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

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Running title: Nitrogen Metabolite Repression

Key words: Nitrogen, Metabolism, Virulence, Cryptococcus neoformans

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ABSTRACT

Proper regulation of metabolism is essential to maximise fitness of organisms in their chosen environmental niche. Nitrogen metabolite repression is an example of a regulatory mechanism in fungi that enables preferential utilisation of easily assimilated nitrogen sources such as ammonium in order 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 utilisation of a wide range of nitrogen sources including uric acid, urea and creatinine – three predominant nitrogen constituents found in *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°C – 40°C). Conversely, Gat1 positively regulates capsule
production. A murine inhalation model of cryptococcosis revealed that the $gat1\Delta$ mutant is slightly more virulent than wild-type, indicating that Gat1 plays a complex regulatory role during infection.
Pioneering studies of fungal nitrogen metabolism have played a crucial role in our current understanding of eukaryotic gene regulation. Until now this classic regulatory phenomenon, known as nitrogen metabolite (or catabolite) repression, has only been observed in members of the phylum *Ascomycota*. The authors show that this mechanism is ancient and conserved in the pathogen *Cryptococcus neoformans* of the phylum *Basidiomycota*. Importantly, beyond using the nitrogen regulatory GATA factor Gat1/Are1 to mediate nitrogen metabolite repression, this deadly pathogen has co-opted this transcription factor into regulating virulence factor production and, by extension, pathogenesis.
INTRODUCTION

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 in order to reduce gaseous nitrogen into a state that is biologically available (POSTGATE 1998). In organisms unable to utilise 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 utilise 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 utilised (MARZLUF 1997). In members of the phylum Ascomycota, the selective utilisation 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 (FRASER et al. 2001; MAGASANIK and KAISER 2002; MARZLUF 1997; MITCHELL and MAGASANIK 1984). 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 maximising the fitness of the organism in its ever-changing local environment (FRASER et al. 2001; MARZLUF 1997; WIAME et al. 1985). 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 (BERGER et al. 2008; BERGER et al. 2006; DAVIS et al. 1993; FENG and MARZLUF 1998). In this way, the fungus only activates the transcription of catabolic genes 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 Cys2/Cys2-type zinc finger motif followed by an adjacent basic region, and are named after the DNA sequence 5'-GATA-3' that they recognise (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 Cryptococcus 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* based on 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 (Berger et al. 2008; Berger et al. 2006; Davis et al. 1993; Feng and Marzluf 1998). 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 utilising nitrogen sources other than ammonium or glutamine (Arst and Cove 1973; Caddick 1992; Fu and Marzluf 1987; Kudla et al. 1990; Marzluf 1997; Stewart and Vollmer 1986). These GATA factors have also been shown to affect virulence in a number of pathogenic fungi. *Aspergillus fumigatus* areAΔ, clinical *Saccharomyces cerevisiae* gln3Δ and *Candida albicans* gat1Δ or gln3Δ mutants all exhibit reduced virulence compared to wild-type in murine virulence assays (Hensel et al. 1998; Kingsbury et al. 2006; Liao et al. 2008; Limjindaporn et al. 2003).

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 \textit{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 (Casadevall and Perfect 1998; Staib \textit{et al.} 1978; Staib \textit{et al.} 1976). In contrast, nitrogen availability becomes scarce upon infection of humans. Beyond these obvious differences in nitrogen availability that \textit{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 \textit{et al.} 1978; Staib \textit{et al.} 1976). 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 \textit{et al.} 2000; Olszewski \textit{et al.} 2004; Shi \textit{et al.} 2010). While there is mounting evidence that nitrogen availability may play a role in pathogenicity of \textit{C. neoformans}, little is known about this process.

In this study we provide the first proof of canonical ascomycete-like nitrogen metabolite repression in the basidiomycete \textit{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 utilisation 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°C) and growth at high body temperature (39°C – 40°C), 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 *gat1Δ* 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 (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 (DSouza et al. 2001). V8 (5% V8 juice, 3 mM KH₂PO₄, 0.1% myo-Inositol, 4% Bacto-agar) and Murashige & Skoog (MS) mating media (PhytoTechnology 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 (Sambrook et al. 1989). _C. elegans_ strain N2 was maintained at 15°C and propagated on its normal laboratory
food source *E. coli* OP50 cells as described previously (Brenner 1974; Garsin *et al.* 2001; Honda *et al.* 1993).

**Bioinformatic analyses**

The genome sequence of *C. neoformans* var. *grubii* reference strain, H99, ([http://www.broadinstitute.org/annotation/genome/cryptococcus_neoformans/MultiHome.html](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 v1.4 within MacVector (Thompson *et al.* 1994). Sequence traces generated at the Australian Genome Research Facility (Brisbane, Queensland) were analysed 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.* 2002; Davidson *et al.* 2000). All primers used for the generation of deletion mutants are listed in Table S2. For example, to construct the *gat1Δ* mutant the 4,239 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 a ~1 kb fragment upstream the *GAT1* start codon, the *NEO* marker and a ~1 kb fragment downstream 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 gat1Δ 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 Ascl/Sacl GAT1 fragment of pIRL1 was subcloned into the Ascl/Sacl sites of the Cryptococcus nourseothricin resistance vector pPZP-NATcc, creating the complementation construct pIRL3 (WALTON et al. 2005). pIRL3 was subsequently linearised with Ascl and Xmal, then biolistically transformed into the gat1Δ 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°C 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 respectively 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°C for 10 min, followed by amplification and quantification in 45 cycles at 95°C for 15 sec and 60°C for 1 min, with melting curve profiling at 95°C for 2 min, 60°C for 15 sec and 95 °C 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) based on the $2^{-\Delta\Delta C_{T}}$ method (Livak and Schmittgen 2001). Several housekeeping genes including GPD1 (glyceraldehyde-3-phosphate dehydrogenase), HHT1 (histone H3) and TUB2 (beta-tubulin) were tested, but the ACT1 (actin) gene was eventually used as a control for normalisation 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 of < 0.05 were considered statistically significant.

**Capsule assays**

Strains were inoculated onto YNB plates supplemented with 10 mM of the specified nitrogen source or into RPMI/DMEM supplemented with 10% fetal
calf serum (Gibco), and incubated at 37°C for 2 days. To visualise 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 greyscale digital camera using the AxioVision AC imaging software. Quantitative analysis of capsule diameter was performed as described previously (ZARAGOZA et al. 2003).

**Growth, melanisation and urease assays**

Starter cultures were prepared by growth in YPD at 30°C overnight with shaking, diluted to OD$_{595}$ nm $= 0.05$ in water, then further diluted 10-fold in series. For nitrogen utilisation, melanisation 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 of the 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°C (nitrogen utilisation, growth test on pigeon guano and urease assays), 30°C and 37°C (melanisation assays), or 37°C – 40°C (growth test at human body temperature).

**Mating assays**

Mating assays were conducted as described previously (NIELSEN et al. 2003). Briefly, strains were pre-grown 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°C overnight with shaking. 10 µL overnight cultures were spread onto both brain heart infusion (BHI) (Becton Dickinson) and 2.5% pigeon guano agar plates (35 mm), and incubated at 25°C overnight. ~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°C 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 Version 5.0c. *p* values of < 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. 2003). Ten mice were inoculated each with a 50 µL drop containing $1 \times 10^5$ 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 pre-infection 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 Version 5.0c. $p$ values of $< 0.05$ 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 number: SCMB/473/09/UQ/NHMRC). Infection and sacrifice were performed under methoxyflurane anaesthesia, 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; FISKIN _et al._ 1990; KMETZSCH _et al._ 2010; POLACHEK and KWON-CHUNG 1980; STÀIB _et al._ 1976). 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/L 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 utilisation of ammonium and more complex macromolecules such as amino acids and purines (HYNES 1970; KINSKY 1961). Only a subset of strains was able to utilise xanthine, lysine and nicotinamide as the sole nitrogen source. None of the strains were able to utilise cadaverine, nitrite or nitrate as sole nitrogen sources. Importantly, while inter-strain 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 characterised fungal nitrate metabolism pathways (CULTRONE *et al.* 2005; HECK *et al.* 2002). Genes encoding homologs of key catabolic enzymes for cadaverine, nitrate and nitrite utilisation 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 utilisation that was observed by Jiang *et al.*, as consistent with our
bioinformatic analyses none of the 16 *Cryptococcus* strains tested were able to utilise 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, favoured 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 analyse 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 *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. 1978; Staib et al. 1976). 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; 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; 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 synthesised the least melanin, proline 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, melanisation on these nitrogen sources was the poorest. Similarly, while ammonium induces melanisation 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 melanised 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.

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 one and two, and always two residues separating cysteines three and four of the zinc finger motif. In contrast, the number of residues comprising the central loop separating cysteines two and three 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 comprises 18 residues (Ballario et al. 1996; Kudla et al. 1990). 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 characterised 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 characterised 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 analysed 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 characterised *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; KMETZSCH et al. 2010; LIU et al. 2008). 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 utilisation in *C. neoformans***

Despite the fact that four GATA factors have been previously characterised 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* (Liao et al. 2008; Limjindaporn et al. 2003; Stanbrough et al. 1995). 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°C (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; Table S5). Consistent with previous studies conducted by Kmetzsch et al., deletion of ORF CNAG00193.2 (*GAT1*) rendered *C. neoformans* incapable of utilising a wide repertoire of nitrogen sources (Kmetzsch et al. 2010). The *gat1Δ* mutant, however, could still utilise a limited selection of amino acids, notably proline, strengthening our hypothesis that proline utilisation is largely independent of nitrogen metabolite repression. Importantly, we found that the *gat1Δ* mutant was also unable to utilise uric acid and creatinine, two predominant nitrogen constituents found in the ecological niche (pigeon guano) of *C. neoformans* (Casadevall and Perfect 1998; Staib et al. 1978; Staib et al. 1976).

**Gat1 mediates nitrogen metabolite repression**

The fact that the *gat1Δ* 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 analyse 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 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 artefacts of the gat1Δ mutant associated with growth in non-utilisable 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 gat1Δ 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 gat1Δ mutant can utilise proline as the sole nitrogen source, albeit less effectively than wild-type. Although the expression of PUT1 was drastically reduced in the gat1Δ 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.

**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 gat1Δ mutant is unable to utilise the most easily assimilated nitrogen source in both ammonium replete and sufficient conditions (1 mM – 100 mM) (data not shown), paradoxically suggesting that the nitrogen metabolite repression mechanism that occurs in the presence of ammonium blocks the ability to utilise 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 analyse 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 $gat1\Delta$ 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 $gat1\Delta$ 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 $gat1\Delta$ 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 *gat1Δ* mutant is unable to utilise 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 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 *gat1Δ* 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 utilisation 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 airborne basidiospores (Giles et al. 2009; Kwon-Chung 1976; Lengeler et al. 2000). To address if Gat1 plays a role in this sexual development, we created a *gat1Δ* derivative of *MATa C. neoformans* strain KN99a (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 *MATα* 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 gat1Δ crosses in comparison to wild-type crosses (Figure 6). Furthermore, deletion of GAT1 in both the MATα and MATa 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 gat1Δ 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 gat1Δ 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 gat1Δ mutant produced reduced amounts of capsule under these serum-induced conditions (Figure 7A; 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 30ºC and 37ºC on L-DOPA medium supplemented with proline as the sole nitrogen source, since proline supports the most robust growth in the gat1Δ mutant on defined medium. Interestingly, the gat1Δ mutant melanised to the same extent as wild-type at 30ºC but produced more melanin than wild-type at 37ºC (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 gat1Δ and negative control ure1Δ mutants are unable to utilise 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. In support of our previous qRT-PCR data indicating that urease is not significantly regulated by nitrogen metabolite repression, the gat1Δ mutant still produces urease even though the mutant is unable to utilise the ammonia resulting from urea catabolism (Figure 7C).

In order to complete a thorough phenotypic analysis of the gat1Δ mutant, we evaluated the role of Gat1 for its ability to grow at high temperature (37ºC – 40ºC), a common stress that C. neoformans encounters both in a mammalian host as well as in its ecological niche. While growth up to 38ºC was equivalent in the wild-type and gat1Δ mutant strains, the mutant exhibited better growth in comparison to wild-type at 39ºC, the febrile temperature commonly
experienced by most patients upon the onset of cryptococcal meningoencephalitis (Figure 7D). Furthermore, at 40°C, growth of the wild-type strain was completely abolished while the \textit{gat1}\textDelta 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.

\textbf{Gat1 is not required for the killing of \textit{C. elegans} but modestly represses virulence in a murine inhalation model of cryptococcosis}

Our ultimate interest in \textit{C. neoformans} lies in better understanding its pathogenicity. In this context our data thus far was confusing, as Gat1 arguably contributes to production of some virulence attributes but repression of others. For example, the \textit{gat1}\textDelta mutant’s reduction in capsule synthesis and abated ability to utilise 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 \textit{C. elegans} killing assays and a murine inhalation model of cryptococcosis.

\textit{C. neoformans} presumably interacts with \textit{C. elegans} in the environment and killing of \textit{C. elegans} by \textit{C. neoformans in vitro} has previously been validated as a model for studying pathogenesis (MYLONAKIS \textit{et al.} 2002). We performed \textit{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 mimic *C. neoformans* ecological niche. Under both growth conditions, killing of *C. elegans* by the *gat1Δ* 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 *gat1Δ* mutant succumbed to infection slightly faster (between 14 and 21 days post infection, median survival = 19 days) than mice infected with the wild-type strain (between 17 and 28 days post infection, median survival = 23 days) (wild-type vs *gat1Δ*, $p = 0.0151$) (Figure 8B). This result suggests that the *gat1Δ* 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 catabolise nutrients from the environment is imperative for survival of an organism. While fungi are well known for their ability to utilise 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 utilisation of nitrogen sources by *C. neoformans* is controlled in a similar fashion. The results we report here represent the first definitive characterisation 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 (Liao *et al.* 2008; Limjindaporn *et al.* 2003; Stan Borough *et al.* 1995). 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 (Gordon *et al.* 2009; Wong *et al.* 2008). *C. neoformans* global nitrogen regulatory circuit instead more closely resembles the single positively-acting GATA factor systems in *A. nidulans* and *N. crassa* (Kudla *et al.* 1990; Stewart and Vollmer 1986). Given that *N. crassa nit-2* was named after the mutant’s inability to utilise nitrate, which *C. neoformans* is unable to do, and the apparent existence of only one GATA factor responsible for nitrogen utilisation, 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 characterised in C. neoformans suggesting that the regulation of GATA factor activity may operate in a similar fashion to these filamentous ascomycetes (ANDRIANOPOULOS et al. 1998; JIANG et al. 2009; PAN et al. 1997). 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 utilise 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 (ANDRIANOPOULOS et al. 1998; PAN et al. 1997; PLATT et al. 1996). 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 (HALL and DIETRICH 2007; MASISON and WICKNER 1995; WONG et al. 2008; XU et al. 1995). 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. 2006; *MONAHAN* et al. 2002; *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 will be required using glutamine, glutamate and perhaps even nitrogen starvation 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 characterised 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 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 and KWON-CHUNG 1998; CHANG et al. 1996). 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 coopted into regulating various aspects of the virulence composite.

It is nevertheless worth noting that the *C. elegans* killing assays were conducted at 25°C instead of mammalian body temperature. Whereas the enhanced growth and melanisation abilities of the *gat1/are1Δ* 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/are1Δ* 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.* recently reported that the *gat1/are1Δ* 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 post infection (KMETZSCH *et al*. 2010). 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/are1Δ* 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 uncharacterised 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; MЕRIKA and ORKIN 1993; RAVAGNANI et al. 1997). Whether these GATA factors antagonise and compete for the same 5'-GATA-3' binding sites in the promoter regions of genes, or 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.
ACKNOWLEDGEMENTS

We thank Bob Simpson for assistance with qRT-PCR, Jason Stajich for assistance with Perl script-based bioinformatic analyses, Jim Kronstad for the provision of the cir1Δ strains and Gary Newell of the Queensland Racing Pigeon Federation Inc. for the provision of pigeon guano.
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FIGURE LEGENDS

FIGURE 1.–The predicted catabolic enzyme-encoding genes of uric acid, URO1 and DAL1, are sensitive to nitrogen metabolite repression. (A) Schematic diagram 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 of 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.

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 co-culture 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). Melanisation increased in the following order: (creatine, uric acid), alanine, proline and (glutamine, asparagine, urea, ammonium). Upon co-culture 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 melanised to the same extent with or without ammonium.

FIGURE 3.–The gat1Δ mutant is unable to utilise a wide variety of nitrogen sources. 10-fold spot dilution assays of wild-type H99 and GATA-type deletion mutants for nitrogen utilisation
showed that the $\text{gat1}\Delta$ mutant is unable to grow on YNB supplemented with 10 mM ammonium, uric acid, urea or creatinine but only exhibits a slight growth defect compared to wild-type on 10 mM proline. Complementation of the $\text{gat1}\Delta$ mutant with the $\text{GAT1}$ gene restored wild-type nitrogen utilisation phenotype (Figure S3).

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

**FIGURE 5.**–$\text{Gat1}$ regulates the expression of the ammonium assimilation enzyme and permease-encoding genes. (A) Schematic diagram representing the predicted pathway of central nitrogen metabolism in *C. neoformans*. (B) cDNA from wild-type H99 and $\text{gat1}\Delta$ mutant grown in YPD were amplified via qRT-PCR using primers against $\text{GDH1}$ (NADPH-GDH), $\text{GLN1}$ (GS), $\text{GLT1}$ (GOGAT), $\text{GDH2}$ (NAD-GDH), $\text{AMT1}$ (ammonium transporter 1), $\text{AMT2}$ (ammonium transporter 2) and the control gene $\text{ACT1}$ (actin). The expression of $\text{GDH1}$, $\text{AMT1}$ and $\text{AMT2}$ was significantly lower in the $\text{gat1}\Delta$ mutant compared to wild-type, while that of $\text{GLN1}$, $\text{GLT1}$ and $\text{GDH2}$ was slightly lower. This difference in level of expression for $\text{GDH1}$ (*** denotes $p < 0.0001$), $\text{AMT1}$ (** denotes $p < 0.01$) and $\text{AMT2}$ (*) denotes $p < 0.05$) was statistically significant. Error bars represent standard errors across three biological replicates.
FIGURE 6.—Gat1 inhibits filament formation during mating. Filamentation assays on V8 and MS mating media (pH 5) showed that filament formation was enhanced in unilateral gat1Δ crosses in comparison to the wild-type crosses. Filamentation was enhanced even further in a bilateral gat1Δ cross. Complementation of the H99α gat1Δ and KN99a gat1Δ mutants with the GAT1 gene restored wild-type mating in all tested crosses (data not shown).

FIGURE 7.—Gat1 is required for capsule synthesis, but negatively regulates melanisation and growth at 39°C or 40°C. (A) India ink cell staining under light microscopy revealed that the wild-type H99 and gat1Δ + GAT1 strains produce enlarged capsules while the gat1Δ mutant produces residual amount of capsule when strains were cultured under serum-induced growth conditions. Scale bar = 10 µm. (B) The gat1Δ mutant produces more melanin compared to both the wild-type and gat1Δ + GAT1 strains when grown on L-DOPA medium supplemented with 10 mM proline at 37°C. In contrast, all three strains melanised to the same extent when grown at 30°C. (C) Unlike the negative control ure1Δ mutant, the wild-type, gat1Δ mutant and gat1Δ + 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) 10-fold spot dilution assays on YPD medium at human body temperature showed that the gat1Δ mutant exhibits enhanced growth compared to both the wild-type and gat1Δ + GAT1 strains at 39°C and 40°C.

FIGURE 8.—The gat1Δ mutant kills C. elegans as efficiently as wild-type H99, but exhibits modestly enhanced virulence in a murine host. (A) ~50 nematode worms were transferred to a lawn of wild-type, gat1Δ or gat1Δ + 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 gat1Δ, p = 0.1066; gat1Δ vs gat1Δ + GAT1, p = 0.9230) and pigeon guano media (wild-type vs gat1Δ, p = 0.0250; gat1Δ vs gat1Δ + GAT1, p = 0.3614). (B) Ten mice were infected intranasally with either 1 × 10⁵ cells of wild-type, gat1Δ or gat1Δ + GAT1 strains, and survival was monitored daily. Mice infected with the wild-type and gat1Δ + GAT1 strains progress to
morbidity at the same rate (wild-type vs $gat1\Delta + GAT1$, $p = 0.6691$), whereas mice infected with the $gat1\Delta$ strain progress to morbidity more rapidly (wild-type vs $gat1\Delta$, $p = 0.0151$).