% yeast extract, 2% Bacto-peptone, 2% glucose) or YNB (0.45% yeast nitrogen base w/o amino acids and ammonium sulfate, 2% glucose, 10 mm nitrogen source) unless specified otherwise. Biolistic transformants were selected on YPD medium supplemented with 100 μl/ml G418 (Sigma) and/or nourseothricin (Werner BioAgents). Pigeon guano medium was prepared as described previously while the derivative pigeon guano (unfiltered) medium was prepared with the exclusion of the filtration step to ensure that insoluble nitrogen sources such as uric acid would not be removed (Nielsen *et al.* 2007). l-3,4-Dihydroxyphenylalanine (l-DOPA) medium was prepared as described previously with the original nitrogen source (asparagine) replaced with 10 mm of the specified nitrogen source (D'souza *et al.* 2001). V8 (5% V8 juice, 3 mm KH_2PO_4, 0.1% *myo*-inositol, 4% Bacto-agar) and Murashige & Skoog (MS) mating media (*Phyto*Technology Laboratories) at pH 5.0 were prepared as described previously

(Kwon-Chung *et al.* 1982; Xue *et al.* 2007). Christensen's urea agar was prepared as described previously (Cox *et al.* 2000). Nematode growth medium (NGM) was prepared as described previously (Brenner 1974). *Escherichia coli* Mach-1 cells served as the host strain for transformation and propagation of all plasmids, which were carried out according to methods of Sambrook *et al.* (1989). *C. elegans* strain N2 was maintained at 15° and propagated on its normal laboratory food source *E. coli* OP50 cells as described previously (Brenner 1974; Honda *et al.* 1993; Garsin *et al.* 2001).

Bioinformatic analyses: The genome sequence of *C. neoformans* var. *grubii* reference strain, H99 (http://www.broadinstitute.org/annotation/genome/cryptococcus_neoformans/MultiHome.html), was used in our study to gain insights into the regulation of nitrogen metabolism and virulence in *C. neoformans*. Sequence analyses were performed using BLAST and MacVector 9.5 (MacVector Inc, Cary, NC) (Altschul *et al.* 1990). Sequence alignments were created using ClustalW v. 1.4 within MacVector (Thompson *et al.* 1994). Sequence traces generated at the Australian Genome Research Facility (Brisbane, Queensland, Australia) were analyzed using Sequencher 4.7 (Gene Codes Corporation, Ann Arbor, MI).

Construction and complementation of mutant strains: GATA-type gene deletion mutants were created using overlap PCR and biolistic transformation as described previously (Davidson *et al.* 2000, 2002). All primers used for the generation of deletion mutants are listed in Table S2. For example, to construct the *gat1*Δ mutant, the 4239-bp *GAT1* (*CNAG00193.2*) coding sequence was replaced with the neomycin phosphotransferase II-encoding selectable marker *NEO* using a construct created by overlap PCR combining an ~1-kb fragment upstream from the *GAT1* start codon, the *NEO* marker, and an ~1-kb fragment downstream from the *GAT1* stop codon. Strain H99 genomic DNA and the plasmid pJAF1 were used as PCR templates (Fraser *et al.* 2003). The construct was transformed into the wild-type strain H99 via particle bombardment. Transformants were selected on YPD plates supplemented with 100 μl/ml G418. Deletion of *GAT1* was confirmed by Southern blot, creating strain RL1 (Southern 2006). To complement the *gat1*Δ mutant, the wild-type *GAT1* gene was amplified from genomic DNA via PCR, the product cloned into pCR2.1-TOPO (Invitrogen) to give pIRL1, and sequenced. The 5.95-kb *Asc*I/*Sac*I *GAT1* fragment of pIRL1 was subcloned into the *Asc*I/*Sac*I sites of the Cryptococcus nourseothricin resistance vector pPZP-NATcc, creating the complementation construct pIRL3 (Walton *et al.* 2005). pIRL3 was subsequently linearized with *Asc*I and *Xma*I, then biolistically transformed into the *gat1*Δ mutant. Stable transformants were selected on YPD plates supplemented with 100 μl/ml nourseothricin and transformants containing a single copy of the wild-type *GAT1* gene were identified by Southern blot.

Quantitative real-time PCR: Strains were grown in YNB supplemented with 10 mm of the specified nitrogen source or YPD and shaken at 30° for 16 hr. Overnight cultures were harvested, cell pellets frozen and lyophilised, total RNA isolated using TRIzol reagent (Invitrogen), and cDNA generated using the SuperscriptIII first-strand synthesis system (Invitrogen). Primers for genes *URO1* (*CNAG04305.2*), *DAL1* (*CNAG00934.2*), *URE1* (*CNAG05540.2*), *GDH1* (*CNAG01577.2*), *GLN1* (*CNAG00457.2*), *GLT1* (*CNAG04862.2*), *GDH2* (*CNAG00879.2*), *AMT1* (*CNAG00235.2*), *AMT2* (*CNAG04758.2*), *PUT1* (*CNAG02049.2*), *PUT5* (*CNAG02048.2*), and *PUT2* (*CNAG05602.2*) (Table S2) were designed to span exon–exon boundaries and tested to verify that they bind specifically to their respective cDNA genes but not H99 genomic DNA (data not shown). Quantitative real-time PCR (qRT-PCR) was performed using SYBR green supermix (Applied Biosystems) and an Applied Biosystems 7900HT fast real time PCR System with the following cycling conditions: denaturation at 95° for 10 min, followed by amplification and quantification in 45 cycles at 95° for 15 sec and 60° for 1 min, with melting curve profiling at 95° for 2 min, 60° for 15 sec, and 95° for 15 sec. Dissociation analysis confirmed the amplification of a single PCR product for each primer pair and an absence of primer dimer formation. Relative gene expression was quantified using SDS software 1.3.1 (Applied Biosystems) on the basis of the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). Several housekeeping genes including *GPD1* (glyceraldehyde-3-phosphate dehydrogenase), *HHT1* (histone H3), and *TUB2* (β-tubulin) were tested, but the *ACT1* (actin) gene was eventually used as a control for normalization since its gene expression demonstrated the highest consistency across all growth conditions tested. Statistical analysis of variance (one-way analysis of variance) was performed using the unpaired, two-tailed *t*-test in GraphPad Prism Version 5.0c. P values <0.05 were considered statistically significant.

Capsule assays: Strains were inoculated onto YNB plates supplemented with 10 mm specified nitrogen source or into RPMI/DMEM supplemented with 10% fetal calf serum (Gibco), and incubated at 37° for 2 days. To visualize capsule, cells were stained with India ink (Becton Dickinson) and observed using a ZEISS Axioplan 2 epifluorescent/light microscope. Pictures were taken with an Axiocam grayscale digital camera using the AxioVision AC imaging software. Quantitative analysis of capsule diameter was performed as described previously (Zaragoza *et al.* 2003).

Growth, melanization, and urease assays: Starter cultures were prepared by growth in YPD at 30° overnight with shaking, diluted to $OD_{595\ nm} = 0.05$ in water, and then further diluted 10-fold in series. For nitrogen utilization, melanization, and urease assays as well as growth test on pigeon guano and at human body temperature, each diluted cell suspension was spotted onto YNB or L-DOPA supplemented with 10 mm specified nitrogen source, Christensen's urea agar, 25% (wt/vol) pigeon guano medium, or YPD medium, respectively. Results were imaged after 2 days' incubation at 30° (nitrogen utilization, growth test on pigeon guano, and urease assays), 30° and 37° (melanisation assays), or 37°–40° (growth test at human body temperature).

Mating assays: Mating assays were conducted as described previously (Nielsen *et al.* 2003). Briefly, strains were pregrown on YPD plates for 2 days, after which a small amount of cells was removed using a flat-end toothpick and patched onto V8 and MS mating media (pH 5.0) either alone or mixed in equal proportions with a strain of the opposite mating-type (*MAT*) (Kwon-Chung *et al.* 1982; Xue *et al.* 2007). Plates were then incubated at room temperature in the dark for 1 week and assessed by light microscopy for formation of filaments and basidia.

***C. elegans* killing assays:** Starter cultures of *C. neoformans* strains were prepared by growth in YPD at 30° overnight with shaking. Overnight cultures, 10 μl, were spread onto both brain–heart infusion (BHI) (Becton Dickinson) and 2.5% pigeon guano agar plates (35 mm), and incubated at 25° overnight. Approximately 50 young adult *C. elegans* worms were then transferred from a lawn of *E. coli* OP50 on NGM to the lawn of BHI and pigeon guano media-grown *C. neoformans* (Mylonakis *et al.* 2002). Plates were incubated at 25° and worms examined for viability at 24-hr intervals using a dissecting microscope, with worms that did not respond to a touch with a platinum wire pick considered dead. Each experimental condition was performed in triplicate. Survival was plotted against time, and P values were calculated by plotting a Kaplan–Meier survival curve and performing a log-rank (Mantel–Cox)

test using Graphpad Prism v. 5.0c. *P* values <0.05 were considered statistically significant.

Murine virulence assays: For murine killing assays, *C. neoformans* strains were used to infect 7-week-old female BALB/c mice by nasal inhalation (Cox *et al.* 2000). Ten mice were inoculated each with a 50-μl drop containing 1×10^5 cells of each strain. Mice were weighed before infection and daily thereafter; animals were sacrificed by cervical dislocation once their body weight had decreased by 20% of the preinfection body weight. Survival was plotted against time, and *P* values were calculated by plotting a Kaplan–Meier survival curve and performing a log-rank (Mantel–Cox) test using Graphpad Prism v. 5.0c. *P* values <0.05 were considered statistically significant.

Ethics statement: This study was carried out in strict accordance with the recommendations in the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes by the National Health and Medical Research Council. The protocol was approved by the Molecular Biosciences Animal Ethics Committee of The University of Queensland (AEC approval no. SCMB/473/09/UQ/NHMRC). Infection and sacrifice were performed under methoxyflurane anesthesia, and all efforts were made to minimize suffering through adherence to the Guidelines to Promote the Wellbeing of Animals Used for Scientific Purposes as put forward by the National Health and Medical Research Council.

RESULTS

Cryptococcus assimilates a limited subset of nitrogen sources: Work on Cryptococcus has demonstrated the ability of this organism to assimilate a number of different nitrogen sources (Chaskes and Tyndall 1975; Staib *et al.* 1976; Polacheck and Kwon-Chung 1980; Fiskin *et al.* 1990; Kmetzsch *et al.* 2010). However, these studies have not taken into consideration the significant advances that have been made in understanding the population structure of the species: the former variety *Cryptococcus gattii* has now been classified as a separate species, and eight distinct Cryptococcus haploid molecular types have been identified, each of which displays distinct physiological properties and virulence features (Kwon-Chung and Varma 2006; Litvintseva *et al.* 2006). To begin to better understand nitrogen metabolism in this pathogen, we sought to extensively identify the panel of usable nitrogen sources in the context of this complex population structure.

We assessed the ability of an array of 16 strains that represent all eight molecular types of the Cryptococcus species complex to assimilate a diverse range of 42 different compounds as sole nitrogen sources on YNB defined medium, including the common L-amino acids as well as a range of purines, pyrimidines, amides, and amines (Table S3). As commercial formulations of agar have been shown to contain substantial concentrations of nitrogen as impurities (up to 4 mM when 20 g/liter is present), we performed all nitrogen assimilation assays using agarose (SeaKem LE) as the gelling agent (Scholten and Pierik 1998). Unlike other well-known fungal genera such as Aspergillus and Neurospora, which can assimilate a remarkably large collection of nitrogen sources, the nitrogen assimilation profile of Cryptococcus more closely resembled that of Saccharomyces; both species are largely restricted to utilization of ammonium and more complex macromolecules such as amino acids and purines (Kinsky 1961; Hynes 1970). Only a subset of strains was able to utilize xanthine, lysine, and nicotinamide as the sole nitrogen source. None of the strains were able to utilize cadaverine, nitrite, or nitrate as sole nitrogen sources. Importantly, while interstrain growth variability on different nitrogen sources was observed, this did not correlate with molecular type.

The explanation for some of these significant differences between Cryptococcus and Aspergillus or Neurospora is evident at the genomic level. As previously reported, analysis of the *C. neoformans* genome confirmed an absence of genes required for molybdenum cofactor biosynthesis, an essential component in all previously characterized fungal nitrate metabolism pathways (Heck *et al.* 2002; Cultrone *et al.* 2005). Genes encoding homologs of key catabolic enzymes for cadaverine, nitrate, and nitrite utilization were also not found in the genome of VNI strain H99 via BLASTp analysis, explaining the lack of growth on these nitrogen sources. We are therefore unable to explain the Cryptococcus growth phenotype associated with nitrate utilization that was observed by Jiang et al.; consistent with our bioinformatic analyses, none of the 16 Cryptococcus strains tested were able to utilize nitrate or nitrite as the sole nitrogen source (Jiang *et al.* 2009).

Nitrogen metabolite repression of transcription exists in *C. neoformans*: In many members of the phylum Ascomycota, nitrogen metabolite repression ensures that of the options available, favored nitrogen sources such as ammonium are assimilated before all others. To address if the same regulatory mechanism exists in the most clinically prevalent form of *C. neoformans* (VNI), we bioinformatically identified several predicted catabolic genes belonging to the uric acid degradation pathway in the unpublished H99 genome and employed qRT-PCR to analyze their transcriptional regulation: *URO1* (encoding urate oxidase), *DAL1* (encoding allantoinase), and *URE1* (encoding the virulence factor urease) (Figure 1A). When compared to an ammonium-grown control, the level of expression of *URO1* and *DAL1* was significantly upregulated (>10-fold) during growth in uric acid as the sole nitrogen source, while that of *URE1* was only slightly upregulated (~3-fold) (Figure 1B). When both uric acid and ammonium were present, this upregulation was abolished, the hallmark of traditional nitrogen metabolite repression (*URO1*, $P = 0.0224$; *DAL1*, $P = 0.0058$; *URE1*, $P = 0.1448$) (Figure 1B). It is worth noting that nitrogen metabolite regulation of *URE1* was not statistically significant, indicating that nitrogen metabolite repression may not affect every nitrogen catabolism-associated gene in *C. neoformans*. However, it is possible that differential expression of

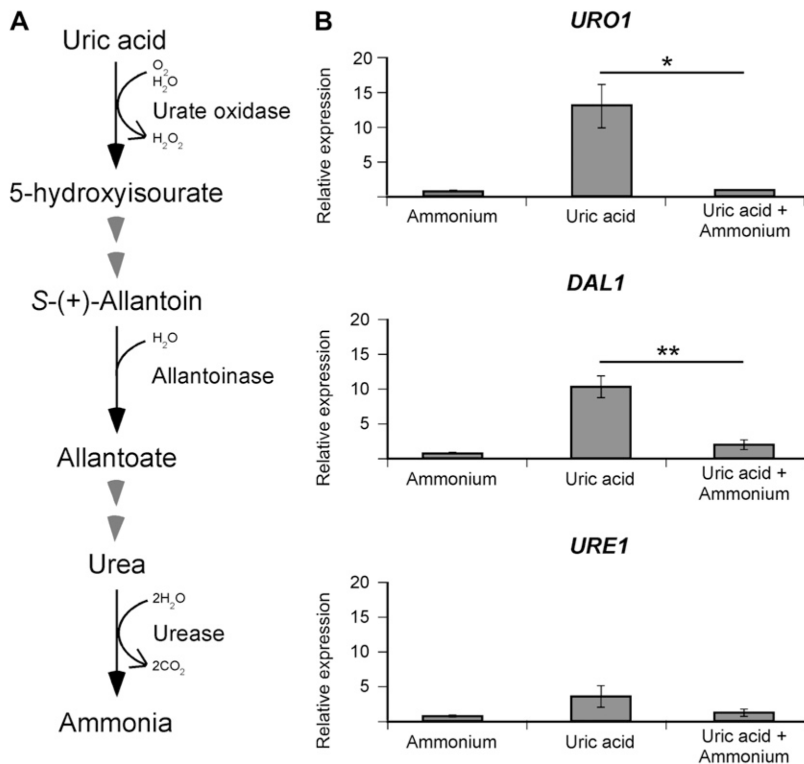


FIGURE 1.—The predicted catabolic enzyme-encoding genes of uric acid, *URO1* and *DAL1*, are sensitive to nitrogen metabolite repression. (A) Scheme representing the predicted (partial) uric acid degradation pathway of *C. neoformans*. (B) cDNA from wild-type H99 grown in YNB supplemented with ammonium, uric acid, or uric acid plus ammonium (10 mM each nitrogen source) were amplified via qRT-PCR using primers against *URO1* (urate oxidase), *DAL1* (allantoinase), *URE1* (urease), and the control gene *ACT1* (actin). In the presence of uric acid as the sole nitrogen source, the expression of *URO1* and *DAL1* was significantly increased while that of *URE1* was slightly increased, but this upregulation was abolished when ammonium was also present. This nitrogen metabolite repression sensitivity of *URO1* (* denotes $P < 0.05$) and *DAL1* (** denotes $P < 0.01$) was statistically significant. Error bars represent standard errors across three biological replicates.

URE1 may be observed under other untested nitrogen conditions such as nitrogen starvation.

Nitrogen metabolite repression regulates well-established virulence factors: Expanding our analysis, we sought to determine whether nitrogen metabolite repression plays a role in regulating the expression of other key virulence factors. Early studies have shown that the virulence factors capsule and melanin are expressed differently during growth on different nitrogen sources (CHASKES and TYNDALL 1975; STAIB *et al.* 1976, 1978). To expand on these classic analyses, we grew *C. neoformans* on a wider selection of easily assimilated (*e.g.*, ammonium) and less easily assimilated (*e.g.*, uric acid) nitrogen sources and found that varying capsule size was indeed observed in the presence of different nitrogen-yielding substrates (Figure 2A and Figure S1). The size of capsule was uniformly small when *C. neoformans* was grown on ammonium, glutamine, proline, or alanine as the sole nitrogen source. In contrast, capsule production was much more prolific when cells were grown on asparagine, compounds from the uric acid degradation pathway (urea, uric acid), or creatinine. Significantly, simultaneous addition of ammonium to each of these nitrogen sources repressed capsule production to a level equivalent to that seen on ammonium alone, providing the first evidence that nitrogen metabolite repression directly influences the formation of the virulence determinant capsule (Figure 2A and Figure S1).

We next investigated *C. neoformans* formation of the dark antioxidant pigment melanin on L-DOPA medium supplemented with the same panel of nitrogen sources. Again, varying virulence factor production was observed in response to the available nitrogen source (Figure 2B). Creatinine, uric acid, and alanine-grown cells synthesized the least melanin, proline synthesized an intermediate amount, and glutamine, asparagine, urea, and ammonium were the most prolific producers of melanin. It was interesting to note that the effect of creatinine and uric acid on capsule production was inversely proportional to melanin production: while uric acid and creatinine induce capsule formation to the largest extent, melanization on these nitrogen sources was the poorest. Similarly, while ammonium induces melanization to the largest extent, capsule formation was extremely poor on this nitrogen source. Consistent with conservation of the nitrogen metabolite repression regulatory paradigm, simultaneous addition of ammonium restored melanin production to the same level as that of the ammonium control (Figure 2B). The only unusual exception occurred with proline-grown cells, which melanized to the same extent with or without ammonium, suggesting that proline catabolism in *C. neoformans* may be insensitive to the repressing effects of ammonium. This observation was not seen with the capsule assays previously, as both proline and ammonium were equally poor inducers of capsule synthesis. Overall, we observed that the general effect of nitrogen metabolite repression on melanin was opposite to that observed for capsule—rather than blocking virulence factor production, the presence of ammonium induces the highest level of melanin formation.

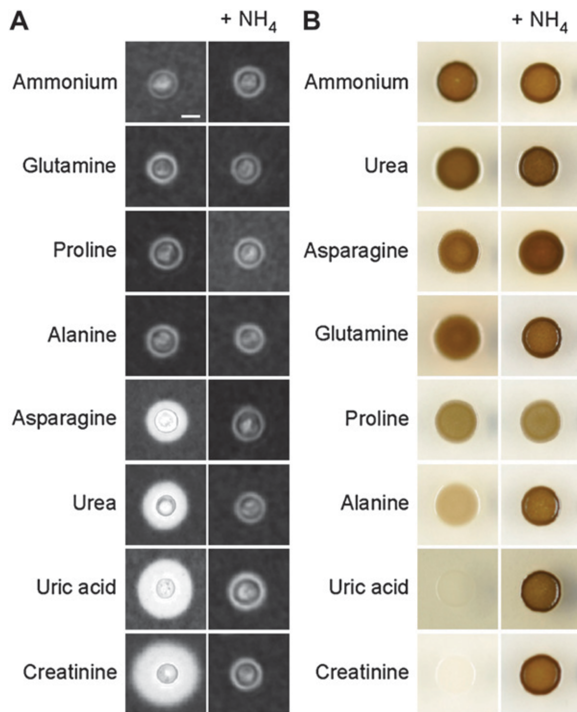


FIGURE 2.—Nitrogen metabolite repression influences capsule and melanin formation. (A) India ink cell staining under light microscopy showed that wild-type H99 produces capsules that vary in size when grown on YNB supplemented with different nitrogen sources (10 mM each). Capsule size increased in the following order: (ammonium, glutamine, proline, alanine), asparagine, urea, uric acid, and creatinine. Upon coculture of each of these nitrogen sources with ammonium, capsule size was restored to that of the ammonium control. Scale bar, 10 μm. (B) Wild-type H99 produces varying amounts of melanin when grown on L-DOPA supplemented with different nitrogen sources (10 mM each). Melanization increased in the following order: (creatinine, uric acid), alanine, proline and (glutamine, asparagine, urea, ammonium). Upon coculture of each of these nitrogen sources with ammonium, melanin production was restored to that of the ammonium control, with the exception of the proline-grown cells, which melanized to the same extent with or without ammonium.

The *C. neoformans* genome encodes up to seven GATA factors: Our phenotypic analyses showed that beyond controlling nitrogen catabolism, nitrogen metabolite repression plays a role in regulating at least two important virulence factors in *C. neoformans*: capsule and melanin. The production of these factors varied on different nitrogen sources, with the addition of ammonium dominating over other nitrogen signals (apart from proline) to either abolish (capsule) or highly activate (melanin) virulence factor production. In the phylum Ascomycota, this dominance of ammonium in controlling a physiological response is mediated by transcription factors belonging to the GATA zinc finger family. We therefore chose to pursue potential GATA factors of *C. neoformans* as likely candidates for the underlying mechanism controlling these responses to nitrogen availability.

GATA transcription factors are distinguished by their highly conserved DNA-binding domain consisting of a Cys_2/Cys_2-type zinc finger motif followed by an adjacent basic region (FU and MARZLUF 1990; MARZLUF 1997). Within the consensus "$Cys–X_2–Cys–X_{variable}–Cys–X_2–Cys$," there are always two residues separating cysteines 1 and 2, and always two residues separating cysteines 3 and 4 of the zinc finger motif. In contrast, the number of residues comprising the central loop separating cysteines 2 and 3 can vary. For example, the zinc finger motif of the *A. nidulans* nitrogen regulatory protein AreA contains a 17-amino-acid central loop while that of *N. crassa* blue light response factor WC1 contains 18 residues (KUDLA *et al.* 1990; BALLARIO *et al.* 1996). We sought to establish a more precise description of this consensus domain in fungi to assist in accurately predicting all of the *bona fide* GATA factor-encoding genes in the genome of *C. neoformans* strain H99.

To more accurately define the sequence constraints associated with fungal GATA factors, we aligned the DNA-binding motif sequences of all 38 known characterized GATA factors, originating from 17 fungal species (Figure S2). By comparing the number of amino acid residues at the central loop of the zinc finger motif and the distribution of basic residues within 25 amino acids adjacent to the zinc finger motif, we were able to define a more stringent GATA motif consensus for fungi: all reported examples matched the motif "$Cys–X_2–Cys–X_{17–20}–Cys–X_2–Cys$ + 5–12 *basic residues within the 25 beyond the most C-terminal cysteine*."

Parallel searches were subsequently taken to identify potential GATA factors in the *C. neoformans* genome. In the first approach, the protein sequences of all 38 known characterized fungal GATA factors were searched against the Broad Institute genome sequence database of strain H99 for potential homologs using BLASTp, leading to the identification of five potential GATA factor-encoding genes (Table S4). However, this approach was heavily biased in requiring the GATA factors detectable in the *C. neoformans* genome to have first been identified in other fungi. In the second approach, we searched the *C. neoformans* genome for the consensus "$Cys–X_2–Cys–X_{17–20}–Cys–X_2–Cys$" using a Perl script-based approach, and hits analyzed for a match with the "5–12 *basic residues within the* 25 *beyond the most C-terminal cysteine*" consensus. This broader analysis identified the same five GATA factor-encoding genes as the BLASTp analysis, plus two additional candidates. In the third approach, we BLASTed all seven hits back against the H99 genome, finding no additional homologs. Through these combined bioinformatic analyses, we therefore identified a total of seven possible GATA factor-encoding genes. Of the seven predicted GATA ORFs identified, all four previously characterized *C. neoformans* members of this family were present: Bwc2, Cir1, Gat201, and Gat1—thus validating the combination approach taken (IDNURM and HEITMAN 2005; JUNG *et al.* 2006; LIU *et al.* 2008;

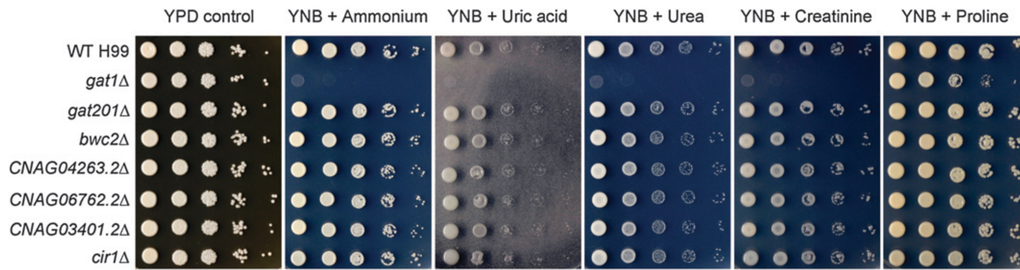


Figure 3.—The *gat1*Δ mutant is unable to utilize a wide variety of nitrogen sources. Tenfold spot dilution assays of wild-type H99 and GATA-type deletion mutants for nitrogen utilization showed that the *gat1*Δ mutant is unable to grow on YNB supplemented with 10 mm ammonium, uric acid, urea, or creatinine but exhibits only a slight growth defect compared to wild type on 10 mm proline. Complementation of the *gat1*Δ mutant with the *GAT1* gene restored wild-type nitrogen utilization phenotype (Figure S3).

Kmetzsch *et al.* 2010). Furthermore, sequence searches of these candidates in Pfam confirmed the presence of a predicted GATA zinc finger, supporting the hypothesis that all seven are *bona fide* GATA factors. Consistent with the observation of Wong *et al.,* all *C. neoformans* GATA factors lack putative leucine zippers that are present in negatively acting nitrogen regulatory GATA factors such as Dal80 and AreB of *S. cerevisiae* and *A. nidulans*, respectively (Wong *et al.* 2008).

Only one positively acting GATA factor controls global nitrogen source utilization in *C. neoformans*: Despite the fact that four GATA factors have been previously characterized in *C. neoformans*, to our knowledge any potential role in nitrogen metabolite repression has not been explored in these transcription factors. It is possible that a GATA factor may play a regulatory role in more than one global response or that more than one GATA transcriptional activator may play a role in nitrogen regulation as in systems of *S. cerevisiae* and *C. albicans* (Stanbrough *et al.* 1995; Limjindaporn *et al.* 2003; Liao *et al.* 2008). All seven ORFs containing our strict consensus for a fungal GATA DNA-binding domain were therefore deleted via homologous recombination.

The seven GATA mutants were viable and had similar growth rate as the wild-type strain on YPD medium at 30° (Figure 3). However, all but one of the *C. neoformans* GATA mutant strains exhibited growth equivalent to wild-type H99 on YNB defined medium supplemented with a wide panel of 38 usable nitrogen sources (Figure 3 and Table S5). Consistent with previous studies conducted by Kmetzsch *et al.* (2010), deletion of ORF *CNAG00193.2* (*GAT1*) rendered *C. neoformans* incapable of utilizing a wide repertoire of nitrogen sources. The *gat1*Δ mutant, however, could still utilize a limited selection of amino acids, notably proline, strengthening our hypothesis that proline utilization is largely independent of nitrogen metabolite repression. Importantly, we found that the *gat1*Δ mutant was also unable to utilize uric acid and creatinine, two predominant nitrogen constituents found in the ecological niche (pigeon guano) of *C. neoformans* (Staib *et al.* 1976, 1978; Casadevall and Perfect 1998).

Gat1 mediates nitrogen metabolite repression: The fact that the *gat1*Δ mutant does not grow on a wide variety of nitrogen sources does not *ipso facto* prove that Gat1 mediates nitrogen metabolite repression. To investigate the regulatory mechanism of Gat1, we performed qRT-PCR to analyze how the deletion of *GAT1* affects transcriptional regulation of genes associated with nitrogen metabolism. The unusual observation that loss of *GAT1* merely reduces growth on the less easily assimilated nitrogen source proline rather than completely abolish it provided an ideal condition under which to study the transcriptional effects of the loss of this regulatory gene. Growth in proline enabled the isolation of RNA from defined medium without concern for starvation-related artifacts of the *gat1*Δ mutant associated with growth in nonutilizable nitrogen sources such as uric acid. Hence, by choosing proline as the sole source of nitrogen for culturing of both the wild-type and mutant strains, we were able to isolate RNA from a steady-state, actively growing culture with a defined nitrogen source.

Bioinformatic analyses of the H99 genome identified several genes predicted to encode enzymes required for degradation of proline: *PUT1* and *PUT5*, two paralogs that each encode proline oxidase, and *PUT2*, which encodes pyrroline-5-carboxylate dehydrogenase (Figure 4A). qRT-PCR using RNA isolated from wild-type cells revealed that only one of these three proline catabolic genes was regulated by nitrogen metabolite repression, with higher levels of transcription (>15-fold) on proline than when both proline and ammonium were present (*PUT1*, $P < 0.0001$) (Figure 4B). In the *gat1*Δ mutant, transcription of *PUT1* was not highly activated but instead showed the same level of transcription irrespective of the presence of ammonium, confirming the role of Gat1 as the mediator of nitrogen metabolite repression (Figure 4B). Unlike *PUT1*, both *PUT5* and *PUT2* were unaffected by nitrogen metabolite repression, explaining why the *gat1*Δ mutant can utilize proline as the sole nitrogen source, albeit less effectively than wild type. Although the expression of *PUT1* was drastically reduced in the *gat1*Δ mutant, *PUT5* was still expressed at wild-type levels sufficient to produce proline oxidase, as was *PUT2* (encoding pyrroline-5-carboxylate dehydrogenase), enabling Gat1-independent growth on proline as the sole nitrogen source.

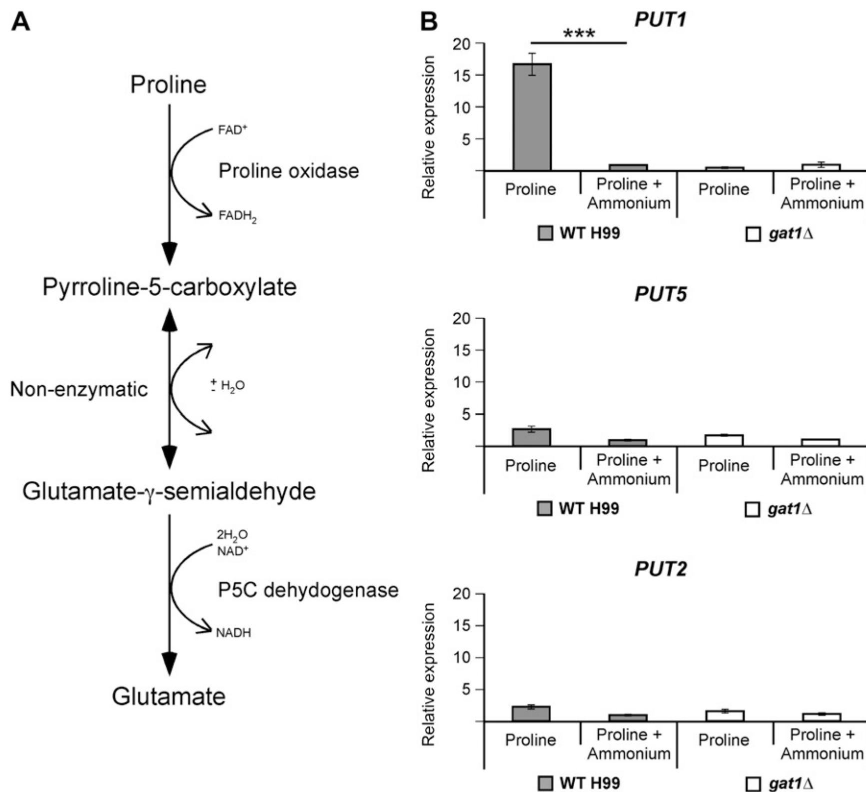


FIGURE 4.—Gat1 regulates nitrogen metabolite repression. (A) Scheme representing the predicted proline degradation pathway of *C. neoformans*. (B) cDNA from wild-type H99 and *gat1*Δ mutant grown in YNB supplemented with proline or proline plus ammonium (10 mM each nitrogen source) were amplified via qRT-PCR using primers against *PUT1* (proline oxidase), *PUT5* (proline oxidase), *PUT2* (pyrroline-5-carboxylate dehydrogenase), and the control gene *ACT1* (actin). One of the predicted catabolic enzyme-encoding genes of proline, *PUT1*, was sensitive to nitrogen metabolite repression in wild type (*** denotes $P < 0.0001$) but not in the *gat1*Δ mutant. The remaining two catabolic genes, *PUT5* and *PUT2*, were nitrogen metabolite repression insensitive in both strains. Error bars represent standard errors across three biological replicates.

Gat1 positively regulates the expression of key ammonium assimilation and permease genes: While certain phenotypic similarities to ascomycete nitrogen regulatory GATA factor mutants were clear, this basidiomycete ortholog mutant exhibited an ammonium phenotype much more extreme than that seen in previously described GATA factor mutants. The *gat1*Δ mutant is unable to utilize the most easily assimilated nitrogen source in both ammonium replete and sufficient conditions (1–100 mM) (data not shown), paradoxically suggesting that the nitrogen metabolite repression mechanism that occurs in the presence of ammonium blocks the ability to utilize ammonium itself. While a slight reduction in growth on ammonium has been observed for other fungal nitrogen regulatory GATA mutants, the complete loss of growth is unprecedented and counterintuitive (CHRISTENSEN *et al.* 1998; HENSEL *et al.* 1998; LIAO *et al.* 2008). To investigate this difference in phenotype, we bioinformatically identified the predicted central nitrogen metabolism genes in the H99 genome: *GDH2* [encoding NAD^+-dependent glutamate dehydrogenase (NAD-GDH)] as well as the ammonium assimilation enzyme-encoding genes *GDH1* [encoding $NADP^+$-dependent glutamate dehydrogenase (NADPH-GDH)], *GLN1* [encoding glutamine synthetase (GS)], and *GLT1* [encoding NAD^+-dependent glutamate synthase (GOGAT)] (Figure 5A).

qRT-PCR was again employed, this time to analyze the transcriptional regulation of these central nitrogen metabolism genes as well as the two known ammonium permease-encoding genes *AMT1* and *AMT2* in both the wild-type and *gat1*Δ mutant strains (Figure 5B) (RUTHERFORD *et al.* 2008). When grown in rich undefined YPD medium that contains a wide array of nitrogen sources including the generally preferred ammonium, glutamine, and glutamate, the level of expression of the major ammonium assimilation enzyme-encoding gene *GDH1* as well as the permease-encoding genes *AMT1* and *AMT2* was significantly lower (∼10–20-fold) in the *gat1*Δ mutant compared to wild type (*GDH1*, $P < 0.0001$; *AMT1*, $P = 0.0086$; *AMT2*, $P = 0.0308$). The level of expression of the other central nitrogen metabolism genes was also consistently lower (∼1.5-fold) in the *gat1*Δ mutant than in wild type, although these differences for the remaining two ammonium assimilation enzyme-encoding genes *GLN1* and *GLT1* were not quite statistically significant (*GLN1*, $P = 0.0666$; *GLT1*, $P = 0.0523$; *GDH2*, $P = 0.1308$). This result indicates that Gat1 controls the expression of the ammonium assimilation enzyme-encoding gene *GDH1* and permease-encoding genes *AMT1* and *AMT2* in *C. neoformans*.

Gat1 is not required for the growth of *C. neoformans* on pigeon guano: The *gat1*Δ mutant is unable to utilize uric acid, urea, or creatinine, the most abundant nitrogen sources found in the natural habitat of *C. neoformans*. This raises the question of whether the *GAT1* gene is essential for optimum growth on pigeon guano, a growth medium previously validated for both *C. neoformans* and *C. gattii* (NIELSEN *et al.* 2007). We therefore

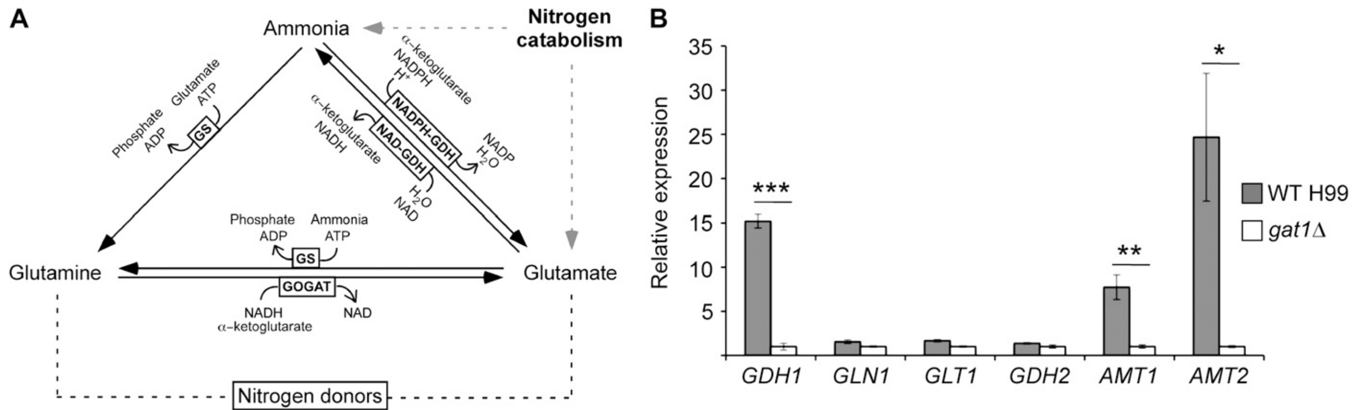


FIGURE 5.—Gat1 regulates the expression of the ammonium assimilation enzyme and permease-encoding genes. (A) Scheme representing the predicted pathway of central nitrogen metabolism in *C. neoformans.* (B) cDNA from wild-type H99 and *gat1*Δ mutant grown in YPD were amplified via qRT-PCR using primers against *GDH1* (NADPH-GDH), *GLN1* (GS), *GLT1* (GOGAT), *GDH2* (NAD-GDH), *AMT1* (ammonium transporter 1), *AMT2* (ammonium transporter 2), and the control gene *ACT1* (actin). The expression of *GDH1, AMT1,* and *AMT2* was significantly lower in the *gat1*Δ mutant compared to wild type, while that of *GLN1, GLT1,* and *GDH2* was slightly lower. This difference in level of expression for *GDH1* (*** denotes $P < 0.0001$), *AMT1* (** denotes $P < 0.01$), and *AMT2* (* denotes $P < 0.05$) was statistically significant. Error bars represent standard errors across three biological replicates.

investigated whether the loss of *GAT1* affected growth on this substrate that mimics the environmental niche. Our growth assays indicate that the wild-type and *gat1*Δ mutant strains displayed equivalent growth on both filtered and unfiltered 25% pigeon guano agar media, revealing that pigeon guano contains at least one nitrogen source that supports robust growth and whose utilization is Gat1 independent (Figure S4).

Gat1 negatively regulates mating in both mating-type α and a strains: During nitrogen limiting or starvation conditions, *C. neoformans* can undergo sexual reproduction that leads to the production of potentially infectious air-borne basidiospores (Kwon-Chung 1976; Lengeler *et al.* 2000; Giles *et al.* 2009). To address whether Gat1 plays a role in this sexual development, we created a *gat1*Δ derivative of *MAT***a** *C. neoformans* strain KN99**a** (VNI) by replacing the *GAT1* ORF with the nourseothricin acetyl-transferase (*NAT*) selectable marker, to serve as a mating partner for our existing *gat1::NEO* of *MAT*α strain H99. Wild type (wild type × wild type), unilateral (*gat1* × wild type), and bilateral (*gat1* × *gat1*) crosses were then performed by mixing strains of opposite mating type on V8 and MS media. The ability to mate was examined by microscopically inspecting the production of filaments and basidia at the edges of the mating patches. Unexpectedly, filamentation and production of basidia were enhanced in both unilateral and bilateral *gat1*Δ crosses in comparison to wild-type crosses (Figure 6). Furthermore, deletion of *GAT1* in both the *MAT*α and *MAT***a** strains led to the most robust filament formation and production of basidia, indicating that the effect of this gene deletion is additive. This result indicates that Gat1 plays a critical role in regulating morphological differentiation in both mating types of *C. neoformans.*

Gat1 positively regulates capsule synthesis, but negatively regulates melanin formation and growth at high body temperature: Given that nitrogen metabolite repression plays a role in regulation of capsule synthesis, we next chose to examine capsule production by the *gat1*Δ mutant. Strains were cultured in amino-acid-rich RPMI and DMEM media supplemented with 10% fetal calf serum; both media supported growth of all strains including the *gat1*Δ mutant (Zaragoza *et al.* 2003). India ink staining revealed that while the wild-type strain produced characteristic halos around its cells representing enlarged capsule, the *gat1*Δ mutant produced reduced amounts of capsule under these serum-induced conditions (Figure 7A and Figure S5). This result indicates that Gat1 plays a positive role in the regulation of capsule synthesis.

Like capsule, melanin synthesis is also under the influence of nitrogen metabolite repression control. The role of Gat1 was therefore evaluated for melanin production at both 30° and 37° on L-DOPA medium supplemented with proline as the sole nitrogen source, since proline supports the most robust growth in the *gat1*Δ mutant on defined medium. Interestingly, the *gat1*Δ mutant melanized to the same extent as wild type at 30° but produced more melanin than wild type at 37° (Figure 7B). This result indicates that Gat1 plays a negative role in regulating melanin production at human body temperature.

We also evaluated the role of Gat1 in regulating the activity of the nitrogen-scavenging enzyme urease on Christensen's urea agar. Although both the *gat1*Δ and negative control *ure1*Δ mutants are unable to utilize urea as the sole nitrogen source, the mutants could still grow on Christensen's medium as impurities found in agar likely provide alternative sources of consumable nitrogen.

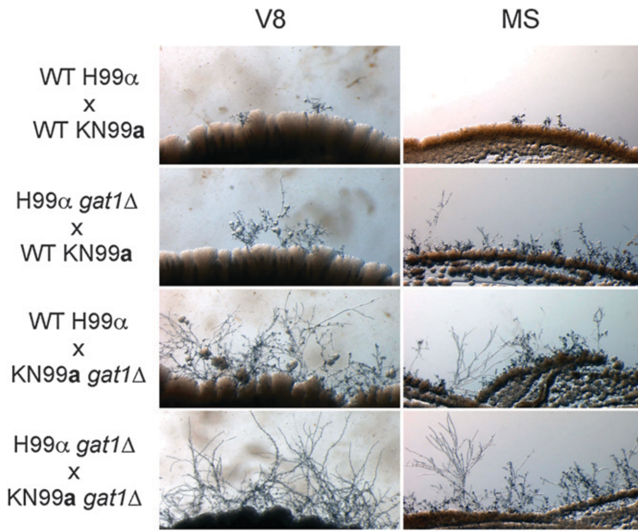


FIGURE 6.—Gat1 inhibits filament formation during mating. Filamentation assays on V8 and MS mating media (pH 5.0) showed that filament formation was enhanced in unilateral *gat1*Δ crosses in comparison to the wild-type crosses. Filamentation was enhanced even further in a bilateral *gat1*Δ cross. Complementation of the H99α *gat1*Δ and KN99**a** *gat1*Δ mutants with the *GAT1* gene restored wild-type mating in all tested crosses (data not shown).

In support of our previous qRT-PCR data indicating that urease is not significantly regulated by nitrogen metabolite repression, the *gat1*Δ mutant still produces urease even though the mutant is unable to utilize the ammonia resulting from urea catabolism (Figure 7C).

To complete a thorough phenotypic analysis of the *gat1*Δ mutant, we evaluated the role of Gat1 for its ability to grow at high temperature (37°–40°), a common stress that *C. neoformans* encounters both in a mammalian host and in its ecological niche. While growth up to 38° was equivalent in the wild-type and *gat1*Δ mutant strains, the mutant exhibited better growth in comparison to wild type at 39°, the febrile temperature commonly experienced by most patients upon the onset of cryptococcal meningoencephalitis (Figure 7D). Furthermore, at 40°, growth of the wild-type strain was completely abolished while the *gat1*Δ mutant was still able to proliferate, albeit at a slow rate. This unanticipated finding indicates that Gat1 plays a negative role in regulating growth at high temperature.

Gat1 is not required for the killing of *C. elegans* but modestly represses virulence in a murine inhalation model of cryptococcosis: Our ultimate interest in *C. neoformans* lies in better understanding its pathogenicity. In this context our data thus far were confusing, as Gat1 arguably contributes to production of some virulence attributes but repression of others. For example, the *gat1*Δ mutant's reduction in capsule synthesis and abated ability to utilize a variety of nitrogen sources suggest that this strain should exhibit a decrease in virulence, just like that seen in equivalent ascomycete GATA factor mutants. In contrast, the increase in melanin production and enhanced growth at high temperature would support an increase in virulence. To determine the effect of Gat1 on virulence, we performed *C. elegans* killing assays and a murine inhalation model of cryptococcosis.

C. neoformans presumably interacts with *C. elegans* in the environment and killing of *C. elegans* by *C. neoformans in vitro* has previously been validated as a model for studying pathogenesis (Mylonakis *et al.* 2002). We performed *C. elegans* virulence assays using two different media: the standard BHI medium for nematode killing experiments, as well as 2.5% pigeon guano medium to

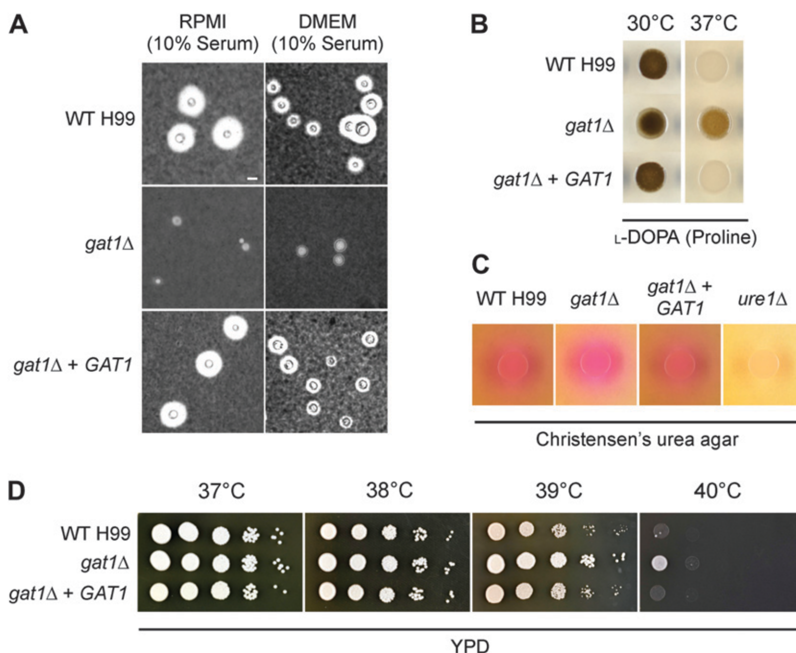
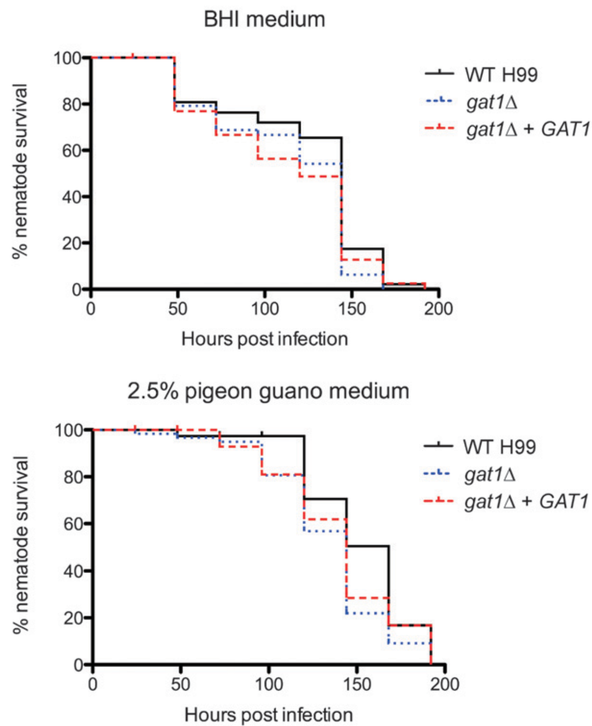


FIGURE 7.—Gat1 is required for capsule synthesis, but negatively regulates melanization and growth at 39° or 40°. (A) India ink cell staining under light microscopy revealed that the wild-type H99 and *gat1*Δ + *GAT1* strains produce enlarged capsules while the *gat1*Δ mutant produces residual amount of capsule when strains were cultured under serum-induced growth conditions. Scale bar, 10 μm. (B) The *gat1*Δ mutant produces more melanin compared to both the wild-type and *gat1*Δ + *GAT1* strains when grown on L-DOPA medium supplemented with 10 mM proline at 37°. In contrast, all three strains melanized to the same extent when grown at 30°. (C) Unlike the negative control *ure1*Δ mutant, the wild-type, *gat1*Δ mutant and *gat1*Δ + *GAT1* strains all had the ability to produce urease when grown on Christensen's urea agar as reflected by the bright pink clearing surrounding the aliquot of spotted cells. (D) Tenfold spot dilution assays on YPD medium at human body temperature showed that the *gat1*Δ mutant exhibits enhanced growth compared to both the wild-type and *gat1*Δ + *GAT1* strains at 39° and 40°.



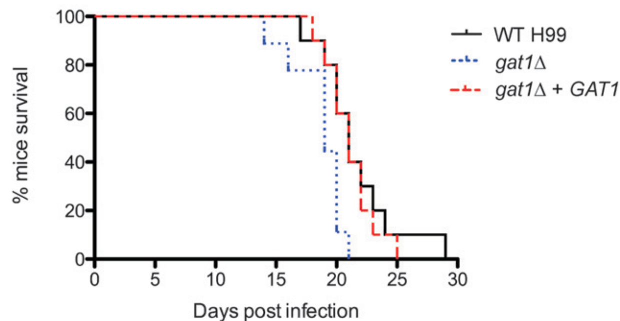


FIGURE 8.—The *gat1*Δ mutant kills *C. elegans* as efficiently as wild-type H99, but exhibits modestly enhanced virulence in a murine host. (A) *C. elegans* infection: ~50 nematode worms were transferred to a lawn of wild-type, *gat1*Δ, or *gat1*Δ + *GAT1* cells as the sole food source on both BHI and 2.5% pigeon guano media, and survival was monitored at 24-hr intervals. There was no observable difference in *C. elegans* killing by all three strains on both BHI (wild type *vs. gat1*Δ, $P = 0.1066$; *gat1*Δ *vs. gat1*Δ + *GAT1*, $P = 0.9230$) and pigeon guano media (wild type *vs. gat1*Δ, $P = 0.0250$; *gat1*Δ *vs. gat1*Δ + *GAT1*, $P = 0.3614$). (B) *Mus musculus* infection: 10 mice were infected intranasally with either 1×10^5 cells of wild type, *gat1*Δ, or *gat1*Δ + *GAT1* strains, and survival was monitored daily. Mice infected with the wild-type and *gat1*Δ + *GAT1* strains progress to morbidity at the same rate (wild type *vs. gat1*Δ + *GAT1*, $P = 0.6691$), whereas mice infected with the *gat1*Δ strain progress to morbidity more rapidly (wild type *vs. gat1*Δ, $P = 0.0151$).

mimic *C. neoformans* ecological niche. Under both growth conditions, killing of *C. elegans* by the *gat1*Δ mutant [LT_{50} (time for half of the worms to die) = 6 days for both BHI and pigeon guano media] was not significantly different to that observed for wild type ($LT_{50} = 6$ days and 7 days, for BHI and pigeon guano media, respectively) (Figure 8A). Gat1 is therefore not required for *C. neoformans*-mediated killing of the invertebrate *C. elegans*.

On the other hand, the murine inhalation model of cryptococcosis more closely mimics human infection by *C. neoformans*—inhaled cryptococcal cells first infect the lungs before disseminating to the brain to cause meningoencephalitis. Interestingly, mice infected with the *gat1*Δ mutant succumbed to infection slightly faster (between 14 and 21 days postinfection, median survival of 19 days) than mice infected with the wild-type strain (between 17 and 28 days postinfection, median survival of 23 days) (wild type *vs. gat1*Δ, $P = 0.0151$) (Figure 8B). This result suggests that the *gat1*Δ mutant is modestly more virulent than wild type during murine infection. Thus, in addition to regulating nitrogen metabolism and virulence factor expression *in vitro*, Gat1 also represses virulence in a vertebrate host.

DISCUSSION

The ability to acquire and catabolize nutrients from the environment is imperative for survival of an organism. While fungi are well known for their ability to utilize a broad range of nitrogen sources, the study of molecular mechanisms controlling nitrogen acquisition and catabolism in model fungi have until now been primarily limited to members of the phylum Ascomycota (WONG *et al.* 2008). Our studies of transcriptional regulation and phenotypic responses to the presence of ammonium have shown that utilization of nitrogen sources by *C. neoformans* is controlled in a similar fashion. The results we report here represent the first definitive characterization of the regulatory phenomenon of nitrogen metabolite repression in a second phylum of the kingdom fungi, the Basidiomycota. In addition to functional conservation between these two phyla, the repertoire of physiological responses controlled by nitrogen metabolite repression in *C. neoformans* has expanded to encompass regulation of virulence factor expression. We have also shown that the GATA factor Gat1 is responsible for mediating nitrogen metabolite repression in *C. neoformans*.

Bioinformatically, the predicted GATA factor of *C. neoformans* Gat1 shows little similarity to nitrogen regulatory GATA factors of the Ascomycota for the majority of the protein, except within the GATA motif itself (Table S6). With 19% overall protein identity, it showed poor but still highest similarity to the various nitrogen regulatory GATA factors of the Ascomycota. This

similarity, however, was largely restricted to the predicted GATA zinc finger where identity to *A. nidulans* AreA and *N. crassa* Nit2 was 88 and 90%, respectively.

The dependence of *C. neoformans* nitrogen metabolism on a single GATA factor is in stark contrast to *S. cerevisiae* or *C. albicans*, where multiple nitrogen regulatory GATA factors are required (Stanbrough *et al.* 1995; Limjindaporn *et al.* 2003; Liao *et al.* 2008). It is important to note that the existence of the two positively acting GATA factors, Gln3 and Gat1, in the hemiascomycete *S. cerevisiae* is not the result of the whole-genome duplication event that occurred around 100 million years ago, since these two orthologs are also present in *C. albicans*, a species that did not undergo this event (Wong *et al.* 2008; Gordon *et al.* 2009). *C. neoformans* global nitrogen regulatory circuit instead more closely resembles the single positively acting GATA factor systems in *A. nidulans* and *N. crassa* (Stewart and Vollmer 1986; Kudla *et al.* 1990). Given that *N. crassa nit-2* was named after the mutant's inability to utilize nitrate, which *C. neoformans* is unable to do, and the apparent existence of only one GATA factor responsible for nitrogen utilization, we therefore recommend an alternative and more informative name to this gene: *ARE1*.

The similarities in the global nitrogen regulatory circuit between *C. neoformans* and *A. nidulans* or *N. crassa* continue when NmrA and Nmr1 are considered. The Nmr proteins are inhibitors of the functions of *A. nidulans* AreA and *N. crassa* Nit2, and a potential homolog of this protein named Tar1 has recently been characterized in *C. neoformans*, suggesting that the regulation of GATA factor activity may operate in a similar fashion to these filamentous ascomycetes (Pan *et al.* 1997; Andrianopoulos *et al.* 1998; Jiang *et al.* 2009). However, this model is confounded by conflicting observations between the studies of Jiang *et al.* and our own. Most notably, we have bioinformatics and phenotypic evidence indicating that *C. neoformans* is unable to utilize nitrate as a nitrogen source. Confusingly, Jiang *et al.* observed growth changes on this same nitrogen source as evidence that Tar1 plays a role in nitrogen metabolism, an observation that we are unable to explain. Furthermore, *C. neoformans* Gat1/Are1 lacks the highly conserved C-terminus sequence of AreA and Nit2, which is involved in Nmr recognition (Platt *et al.* 1996; Pan *et al.* 1997; Andrianopoulos *et al.* 1998). Nevertheless, Nmr proteins also interact with the DNA-binding domain of AreA and Nit2, and further study will be required to determine if Tar1 is a true functional ortholog of NmrA/Nmr1.

Unlike regulation of AreA and Nit2 by Nmr, the *S. cerevisiae* GATA transcriptional activators Gln3 and Gat1 are negatively regulated by the structurally unrelated prion-forming glutathione-*S*-transferase Ure2, predicted to have been horizontally transferred from the bacterial species *Burkholderia vietnamiensis* (Masison and Wickner 1995; Xu *et al.* 1995; Hall and Dietrich 2007; Wong *et al.* 2008). Our analyses did not identify a Ure2-like candidate encoded in the *C. neoformans* genome. Together, these data support the model that *C. neoformans* and the filamentous ascomycetes share a nitrogen regulatory mechanism that more closely resembles the ancestral nitrogen metabolism regulatory pathway than that seen in *S. cerevisiae.* We therefore propose that the last common ancestor of the Ascomycota and Basidiomycota likely had one positively acting GATA factor (the AreA/Nit2/Gat1/Are1 ortholog) and corepressor protein Nmr; after the phyla separated, a second GATA factor (Gln3) and Ure2 coevolved in *S. cerevisiae*, and the *nmr* gene was lost.

While the effect of the loss of *GAT1/ARE1* on nitrogen metabolism largely met our predicted phenotype, a key aspect of it did not. One of the most confusing, and seemingly contradictory, phenotypes of the *gat1/are1*Δ mutant is its inability to grow on ammonium as the sole nitrogen source. We subsequently gained insights into this paradox by proving that along with its established role as an activator of secondary catabolic gene expression in the absence of ammonium, Gat1/Are1 is also functional in the presence of ammonium. Like *A. nidulans* AreA, *C. neoformans* Gat1/Are1 also regulates the expression of the major ammonium assimilation enzyme-encoding gene *GDH1* as well as the permease-encoding genes *AMT1* and *AMT2* (Christensen *et al.* 1998; Monahan *et al.* 2002, 2006; Rutherford *et al.* 2008). Since the alternative pathway of ammonium assimilation (via *GLN1* and *GLT1*) is not significantly impaired in the *gat1/are1*Δ mutant, we speculate that the basal levels of transcription of *AMT1* and *AMT2* may be insufficient for ammonium uptake. It is worth noting that our nitrogen metabolite repression study followed the traditional use of ammonium as the "repressing" nitrogen source. In reality, it is likely that the true signal affecting Gat1/Are1 activity may be intracellular concentrations of glutamine and/or glutamate since these metabolites reflect the nitrogen status of the cell in other species. Further work using glutamine, glutamate, and perhaps even nitrogen starvation will be required to dissect this aspect of the nitrogen-sensing mechanism.

The opposing regulatory effect of Gat1/Are1 on melanin and capsule production has been observed in previously characterized GATA factors of *C. neoformans*: Cir1 and Gat201 (Jung *et al.* 2006; Liu *et al.* 2008). Notably, although *LAC1* is the major contributor to melanin biosynthesis, we were unable to find putative Gat1/Are1 HGATAR binding sites within a 1-kb region upstream from the start codon of *LAC1* (Pukkila-Worley *et al.* 2005). In contrast, numerous HGATAR sites were found in the promoter regions of *CAP60* and *CAP64*, two key genes that are regulated to control capsule biosynthesis (Chang *et al.* 1996; Chang and Kwon-Chung 1998). Together, these data suggest that Gat1/

Are1 may activate the transcription of capsule biosynthesis genes directly, but indirectly regulate melanin production, perhaps by regulating the expression of a repressor of *LAC1* transcription.

To gain insights into the role of Gat1/Are1 in the environment we employed a novel approach, combining an established virulence model based on a known predator (*C. elegans*) with a medium designed to emulate the environmental niche (pigeon guano). This study enabled us to make an important observation: Gat1/Are1 does not appear to have a significant effect on virulence in the environmental niche; instead it may play a role in nitrogen scavenging. However, in a murine host, this gene has likely been co-opted into regulating various aspects of the virulence composite.

It is nevertheless worth noting that the *C. elegans* killing assays were conducted at 25° instead of mammalian body temperature. Whereas the enhanced growth and melanization abilities of the *gat1/are1*Δ mutant may be exhibited during the infection process in mice, these virulence attributes could not be displayed in *C. elegans*. While the slightly quicker progression to morbidity in mice infected with the *gat1/are1*Δ mutant would not be considered great enough to classify this strain as "hypervirulent," it certainly highlights the complexity of the role of Gat1/Are1 in gene regulation of *C. neoformans* during infection. We note that Kmetzsch *et al.* (2010) recently reported that the *gat1/are1*Δ mutant exhibited equivalent virulence to wild type when high doses (1×10^7 cells per strain) were used, which resulted in mice succumbing to infection as early as the third day postinfection. In contrast, we employed the more traditional inoculum of 1×10^5 cells to ensure the gradual progression of disease. We believe this increased sensitivity helped identify the subtle difference in virulence between the wild-type and *gat1/are1*Δ mutant strains that had previously been missed.

Notably, all other known GATA factors of *C. neoformans* (Bwc2, Cir1, and Gat201) play a role in virulence factor expression *in vitro* and affect virulence during murine infection (Idnurm and Heitman 2005; Jung *et al.* 2006; Liu *et al.* 2008). Bwc2, Cir1, Gat201, and Gat1/Are1 together with the three other uncharacterized GATA factors (CNAG03401.2, CNAG04263.2, CNAG06762.2) we have identified are predicted to bind to 5′-GATA-3′ sites in the genome (Ko and Engel 1993; Merika and Orkin 1993; Ravagnani *et al.* 1997). Whether these GATA factors antagonize and compete for the same 5′-GATA-3′ binding sites in the promoter regions of genes, act in synergy to activate transcription of genes, or act interchangably remains to be determined. Notwithstanding, it is becoming increasingly apparent that Bwc2, Cir1, Gat201, and Gat1/Are1 can act in parallel to regulate multiple virulence pathways in *C. neoformans*.

In summary, Gat1/Are1 not only regulates nitrogen metabolite repression and the expression of catabolic enzyme and permease-encoding genes required for nitrogen assimilation, it is also a key regulator of essential virulence traits in this important human pathogen. While the significance of Gat1/Are1 is now clearly demonstrated, full appreciation of its role awaits further analysis of the gene targets and processes regulated by this global transcription factor as well as its potential interactions with other GATA factors. Certainly, a more complete understanding of the complex regulatory circuit governing nitrogen metabolism and virulence mechanisms in *C. neoformans* will require further study.

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GENETICS

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Nitrogen Metabolite Repression of Metabolism and Virulence in the Human Fungal Pathogen *Cryptococcus neoformans*

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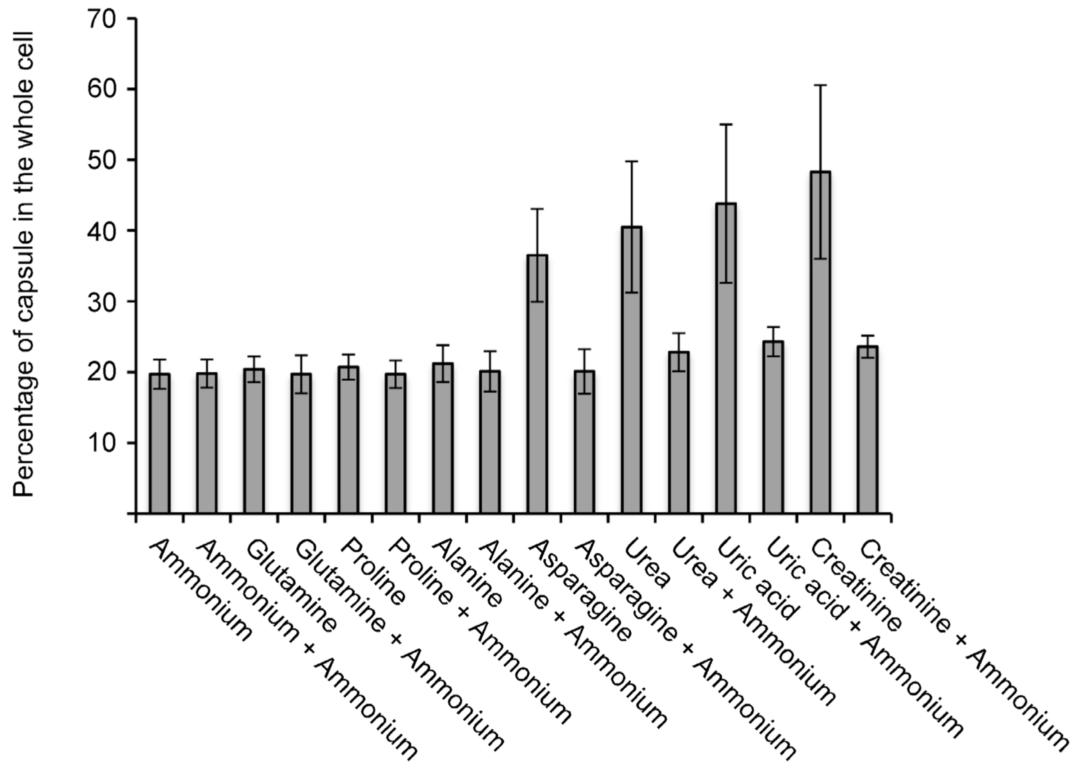

FIGURE S1.–Quantitative measurements of relative capsule diameters of wild-type H99 cells grown on YNB supplemented with various nitrogen sources (10 mM). A total of 60 cells (20 cells from three independent experiments) were measured for each nitrogen condition. Error bars represent standard errors.

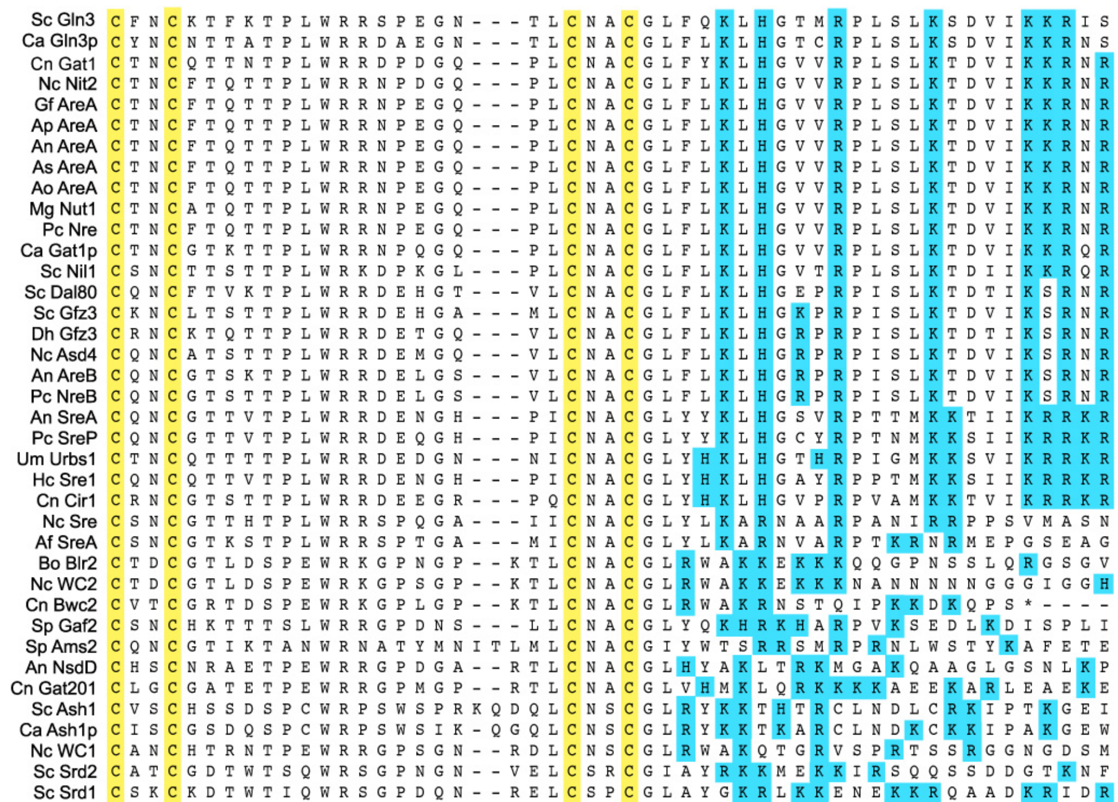

FIGURE S2.–ClustalW multiple sequence alignment of GATA DNA-binding domains from various fungi retrieved either from GenBank or Broad Institute H99 database. Residues highlighted in yellow represent the universally conserved cysteines involved in zinc ion chelation. Amino acids at the central loop separating the second and third cysteines are in the range of 17-20 residues in length. Residues highlighted in blue indicate basic amino acids within 25 residues beyond the most C-terminal cysteine. Accession numbers or Broad annotations of protein sequences: *Saccharomyces cerevisiae* (Sc) Gln3 P18494.2, *Candida albicans* (Ca) Gln3p XP_721981.1, *Cryptococcus neoformans* (Cn) Gat1 CNAG00193.2, *Neurospora crassa* (Nc) Nit2 P19212.2, *Gibberella fujikuroi* (Gf) AreA P78688.1, *Aspergillus parasiticus* (Ap) AreA AAD37409.1, *Aspergillus nidulans* (An) AreA XP_681936.1, *Aspergillus niger* (As) AreA O13412.1, *Aspergillus oryzae* (Ao) AreA XP_001816951.1, *Magnaporthe grisea* (Mg) Nut1 XP_366679.1, *Penicillium chrysogenum* (Pc) Nre Q01582.1, Ca Gat1p AAP50501.1, Sc Nil/Gat1 NP_116632.1, Sc Dal80 CAA42757.1, Sc Gfz3 NP_012425.1, *Debaryomyces hansenii* (Dh) Gfz3 CAJ78410.1, Nc Asd4 Q9HEV5.1, An AreB XP_663825.1, Pc NreB XP_002557977.1, An SreA XP_657780.1, Pc SreP XP_002567610.1, *Ustilago maydis* (Um) Urbs1 XP_757197.1, *Histoplasma capsulatum* (Hc) Sre1 ABY66603.1, Cn Cir1 CNAG04864.2, Nc Sre XP_961978.1, *Aspergillus fumigatus* (Af) SreA XP_753523.1, *Bipolaris oryzae* (Bo) Blr2 BAF47401.1, Nc WC2 P78714.1, Cn Bwc2 CNAG02435.2, *Schizosaccharomyces pombe* (Sp) Gaf2 AAB38022.1, Sp Ams2 NP_588400.2, An NsdD XP_660756.1, Cn Gat201 CNAG01551.2, Sc Ash1 NP_012736.1, Ca Ash1p CAC48044.1, Nc WC1 Q01371.2, Sc Srd2 NP_015304.1 and Sc Srd1 NP_009944.2.

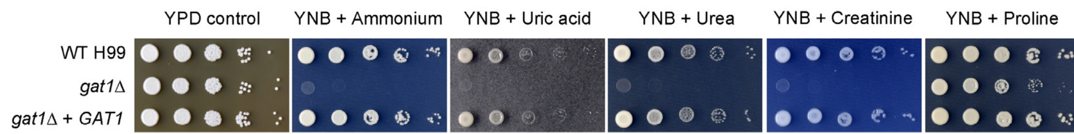

FIGURE S3.–Complementation of nitrogen utilisation phenotype to wild-type levels upon the re-introduction of *GAT1* into the *gat1*Δ mutant. 10-fold spot dilution assays for nitrogen utilisation showed that the *gat1*Δ + *GAT1* strain exhibited wild-type growth on 10 mM ammonium, uric acid, urea, creatinine and proline.

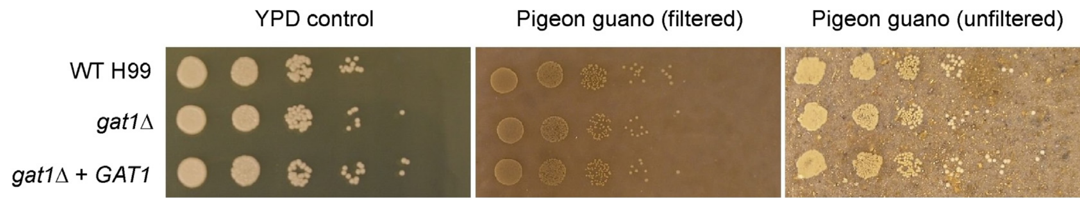

FIGURE S4.–Gat1 is dispensable for growth on pigeon guano. 10-fold spot dilution assays on 25% (wt/vol) pigeon guano media showed that both the *gat1*Δ and *gat1*Δ + *GAT1* strains exhibited similar growth rates to wild-type H99.

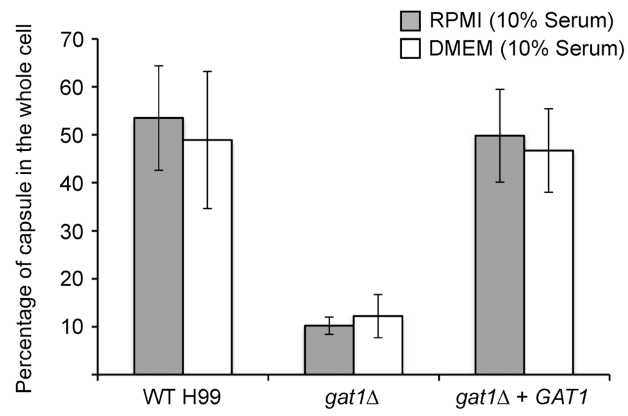

FIGURE S5.–Quantitative measurements of relative capsule diameters of wild-type H99, *gat1Δ* and *gat1Δ + GAT1* cells grown in RPMI and DMEM (10% fetal calf serum). A total of 60 cells (20 cells from three independent experiments) of each strain were measured. Error bars represent standard errors.

TABLE S1

***Cryptococcus* strains used in this study**

Strain	Strain details	Molecular type	Country of origin	Source
H99	Laboratory strain	VNI	USA	J. Perfect
RL1	H99 *gat1*Δ	VNI	N/A	This study
CM127	H99 *gat1*Δ+*GAT1*	VNI	N/A	This study
RL2	H99 *gat201*Δ	VNI	N/A	This study
RL3	H99 *bwc2*Δ	VNI	N/A	This study
RL4	H99 *CNAG04263.2*Δ	VNI	N/A	This study
RL5	H99 *CNAG06762.2*Δ	VNI	N/A	This study
RL6	H99 *CNAG03401.2*Δ	VNI	N/A	This study
H9CIR4	H99 *cir1*Δ	VNI	N/A	J. Kronstad
RL8	KN99**a** *gat1*Δ	VNI	N/A	This study
YL5	H99 *ure1*Δ	VNI	N/A	This study
125.91	Clinical isolate	VNI	Tanzania	J. Heitman
8-1	Clinical isolate	VNII	USA	J. Heitman
I57	Clinical isolate	VNII	India	B. Fries
JEC21	Laboratory strain	VNIV	USA	J. Kwon-Chung
NIH430	Clinical isolate	VNIV	Denmark	Kavanaugh
Bt33	Clinical isolate	VNB	Botswana	A. Litvintseva
Bt63	Clinical isolate	VNB	Botswana	A. Litvintseva
WM276	Environmental isolate	VGI	Australia	W. Meyer
E566	Environmental isolate	VGI	Australia	J. Fraser
R265	Environmental isolate	VGII	Canada	J. Fraser
CBS 1930	Veterinary isolate	VGII	Aruba	W. Meyer
NIH312	Clinical isolate	VGIII	USA	J. Kwon-Chung

B4546	Environmental isolate	VGIII	Australia	J. Fraser
MMRL2651	Clinical isolate	VGIV	India	W. Schell
Bt201	Clinical isolate	VGIV	Botswana	A. Litvintseva

C. neoformans consists of variety *grubii* and variety *neoformans*, comprising four molecular types, VNI, VNII, VNB and VNIV (**v**ariety ***n****eoformans*). *C. gattii* comprises molecular types VGI, VGII, VGIII and VGIV (**v**ariety ***g****attii*).

TABLE S2

Primers used in this study

Primer name	Purpose	Sequence (5'-3')
UQ613	*URO1* qRT-PCR	TGAGTACACCCTCCGAGTCCTT
UQ733	*URO1* qRT-PCR	TTTACAGTGTCGGTAGCAACGAC
UQ609	*DAL1* qRT-PCR	CTTATCGACACCCACGTCCA
UQ736	*DAL1* qRT-PCR	GCATGTCGATCAGGGTGGT
UQ739	*URE1* qRT-PCR	TAAGATCAAACTCGCTGACATGG
UQ740	*URE1* qRT-PCR	CTCGAATGACCTTACCTCCACC
UQ1070	*PUT1* qRT-PCR	AAAAGCTAAAGAGAACGACGTTGC
UQ1071	*PUT1* qRT-PCR	TCTCACTCTTTGACTTTGGAGGTTT
UQ1072	*PUT5* qRT-PCR	AGAAGGCTAAGGAGAACAACATCATT
UQ1073	*PUT5* qRT-PCR	GTCCAGATCTCCTCCTTGGAAG
UQ1074	*PUT2* qRT-PCR	TCATTAACGGTGAGGAGGTCAAG
UQ1075	*PUT2* qRT-PCR	GCAAGAGCACCGTCAATGG
UQ1586	*GDH1* qRT-PCR	GCTTCCCAGAATGAGCTTAACG
UQ1587	*GDH1* qRT-PCR	CGTCAAGTGTACAGCCCATGTT
UQ1588	*GDH2* qRT-PCR	CTTATGCTTATGTCGGATGGATCTT
UQ1589	*GDH2* qRT-PCR	TGGACTCATCCAGAGCATCCT
UQ1590	*GLN1* qRT-PCR	TCGAATCCCCAGGCATGT
UQ1591	*GLN1* qRT-PCR	CGACGAGGATGGCTGTGACT
UQ1592	*GLT1* qRT-PCR	CCGTTGGATCAGAGAAATTCTACC
UQ1593	*GLT1* qRT-PCR	CTGCTGAGGACCGAGGAAAC
UQ1630	*AMT1* qRT-PCR	TTATGTTTTGCTGGACTACCTTGGT
UQ1631	*AMT1* qRT-PCR	CGCCACCTGCATAGTCCAAT
UQ1632	*AMT2* qRT-PCR	TGAGGTTAAGGGCTAGCGAAGA

UQ1633	*AMT2* qRT-PCR	CGGGATCTGTGCCAACATAGT
UQ482	*ACT1* qRT-PCR	CCTACAACTCTATCATGAAGTGTGATCTC
UQ728	*ACT1* qRT-PCR	TCTGCATACGGTCGGCAATAC
UQ856	*GAT1* deletion	GTTGCGGGATGCTGGTGAAAT
UQ857	*GAT1* deletion	AGCTCACATCCTCGCAGCCCTAGCCGCCGCTAGCTG
UQ858	*GAT1* deletion	GTGTTAATACAGATAAACCGCGAATGAATGGAAGCA
UQ859	*GAT1* deletion	GCCCAGCAACTACCATCGTGT
UQ946	*GAT1* deletion	CAGCTAGCGGCGGCTAGGGCTGCGAGGATGTGAGCT
UQ947	*GAT1* deletion	TTGCTTCCATTCATTCGCGGTTTATCTGTATTAACA
UQ864	*GAT201* deletion	CCGCTAAGGAAGGAAGTGGTG
UQ865	*GAT201* deletion	AGCTCACATCCTCGCAGCTGTGCTGGCAAGTAGGGA
UQ866	*GAT201* deletion	GTGTTAATACAGATAAACCTGAGAATAGGAAAGAAC
UQ867	*GAT201* deletion	CAGGGTAAAGGCCGTTAGATA
UQ963	*GAT201* deletion	TCCCTACTTGCCAGCACAGCTGCGAGGATGTGAGCTGGAGAGCG
UQ964	*GAT201* deletion	GCTGTTCTTTCCTATTCTCAGGTTTATCTGTATTAACACGGAAGAGATGTAG
UQ872	*BWC2* deletion	TACTCCAGTATCGCTGTCTCC
UQ873	*BWC2* deletion	AGCTCACATCCTCGCAGCTGGCCGTGCTATAGGTGA
UQ874	*BWC2* deletion	GTGTTAATACAGATAAACCGAGTAGTACTTTGGACT
UQ875	*BWC2* deletion	TCAGCCTCGCACATAATCTAA
UQ966	*BWC2* deletion	TCACCTATAGCACGGCCAGCTGCGAGGATGTGAGCTGGAGAGCG
UQ967	*BWC2* deletion	CAGTCCAAAGTACTACTCGGTTTATCTGTATTAACACGGAAGAGATGTAG
UQ876	*CNAG04263.2* deletion	CTTGAAGCTGCGTTAGAACAT
UQ877	*CNAG04263.2* deletion	CTCACATCCTCGCAGCTGTGGTTTTTGTAGGC
UQ878	*CNAG04263.2* deletion	GTTAATACAGATAAACCGGAGTGATTAAGAGG
UQ879	*CNAG04263.2* deletion	TTAGCTTCTGGCGTCTGATGA
UQ968	*CNAG04263.2* deletion	CCGCCTACAAAAACCACAGCTGCGAGGATGTGAGCTGGAGAGCG
UQ969	*CNAG04263.2* deletion	CCACCTCTTAATCACTCCGGTTTATCTGTATTAACACGGAAGAGATGTAG

UQ888	*CNAG06762.2* deletion	GGGAAGATTAGCGCACCAAAC
UQ889	*CNAG06762.2* deletion	AGCTCACATCCTCGCAGCTGTTGGGGAAAAAGGAAA
UQ890	*CNAG06762.2* deletion	GTGTTAATACAGATAAACCGTGTTGTAACGAGTCGA
UQ891	*CNAG06762.2* deletion	CGCTGCTCTTATCATGTCCAT
UQ974	*CNAG06762.2* deletion	TTTCCTTTTTCCCCAACAGCTGCGAGGATGTGAGCTGGAGAGCG
UQ975	*CNAG06762.2* deletion	ATCGACTCGTTACAACACGGTTTATCTGTATTAACACGGAAGAGATGTAG
UQ852	*CNAG03401.2* deletion	CTTGAAGGTTAATCGTGACGG
UQ853	*CNAG03401.2* deletion	AGCTCACATCCTCGCAGCCCTATAATTCTCTTTTAT
UQ854	*CNAG03401.2* deletion	TGTTAATACAGATAAACCAATGCCAGGGAGAGAATT
UQ855	*CNAG03401.2* deletion	ATCCGCCAACAACAGTACCAC
UQ960	*CNAG03401.2* deletion	ATAAAAGAGAATTATAGGGCTGCGAGGATGTGAGCTGGAGAGCG
UQ961	*CNAG03401.2* deletion	AATTCTCTCCCTGGCATTGGTTTATCTGTATTAACACGGAAGAGATGTAG
UQ1234	*GAT1* complementation	GTGGCGCGCCGCTGGTGAAAT
UQ1235	*GAT1* complementation	GCCCAGGAGCTCCCATCGTGT
M13F	General sequencing	GTAAAACGACGGCCAG
M13R	General sequencing	CAGGAAACAGCTATGAC
UQ1027	*GAT1* sequencing	GAAAGGCGAATAAAGGCTGTT
UQ1028	*GAT1* sequencing	TCTGCCGCGTCTTCCCGGCCC
UQ1029	*GAT1* sequencing	CCAAAAGCTGCTAGGGGCACC
UQ1030	*GAT1* sequencing	TTGCCAAACGGTTTGTCTCTT
UQ1031	*GAT1* sequencing	GCAGCCGCAGCAACGGCTGCG
UQ1032	*GAT1* sequencing	AAAAAAAAGCATAAATAAAAA
UQ1033	*GAT1* sequencing	TGACCGCACAGACGTATTTTT
UQ1034	*GAT1* sequencing	AGCTTTCGGTAATGGCCGGAT
UQ1035	*GAT1* sequencing	GTGGTCGAAGGATGTCTTCCG

qRT-PCR primers were designed using Primer Express software 2.0 (Applied Biosystems). All remaining primers were designed using Oligo 6.8 (Molecular Biology Insights, West Cascade CO).

TABLE S3

Nitrogen utilisation profile of various *Cryptococcus* strains

Molecular type	VNI		VNII		VNIV		VNB		VGI		VGII		VGIII		VGIV	
Strains / Nitrogen sources	H99	125.91	8-1	I57	JEC21	NIH430	Bt33	Bt63	WM276	E566	R265	CBS 1930	NIH312	B4546	MMRL2651	Bt201
Proline[a]	+++	+++	++	+++	++	+++	+	+++	+++	+++	+++	+++	++	++	+++	+++
Glutamine[a]	+++	+++	++	++	+++	+++	+	+++	+++	+++	+++	+++	++	+	+++	+++
Asparagine[a]	+++	+++	++	+++	+++	+++	+	++	++	+++	+++	+++	+++	+	+++	+++
Glutamate[a]	+++	+++	++	+++	+++	+++	+	+++	+++	+++	+++	+++	++	+	++	++
GABA[a]	+++	+++	+	+++	+++	+++	+/-	+++	+++	+++	+++	+++	++	+	+++	+++
Creatinine[a]	+++	+	++	++	+++	+++	+	+	+++	+++	+++	+++	+++	+	+++	+++
Glycine[a]	+++	+++	+	++	++	+++	+	++	+++	+++	+++	+++	++	+	+++	+++

Ammonium[a]	+++	+++	+	++	+++	+++	+	+++	+++	++	+++	+++	+	+/-	+++	++
Arginine[a]	++	++	+	++	+	++	++	++	+++	+++	+++	+++	++	+	+++	++
Aspartic acid[b]	++	++	+	+	+	+/-	+	+++	+++	+++	+++	+++	+	/-	+++	+++
Urea[b]	++	++	++	++	+	+	++	++	++	++	++	++	+	+/-	++	++
Alanine[b]	++	+	+	++	++	++	+/-	+	++	++	++	++	+	+/-	++	++
Tyrosine[b]	++	++	+	++	+	++	+	++	++	++	+	++	+/-	/-	++	++
Guanine[b]	++	++	+	+	++	+	+/-	+	+	++	++	++	+	+/-	++	++
Serine[b]	+	+	+/-	+	++	++	+/-	+	+	++	++	++	++	+	++	+
Uric acid[b]	++	++	+	+	/-	/-	+	++	++	++	++	++	+	++	++	++
Kynurenine[b]	+	+	+	+	+	+	+/-	+	++	++	++	++	+	+/-	++	+
Citrulline[b]	++	++	+/-	+	+	+	++	++	+	+	+	+	+/-	+/-	+	+
Histidine[b]	+	+	+	+	+	+	+	+	++	+	+	+	+	+/-	+	+
Tryptophan[c]	++	+/-	+	+	+/-	+	+/-	++	+	+	+	+	+/-	+/-	+	+
Threonine[c]	+	+	+	+	+	+	+/-	+	+	+	+	+	+/-	+/-	+	+
Leucine[c]	+	+/-	+	+	+	+	+/-	+	++	+	+	+	+/-	/-	+	+

Ornithine[c]	+	+	+/-	+/-	+/-	+/-	++	+	+	+	+	+	+/-	+/-	++	+
Allantoin[c]	+	+	+	+	+/-	+/-	+	+/-	+	+	+	+	+/-	+/-	+	+
Lysine[c]	++	++	+	+	+/-	+/-	+/-	++	+	+	+	+	-	-	+	+
Isoleucine[c]	+	+	+	+/-	+	+	+/-	+/-	+	+	+/-	+	+/-	+/-	+/-	+
Methionine[c]	+	+/-	+/-	+/-	+/-	+	+/-	+/-	+	+	+	+	+/-	+/-	+	+
Valine[c]	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	++	+	+	+	+	+/-	+	+
Guanosine[c]	/-	/-	+	+	+	+	+	+	+	+	+/-	+/-	+/-	+/-	+	+
Phenylalanine[c]	+	+	+/-	+/-	+/-	+/-	+/-	+	+	+/-	+/-	+	/-	/-	+	+/-
Adenine[c]	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-
Adenosine[c]	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-
Glucosamine[d]	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	/-	/-	+/-	+/-
Acetamide[d]	+/-	+/-	+/-	+/-	+/-	+/-	/-	+/-	+/-	+/-	+/-	+/-	+/-	/-	+/-	+/-
Formamide[d]	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	/-	/-	/-	+/-
Nicotinamide[d]	+/-	+/-	/-	+/-	+/-	+/-	/-	+/-	-	+/-	+/-	+/-	/-	/-	+/-	+/-
Uracil[d]	+/-	+/-	+/-	+/-	/-	/-	/-	+/-	+/-	+/-	+/-	+/-	/-	/-	+/-	+/-

Niacinamide[d]	+/-	+/-	/-	+/-	+/-	/-	/-	+/-	/-	/-	/-	+/-	/-	/-	+/-	+/-
Xanthine[d]	-	-	/-	/-	-	-	/-	/-	-	/-	/-	/-	/-	/-	-	-
Cadaverine[e]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Nitrite[e]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Nitrate[e]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

[a]Good nitrogen source; [b]Average nitrogen source; [c]Poor nitrogen source; [d]Very poor nitrogen source; [e]Not a nitrogen source

+++ denotes excellent growth; ++ denotes good growth; + denotes moderate growth; +/- denotes poor growth; /- denotes very poor growth; - denotes no growth

10-fold spot dilution assays revealed that the strains exhibited varying growth rates on different nitrogen sources (10 mM) that were mainly limited to ammonium, purines and amino acids.

TABLE S4

Reciprocal BLASTp analyses of characterised fungal GATA factors against the *C. neoformans* genome

Species	Protein	Function	Reference	Best hit in *C. neoformans*	E value
N. crassa	Nit2	Nitrogen metabolism (Positively – acting)	Stewart 1986	CNAG00193.2 (Gat1)	1.12689E-21
G. fujikuroi	AreA		Mihlan 2003		1.28176E-19
A. parasiticus	AreA		Chang 2000		1.16799E-24
A. nidulans	AreA		Kudla 1990		9.05971E-25
A. niger	AreA		MacCabe 1998		2.24874E-23
A. oryzae	AreA		Christensen 1998		1.16799E-24
A. fumigatus	AreA		Hensel 1998		1.23235E-21
M. grisea	Nut1		Froeliger 1996		1.81053E-34
P. chrysogenum	Nre		Haas 1995		3.09042E-23
C. albicans	Gat1p		Limjindaporn 2003		1.14722E-20
S. cerevisiae	Gln3		Minehart 1991		5.14654E-18
C. albicans	Gln3p		Liao 2008		1.29509E-15
S. cerevisiae	Gat1		Stanbrough 1995		2.77123E-20
C. neoformans	**Gat1**		Kmetzsch 2010		0.0
D. hansenii	DhGzf3	Nitrogen metabolism (Negatively – acting)	Garcia-Salcedo 2006		9.62535E-19
S. cerevisiae	Gzf3		Soussi-Boudekou 1997		5.37613E-17
A. nidulans	AreB		Conlon 2001		9.70406E-20
P. chrysogenum	NreB		Haas 1997		1.3125E-18
S. cerevisiae	Dal80		Cunningham 1992		6.18739E-16
H. capsulatum	Sre1	Iron uptake	Chao 2008		8.20534E-17
A. fumigatus	SreA		Schrettl 2008		2.02048E-16
N. crassa	Asd4	Sexual development	Feng 2000		3.01803E-17
U. maydis	Urbs1	Iron uptake	Voisard 1993	CNAG04864.2 (Cir1)	4.96038E-20
A. nidulans	SreA		Haas 1999		1.23395E-13
P. chrysogenum	SreP		Haas 1997		1.35569E-17
N. crassa	Sre		Zhou 1998		3.6177E-19
S. pombe	Gaf2		Hoe 1996		1.00542E-26
C. neoformans	**Cir1**		Jung 2006		0.0

N. crassa	WC1	Blue light signal	Ballario 1996	CNAG05181.2 (Bwc1) *	0.0
N. crassa	WC2		Linden 1997	CNAG02435.2 (Bwc2)	2.91186E-12
B. oryzae	Blr2		Moriwaki 2008		2.86005E-13
C. neoformans	**Bwc2**		Idnurm 2005		0.0
S. cerevisiae	Ash1	Pseudohyphal growth	Chandarlapaty 1998		0.00163479
A. nidulans	NsdD	Sexual development	Han 2001	CNAG01551.2 (Gat201)	6.06295E-11
S. cerevisiae	Srd1	Pre-rRNA processing	Hess 1994		4.0921E-5
S. cerevisiae	Srd2	Pseudohyphal growth	Canizares 2002		2.55412E-7
C. neoformans	**Gat201**	Anti-phagocytosis	Liu 2008		0.0
S. pombe	Ams2	Cell cycle	Chen 2003	CNAG03401.2	4.1389E-9
C. albicans	Ash1p	Pseudohyphal growth	Munchow 2002		0.00266078

* *C. neoformans* Bwc1 is not strictly a GATA factor due an absence of the zinc finger DNA-binding domain in this ortholog

Protein sequences of fungal GATA factors retrieved from GenBank were queried against the *C. neoformans* strain H99 genome sequence available at the Broad Institute.

TABLE S5

Growth responses of wild-type H99 and GATA-type deletion mutants on various utilisable nitrogen sources

	Strains							
Nitrogen source	H99	*gat1*Δ	*gat201*Δ	*bwc2*Δ	*CNAG04263.2*Δ	*CNAG06762.2*Δ	*CNAG03401.2*Δ	*cir1*Δ
Proline	+++	++	+++	+++	+++	+++	+++	+++
Glutamine	+++	+/-	+++	+++	+++	+++	+++	+++
Asparagine	+++	+/-	+++	+++	+++	+++	+++	+++
Creatinine	+++	-	+++	+++	+++	+++	+++	+++
Glutamate	+++	+/-	+++	+++	+++	+++	+++	+++
GABA	+++	+/-	+++	+++	+++	+++	+++	+++
Glycine	+++	-	+++	+++	+++	+++	+++	+++
Ammonium	+++	-	+++	+++	+++	+++	+++	+++
Arginine	++	+	++	++	++	++	++	++
Uric acid	++	-	++	++	++	++	++	++
Urea	++	-	++	++	++	++	++	++
Aspartic acid	++	+/-	++	++	++	++	++	++
Alanine	++	+/-	++	++	++	++	++	++
Tyrosine	++	-	++	++	++	++	++	++
Guanine	++	-	++	++	++	++	++	++
Citrulline	++	-	++	++	++	++	++	++
Tryptophan	++	-	++	++	++	++	++	++
Lysine	++	+/-	++	++	++	++	++	++
Serine	+	-	+	+	+	+	+	+
Kynurenine	+	-	+	+	+	+	+	+
Histidine	+	-	+	+	+	+	+	+
Threonine	+	-	+	+	+	+	+	+
Leucine	+	-	+	+	+	+	+	+
Ornithine	+	+/-	+	+	+	+	+	+
Isoleucine	+	-	+	+	+	+	+	+
Allantoin	+	-	+	+	+	+	+	+
Methionine	+	-	+	+	+	+	+	+
Phenylalanine	+	-	+	+	+	+	+	+
Valine	+/-	-	+/-	+/-	+/-	+/-	+/-	+/-
Adenine	+/-	-	+/-	+/-	+/-	+/-	+/-	+/-
Adenosine	+/-	-	+/-	+/-	+/-	+/-	+/-	+/-
Guanosine	+/-	-	+/-	+/-	+/-	+/-	+/-	+/-
Glucosamine	+/-	-	+/-	+/-	+/-	+/-	+/-	+/-
Formamide	+/-	-	+/-	+/-	+/-	+/-	+/-	+/-

Nicotinamide	+/-	-	+/-	+/-	+/-	+/-	+/-	+/-
Uracil	+/-	-	+/-	+/-	+/-	+/-	+/-	+/-
Acetamide	+/-	-	+/-	+/-	+/-	+/-	+/-	+/-
Niacinamide	+/-	-	+/-	+/-	+/-	+/-	+/-	+/-

- denotes no growth; +/- denotes poor growth; + denotes moderate growth; ++ denotes good growth; +++ denotes excellent growth

Media was supplemented with 10 mM of each nitrogen source. Summarised results of the nitrogen utilisation assays.

TABLE S6

Sequence identity of characterised fungal GATA factors in comparison to predicted *C. neoformans* members

			% DNA-binding domain (Protein) identity to *C. neoformans* GATA factor candidates						
Species	Protein	Function	CNAG00193.2 (Gat1)	CNAG01551.2 (Gat201)	CNAG02435.2 (Bwc2)	CNAG03401.2	CNAG04263.2	CNAG04864.2 (Cir1)	CNAG06762.2
N. crassa	Nit2	Nitrogen metabolism (Postively-acting)	**90 (19)**	31 (14)	32 (13)	40 (18)	36 (17)	64 (20)	28 (15)
G. fujikuroi	AreA		**88 (19)**	31 (17)	32 (15)	40 (18)	36 (18)	66 (19)	26 (18)
A. parasiticus	AreA		**88 (19)**	31 (18)	32 (15)	34 (18)	36 (19)	66 (21)	26 (17)
A. nidulans	AreA		**88 (18)**	31 (17)	32 (16)	40 (17)	36 (20)	66 (21)	26 (17)
A. niger	AreA		**88 (18)**	31 (19)	32 (15)	40 (18)	36 (19)	66 (20)	26 (19)
A. oryzae	AreA		**88 (18)**	31 (18)	32 (15)	34 (17)	36 (17)	66 (20)	26 (17)
M. grisea	Nut1		**88 (18)**	31 (16)	32 (15)	34 (18)	36 (18)	66 (21)	26 (16)
P. chrysogenum	Nre		**88 (17)**	31 (17)	32 (18)	34 (18)	36 (19)	66 (19)	26 (17)
C. albicans	Gat1p		**86 (15)**	39 (15)	34 (14)	44 (18)	36 (17)	66 (18)	38 (16)
S. cerevisiae	Gat1		**78 (13)**	29 (16)	38 (17)	40 (15)	38 (16)	64 (13)	28 (19)
C. albicans	Gln3p		**76 (17)**	39 (17)	34 (16)	32 (17)	36 (16)	60 (19)	26 (19)
S. cerevisiae	Gln3		**72 (18)**	39 (17)	34 (16)	32 (17)	36 (17)	58 (18)	16 (17)

D. hansenii	DhGzf3	Nitrogen metabolism (Negatively-acting)	**74 (17)**	31 (21)	34 (20)	36 (20)	38 (18)	66 (16)	22 (17)
S. cerevisiae	Gzf3		**74 (16)**	29 (18)	32 (19)	32 (17)	38 (18)	68 (18)	26 (17)
A. nidulans	AreB		**74 (10)**	21 (20)	38 (19)	34 (15)	40 (7)	68 (12)	28 (17)
P. chrysogenum	NreB		**74 (8)**	37 (19)	38 (20)	34 (16)	40 (16)	70 (12)	28 (18)
S. cerevisiae	Dal80		**72 (4)**	29 (11)	32 (3)	36 (8)	38 (5)	62 (4)	24 (10)
U. maydis	Urbs1	Iron uptake	68 (22)	35 (10)	34 (15)	36 (19)	42 (19)	**76 (19)**	22 (16)
A. nidulans	SreA		64 (16)	35 (18)	32 (19)	38 (20)	36 (20)	**76 (17)**	24 (22)
H. capsulatum	Sre1		64 (17)	35 (20)	30 (18)	38 (22)	40 (17)	**74 (17)**	22 (19)
P. chrysogenum	SreP		**74 (17)**	35 (21)	30 (17)	36 (18)	36 (18)	**74 (17)**	26 (20)
A. fumigatus	SreA		44 (16)	39 (18)	36 (18)	42 (19)	32 (20)	**46 (16)**	28 (19)
N. crassa	Sre		**44 (16)**	37 (18)	38 (19)	34 (18)	34 (17)	**44 (16)**	28 (18)
S. pombe	Gaf2		**46 (17)**	33 (20)	38 (18)	28 (19)	38 (18)	40 (17)	30 (22)
C. neoformans	**Cir1**		64 (16)	39 (11)	32 (8)	36 (14)	36 (13)	**100 (100)**	24 (10)
B. oryzae	Blr2	Blue light signal transduction	32 (12)	47 (23)	**63 (21)**	50 (19)	32 (18)	28 (17)	36 (18)
N. crassa	WC2		36 (16)	47 (21)	**58 (22)**	46 (20)	28 (18)	28 (16)	34 (19)
C. neoformans	**Bwc2**		34 (9)	45 (19)	**100 (100)**	50 (15)	34 (12)	32 (8)	32 (15)
N. crassa	WC1		**44 (17)**	39 (14)	40 (11)	38 (17)	34 (17)	38 (17)	30 (14)
N. crassa	Asd4	Sexual development	**74 (13)**	37 (16)	33 (21)	35 (18)	37 (17)	66 (12)	25 (18)
A. nidulans	NsdD		41 (15)	**54 (27)**	41 (19)	37 (20)	35 (19)	31 (17)	43 (18)
S. cerevisiae	Srd1	Pre-rRNA processing	34 (6)	**41 (16)**	36 (19)	26 (14)	28 (9)	24 (7)	32 (15)

C. neoformans	**Gat201**	Anti-phagocytosis	33 (12)	**100 (100)**	45 (19)	43 (16)	33 (15)	39 (10)	41 (17)
S. pombe	Ams2	Cell cycle	29 (18)	25 (18)	27 (17)	31 (18)	31 (18)	31 (17)	**25 (21)**
S. cerevisiae	Ash1	Pseudohyphal growth	38 (17)	29 (19)	**38 (21)**	38 (19)	22 (18)	36 (18)	32 (19)
S. cerevisiae	Srd2		34 (4)	35 (15)	34 (18)	26 (12)	32 (8)	22 (4)	**40 (13)**
C. albicans	Ash1p		36 (12)	29 (19)	36 (17)	**38 (20)**	22 (18)	32 (16)	26 (21)

Protein sequences of fungal GATA factors retrieved from GenBank were individually aligned with each *C. neoformans* strain H99 GATA factor candidate using ClustalW to obtain the percentage (%) DNA-binding domain/protein identity. Values highlighted in bold represent the highest % identity with DNA-binding domain identity given priority over protein identity, due to the established fact of high conservation at each function-specific GATA DNA-binding domain.