Nitrogen Source-Dependent Capsule Induction in Human-Pathogenic Cryptococcus Species

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Cryptococcus neoformans and C. gattii cause meningoencephalitis and are an increasing human health threat. These pathogenic Cryptococcus species are neurotropic and persist in the cerebrospinal fluid (CSF) of the mammalian host during infection. In order to survive in the host, pathogenic fungi must procure nutrients, such as carbon and nitrogen, from the CSF. To enhance our understanding of nutrient acquisition during central nervous system infection by Cryptococcus species, we examined the utilization of nitrogen sources available in CSF. We screened for the growth and capsule production of 817 global environmental and clinical isolates on various sources of nitrogen. Both environmental and clinical strains grew robustly on uric acid, Casamino Acids, creatinine, and asparagine as sole nitrogen sources. Urea induced the greatest magnitude of capsule induction. This induction was greater in Cryptococcus gattii than in C. neoformans. We confirmed the ability of nonpreferred nitrogen sources to increase capsule production in pathogenic species of Cryptococcus. Since urea is metabolized to ammonia and CO$_2$ (a known signal for capsule induction), we examined urea metabolism mutants for their transcriptional response to urea regarding capsule production. The transcriptional profile of C. neoformans under urea-supplemented conditions revealed both similar and unique responses to other capsule-inducing conditions, including both intra- and extracellular urea utilization. As one of the most abundant nitrogen sources in the CSF, the ability of Cryptococcus to import urea and induce capsule production may substantially aid this yeast’s survival and propagation in the host.

Cryptococcus neoformans is a pathogenic, basidiomycete yeast responsible for causing meningoencephalitis in immunocompromised individuals. Distributed worldwide, it is the fourth largest infectious disease killer in sub-Saharan Africa as a complication of the AIDS epidemic (1). Because of the mortality and increasing incidence of cryptococcosis, it is imperative that we understand all aspects of this pathogen-host interaction and the methods of C. neoformans survival in an attempt to disrupt them.

As with carbon assimilation (2), nitrogen metabolism is fundamentally critical for the survival of fungal pathogens within their plant or animal hosts. For instance, Lau and Hamer reported the influence of nitrogen regulatory elements on the expression of the Magnaporthe grisea pathogenicity gene MPG1 (3). In their study, novel nitrogen regulatory elements NPR1 and NPR2 were shown to be required for expression of MPG1 and the npr1 and npr2 mutant strains were avirulent on barley. Lau and Hamer also demonstrated that the global nitrogen regulatory transcription factor NUT1, a homolog of the Neurospora crassa NIT2 and Aspergillus nidulans areA transcription factor genes, was also partly responsible for MPG1 expression under nitrogen starvation. However, the complexity of the regulation of pathogenicity through nitrogen pathways in fungi is apparent, as strains with deletion of NUT1 showed no loss of pathogenicity (4), but deletion of the relevant NIT2/areA homolog resulted in attenuated virulence (5).

The role of nitrogen metabolism in fungal pathogenicity of animals has also been investigated. Deletion of the global nitrogen regulatory transcription factor areA results in attenuation of virulence in the murine model of invasive pulmonary aspergillosis (IPA) (6). While the various components of the nitrate assimilation pathway have yet to be tested for pathogenicity in A. fumigatus, another regulatory protein, RhbA, was shown to facilitate growth on nitrate, histidine, and proline and to promote virulence (7). The link between nitrogen utilization and pathogenicity has also been observed in human-pathogenic yeasts, such as Candida albicans. Limjindaporn and colleagues showed that the areA homolog GAT1 regulates nitrogen utilization in C. albicans and is required for pathogenicity in a murine model of candidiasis, even in the absence of defects in dimorphism or growth in serum (8).

Nitrogen metabolism has begun to be understood in C. neoformans in the context of pathobiology. Early studies on nitrogen utilization in C. neoformans focused on the metabolism of nitrogenous compounds influencing melanin production during growth in areas of ecological or medical importance. Recently, Lee and colleagues demonstrated the necessity of the Gat1 global transcription factor for utilization of uric acid, creatinine, and urea (9). Indeed, the researchers noted the ability of various nitrogen sources to greatly enhance capsule production, with the greatest increase shown with creatinine in minimal medium. Underlining the differences between pathogenic Cryptococcus species, Ngamskulrungroj and colleagues showed that GAT1 regulation of amino acid utilization differs between C. neoformans and C. gattii (10). Regulation of nitrogen metabolism and its impact on virulence in cryptococcosis have begun to be appreciated. Results ob-

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tained after deletion of the C. neoformans GAT1 gene, which is homologous to areA in Aspergillus spp., the deletion of which resulted in decreased virulence (9), are mixed; one study showed increased thermostability and slightly increased virulence in a murine model of cryptococcosis (9), whereas another study showed no effect of this mutation on virulence (11). Mechanistically, Wilson and colleagues showed in Magnaporthe that control of nitrogen metabolism and pathogenicity was integrated with trehalose metabolism; Tps1 is required for proper regulation of nitrogen repressor NMRI (12). The latter finding is intriguing, as recent data regarding the NMRI homolog TAR1 in C. neoformans demonstrate the relationship of nitrogen regulation to melanin production, which is a major virulence factor for C. neoformans (13), and the trehalose pathway has signal control on the cryptococcal virulence composite (14, 15).

Urea is a product of metabolism of proteins and exists in copious amounts in cerebrospinal fluid (CSF) (16). Extracellular urease (URE1) production by C. neoformans has been shown to be a virulence factor in cryptococcosis (17). Cox and colleagues showed that a urease-deficient mutant (ure1Δ) was impaired for virulence in murine models of both inhalation and intravenous disease, yet the mutant strain was completely virulent in the rabbit CSF model of cryptococcosis (17). The explanation for the difference in virulence of the ure1Δ mutant between animal models of disease was tentatively explained by a study showing that Ure1 is important for sequestering Cryptococcus yeasts in the microvasculature of the brain and promoting the blood-brain barrier crossing of the yeast into the central nervous system (CNS) (18). This requirement for urease was bypassed in the rabbit CSF model, where the CSF was inoculated directly. Though rare, urease-deficient strains of C. neoformans have been isolated from patients with invasive cryptococcosis (19, 20).

Like melanin production (21), capsule production in C. neoformans has been a topic of great interest in understanding cryptococcal pathobiology, and certain aspects of capsule synthesis are known. Increased CO2 concentrations, iron deprivation, and serum have all been shown to induce capsule production by this yeast (22–24). A carbon substrate, the availability of vitamins, and amino acid availability have also been described to promote capsule growth (25). In vivo infections produce differently sized capsules, depending on the organ site of infection; the lung and the brain show greater induction than other organs (26). This is important, as increased capsule size has been linked to decreased phagocytosis (27).

Nitrogen has also been shown to influence the production of capsule to various degrees, and this induction is subject to nitrogen catabolite repression (9). Furthermore, differences between C. neoformans and C. gattii in the utilization of nitrogen sources, including d-proline, phenylalanine, tryptophan, and d-alanine, exist (10, 28), but the extension of these differences to capsule production has not been studied. In order to enhance our understanding of the relationship between nitrogen acquisition and capsule production during infection by C. neoformans, we evaluated capsule production during growth on five different nitrogen sources: asparagine-Casamino Acids, creatinine, ammonium, uric acid, and urea. In our study, the nitrogen source was shown to profoundly affect capsule size, with urea being the most dramatic inducer of capsule production in this pathogenic yeast.

MATERIALS AND METHODS

Strains and media. Yeast extract-peptone-dextrose (YPD; 1% yeast extract, 2% peptone, 2% dextrose) medium was used for maintaining the strains. Cultures were grown with continuous shaking (220 rpm) at 30°C on agar plates unless otherwise noted. Five strains representing four serotypes of Cryptococcus pathogenic species, serotype A strain H99, serotype B strain R265, serotype B strain WM276, serotype C strain DUMC106.97, and serotype D strain JEC21, were selected for study in assays, unless stated otherwise. H99-derived deletion mutants ure1Δ and amtl/2Δ (JR5), obtained as a gift from Joe Heitman, were used for urea metabolism pathway experiments (17, 29).

Cryptococcus sp. strain library. Cryptococcus sp. isolates were obtained from our laboratory collection or as a generous gift from Joe Heitman. The isolates in the combined collection of 817 Cryptococcus isolates were genotyped using multilocus sequence typing (MLST) strategies, and the isolates are described in Table S1 in the supplemental material. The strain collection was propagated from ~80°C freezer stocks in 1-ml cultures of YPD plus Hognes freezing medium (30) in 96-well growth blocks at 37°C for 48 h. The strains were then plated onto 6 different nitrogen source plates (ammonium sulfate, sodium nitrate, urea, uric acid, asparagine, and Casamino Acids). Agar plates were composed of yeast carbon base (YCB) minimal medium and 5 g/liter of nitrogen source. The plates were incubated for 72 h at 37°C and subsequently analyzed by inspection for growth and mucoid and filamentous appearance. The plates were also observed following 120 h growth.

Capsule induction by nitrogen source. The four media described above, YCB plus asparagine, YCB plus creatinine, YCB plus ammonium, and YCB plus urea, were induced with each of the 5 strains, H99, R265, WM276, DUMC 106.97, and JEC21, in a 32-well plate. Nitrogen source concentrations were 5 g/liter. The cultures were grown for 48 h, and images were taken using a Zeiss Axioskop microscope. Capsule measurements were calculated as previously described (22). Briefly, 2 μl of India ink (Becton, Dickinson) and 3 μl of culture were placed on glass slides. Images were captured with a ×63 differential interference contrast oil lens using AxioVision AC imaging software. Capsule-to-cell-diameter ratios were measured for ≥50 cells per strain culture using the Ruler tool in Adobe Photoshop CS4 (Adobe Systems Inc., San Jose, CA).

Growth curve. Growth curves were assessed for Cryptococcus yeast strains H99, R265, and WM276 in YCB plus ammonium, YCB plus urea, and YCB plus uric acid broth. The nitrogen concentration in the medium in each culture was 0.5 g/liter. Fifty milliliters of overnight cultures of each strain in YPD was diluted to 1 × 10^3 CFU/ml and used to inoculate the 3 types of YCB broth. The optical density at 600 nm was determined in triplicate three times per day for 70 h and plotted on a log_10 scale.

Benomyl assay. Strains R265 and H99 were grown for 24 h in 40 ml of YCB plus ammonium medium (5 g/liter) at 37°C. After 24 h, the cultures were divided into 10-ml aliquots and washed with sterile water. Cells were then resuspended in 50 ml of either YCB plus ammonium or YCB plus urea at 5 g/liter nitrogen source. Two microliters of 50 mM benomyl was added to one of each of the media and strain types. Cells were grown for 24 more hours and were imaged using the method described above for Zeiss Axioskop capsule measurement.

Urea titration assay. Strains R265 and H99 were grown at 37°C for 24 h in 40 ml YCB plus uric acid (0.5 g/liter) medium. After 24 h, the cultures were washed with sterile water and placed in new medium with incrementally increasing amounts of urea added to YCB plus uric acid medium. The concentrations of urea tested were 0 g/liter, 0.05 g/liter, 0.5 g/liter, and 5 g/liter. After growing for 24 h, the cells were imaged utilizing the method described above for capsule measurement using a Zeiss Axioskop microscope.

Urea mutant capsule induction assay. Mutants of the urea metabolism pathway were grown in YCB plus proline (0.25 g/liter) liquid medium for 18 h at 30°C. Cultures were then spun down and washed three times with deionized water and resuspended in YCB plus proline (0.25 g/liter) and urea (5 g/liter) liquid medium. The capsules of the H99
amtl/Δ and ure1/Δ mutants and wild-type (WT) strain H99 were measured as described above. Additionally, the amtl/Δ, ure1/Δ, and H99 strains were grown in YPD, washed, and then transferred to Dulbecco’s modified Eagle’s medium (DMEM) or DMEM plus urea (0.25 g/liter) in 12-well plates and grown for 48 h at 37°C in a CO2 incubator (data not shown). Capsule images were made as described above.

Microarrays. YCB plus proline (0.25 g/liter) medium was inoculated with the wild-type H99 and the ure1/Δ and amtl/Δ2 mutant strains and grown overnight at 30°C. Cells were washed in sterile water and resuspended in 25 ml of either YCB plus proline (0.25 g/liter) or YCB plus proline (0.25 g/liter) and urea (0.25 g/liter). All media were pH 5 ± 0.1. H99 was also resuspended in YCB plus 6 mM ammonium and 10 mM urea at the concentrations outlined by Lee et al. (9). The cultures were grown for 1 h at 30°C, pelleted, and frozen at −80°C. RNA for all samples was prepared for microarray analysis as previously described (2). Following RNA extraction, data acquisition and microarray hybridizations were executed at the Duke Microarray Facility (http://www.genome.duke.edu/cores/microarray/) according to established protocols for custom-spotted arrays. Data were normalized using the analysis of variance process in JMP Genomics (v6.0) software (31), and calculations of fold change were performed using the Adaptive Holm multiple-testing correction to set statistical significance at a P value of <0.000024434.

Gene transcriptional response comparisons. Lists of genes with statistically significant responses generated from the microarrays were compiled into Venn diagram comparisons using the JMP Genomics (v6.0) Venn diagram function. Gene lists were generated from a comparison of WT H99 growth under YCB plus proline or YCB plus proline and urea-supplemented conditions and a comparison of the growth of the WT, ure1/Δ, and amtl/Δ strains in YCB plus proline and urea medium. The 2 lists and the gene list in Table S2 in the work of Haynes (32) were compared for determination of identical locus identifiers.

GO enrichment. Gene ontology (GO) enrichment analysis was carried out by utilizing the R package TopGo. Gene ontology information for C. neoformans H99 was downloaded from http://genome.jgi-psf.org/Cryne_H99_1/Cryne_H99_1.download ftp.html. P values were corrected for multiple-hypothesis testing, and significance was determined using a cutoff value of 0.01.

Statistical analysis. All data analysis was executed in the Microsoft Excel program (Microsoft Inc., Redmond, WA), unless otherwise noted. Data were expressed as the mean ± standard deviation of at least three repeat experiments. Each experiment was performed in triplicate. Differences in capsule sizes were assessed using Student’s t test or repeated-measures analysis of variance to determine the statistical significance between the parental strain control and mutant treatment groups. A P value of <0.05 was considered statistically significant.

Microarray data accession number. The accession number assigned to the microarray data is GSE46829.

RESULTS

Nitrogen assimilation varies by serotype, as revealed in a survey of North American and African Cryptococcus isolates. Using a collection of 817 Cryptococcus strains from numerous locations in Africa and North America (Table 1), we evaluated the ability of the different pathogenic serotypes to assimilate ecological and CNS-relevant nitrogen sources and assess their impact on gross morphology and virulence trait expression. A qualitative assessment of growth using ammonium, nitrate, urea, uric acid, asparagine, or Casamino Acids revealed some interesting observations (Table 2); while C. neoformans and C. gattii strains share aspects of nitrogen utilization, stark differences also exist. Uric acid was utilized as a nitrogen source by at least 96% of the C. neoformans and C. gattii strains. Interestingly, more C. gattii strains (76.96%) than either C. neoformans (11.54%) or hybrid serotype (39.30 and 33.33%) isolates grew robustly on urea. Casamino Acids and ammonium supported the growth of 95.90% of all the non-hybrid serotype isolates.

The nitrogen source also affected the colony morphology different among the strains (Fig. 1). First, isolates in this collection exhibited a wrinkled colony appearance and filamentous behavior (see Table S1 in the supplemental material), and these were pronounced along serotype lines. These phenotypes were primarily seen when strains were grown on uric acid and were equally observed in both clinical and environmental C. gattii isolates. Filamentous growth could indicate hyphal formation or haploid fruiting, and these observations could be further explored, but evidence of spores was not examined, since these cultures were not protected from light. Second, the different nitrogen sources induced a mucoid colony phenotype at various frequencies among the different serotypes. This phenotype was most pronounced in C. gattii and C. neoformans var. grubii strains, where 66.80% and 57.10%, respectively, exhibited a mucoid appearance. Although no serotype BD isolates exhibited mucoid colonies on any of the nitrogen sources selected, this observation may be due to the small sample size of BD isolates included in this study. Overwhelmingly, urea as a nitrogen source produced the most mucoid colonies in all strains (54.5%), followed by asparagine at 23.1%.

The mucoid colony phenotype has been associated with increased virulence in strains (33). We found that this mucoid phenotype was correlated with increased capsule size, a known virulence trait, when a selection of these strains was mixed with India ink and viewed microscopically (data not shown). This observation prompted us to focus on the impact of the nitrogen source on capsule production.

Nitrogen sources present in CSF affect capsule production differently between pathogenic Cryptococcus species. The global screen of Cryptococcus isolates on various nitrogen sources showed that urea induces a mucoid colony appearance and the resulting increased capsule size in a wide breadth of strains. Urea exists throughout the body at various concentrations. It is the most prevalent nitrogen source in the CSF, the critical site of cryptococcal infection (16). Previously, our laboratory defined the differences in C. neoformans carbon metabolism during growth at different body sites, such as the CSF, during infection (2). In the interest of better defining the nitrogen metabolites’ importance for growth and survival within this host site, we assessed urea and 3 other readily assimilated nitrogen sources available in CSF (cre-
atinine, ammonium, and asparagine) for their impact on growth and virulence factor expression in *C. neoformans* and *C. gattii* (34). Furthermore, we aimed to determine whether differences in capsule size for the most CNS-relevant nitrogen sources correlated with a mucoid colony appearance in the isolate screen. As shown in Fig. 2, nitrogen source influences capsule production differently in isolates representing the 4 major serotypes. Overall, urea was the most potent inducer of capsule across the strains. Interestingly, the *C. gattii* strains, including Vancouver outbreak strain R265, produced the most robust capsules in urea-containing medium. Indeed, the capsules produced by these *C. gattii* strains in urea were >33% larger than the capsule produced by serotype A strain H99 (*C. neoformans*) in creatinine, the nitrogen source shown to induce its largest capsule, confirming published results (9). While we found that urea induced the greatest capsule induction response overall, there was some variability among the strains.

We noticed in our screens that the strains did not grow as rapidly on urea as on other nitrogen sources at the levels used in this study. Therefore, growth rates on urea compared to those on ammonium were measured for serotype A and B strains, and the results precisely confirmed this observation (Fig. 3). Poor growth conditions have been shown to induce a larger capsule size. Therefore, we wanted to separate the impact of inhibited or slow cell growth on capsule production from that of the potential direct capsule signaling produced by the urea molecule. To confirm that the observed impact of urea on the capsule was not solely due to reduced growth, we compared capsule production for both H99 and R265 on ammonium and urea in the presence and absence of benomyl. Benomyl, a benzimidazole fungicide, blocks mitosis by

| Species identification or type | % of isolates with growth ability (% of growing isolates that were mucoid) |
|-------------------------------|--------------------------------------------------------------------------|
|                              | NH₄ | Nitrato | Urea | Uric acid | Asparagine | Casamino Acids | % mucoid⁶ |
| *C. neoformans* var. grubii   | 99.60 (1.59) | 27.63 (2.78) | 73.36 (57.10) | 99.80 (4.20) | 100.00 (7.20) | 99.60 (3.00) | 59.64 |
| *C. neoformans* var. neoformans | 100.00 (0.00) | 26.92 (7.69) | 11.54 (0.00) | 100.00 (0.00) | 96.15 (0.00) | 100.00 (0.00) | 7.69 |
| *C. gattii*                   | 96.31 (5.07) | 81.11 (44.20) | 76.96 (66.80) | 96.31 (57.00) | 95.85 (67.00) | 92.17 (49.00) | 84.79 |
| Serotype AD                   | 100.00 (0.00) | 14.29 (0.00) | 39.29 (16.10) | 100.00 (0.00) | 100.00 (0.00) | 100.00 (0.00) | 16.07 |
| Serotype BD                   | 66.67 (0.00) | 33.33 (0.00) | 33.33 (0.00) | 33.33 (0.00) | 33.33 (0.00) | 33.33 (0.00) | 0.00 |
| Unknown                       | 91.67 (16.70) | 75.00 (33.33) | 66.67 (33.33) | 83.33 (42.00) | 91.67 (58.00) | 91.67 (50.00) | 58.33 |

⁵ Strains were grown on YCB plus NH₄, NO₃, urea, uric acid, asparagine, and Casamino Acids. Overall viability, filamentous growth, and mucoid colony appearance were assessed on a +++ +, +, and − scale.

⁶ Overall percentage of isolates within each species identification or type that displayed a mucoid colony appearance.

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**FIG 1** Environmental and clinical isolate screen findings. (A) *C. gattii* serotype C strains on uric acid; (B) a *C. gattii* strain on uric acid, capsule production; (C) *C. neoformans* serotype A strains on urea, mucoid appearance; (D) *C. gattii* on uric acid, filamentous growth; (E) *C. neoformans* serotype A strains on urea, growth differences on a white light box; (F) an African clinical isolate on urea, capsule production.
disrupting microtubule formation. It was therefore useful to assess the impact of impeded growth on capsule production independently of any effect caused by urea as a nonpreferred nitrogen source. Capsule production was consistently higher in the presence of urea, regardless of the presence or absence of benomyl, indicating that urea itself directly increases or signals capsule production in pathogenic *Cryptococcus* species. Nutrient starvation is an unlikely cause of the observed large capsule phenotype in the urea-containing medium.

**Urea acts as a signal for increased capsule production in *Cryptococcus* species.** Since urea was shown to prominently increase capsule production, we sought to describe how the urea molecule actually signaled the observed increase in capsule production. This is important to understand, as urea exists at concentrations comparable to those for glucose in human CSF (16) and may be a factor contributing to the formation of the protective large capsules observed in patients with cryptococcosis. Therefore, we increased urea concentrations in the presence of a constant concentration of an alternate nonpreferred nitrogen source (uric acid). Indeed, increasing urea concentrations in culture yielded increased capsule sizes in both the H99 and R265 strains (Fig. 4), indicating that capsule induction by urea is dose responsive.

Since urea can be broken down to ammonia and CO₂, we examined previously described mutants for their response to urea regarding capsule production (Fig. 5). The urease gene *URE1* encodes the major urease in *C. neoformans* and has previously been

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**FIG 2** Capsule thickness in serotypes A, B, C, and D in YCB medium plus a nitrogen source. Serotypes A, B, C, and D were grown in YCB medium with ammonium, asparagine, creatinine, or urea as the nitrogen source. The ratios of the cell diameter (dia.) to the total diameter including the capsule for ≥50 cells per group are shown. Bars represent standard errors.

**FIG 3** Growth on different nitrogen sources. Strains were grown overnight and then used to inoculate either YCB plus ammonium, YCB plus urea, or YCB plus uric acid medium (not shown). The optical density at 600 nm (OD₆₀₀) was recorded three times per day until the cultures entered stationary phase.
shown by us to yield no impact on CNS virulence in a rabbit CNS model of cryptococcosis (17). Likewise, a strain lacking both ammonia transporters (Amt1 and Amt2) was shown to have no virulence defects in mice, lacks the ability to take up ammonia from the extracellular milieu (29), and thus helps control urea breakdown product effects. However, CAN2, encoding the final step in the conversion of CO₂ to bicarbonate, was not evaluated due to the strict requirement of the mutant strain for elevated CO₂ levels for growth that would confound the effect of urea on capsule induction. The ure1Δ and amt1Δ amt2Δ mutants were compared to wild-type strain H99 for capsule formation in the presence of urea as the sole nitrogen source. The ure1Δ mutant strain showed elevated capsule production when grown in defined medium with urea and a low level of CO₂ (that found in ambient air) (Fig. 6) relative to that of both the WT and the amt1/2 mutant. This finding supports the hypothesis that the urea molecule itself acts as an external or internal signal for capsule production in Cryptococcus species.

A variety of conditions present in vivo, including the CO₂ concentration and the presence of serum, can induce capsule production. In order to determine whether urea can further enhance capsule production when cells are already under capsule-inducing conditions, we grew the urea pathway mutants and WT at 5% CO₂ in either DMEM or DMEM plus urea (0.25 g/liter). No discernible capsule size difference was detected between strains grown with or without urea at the concentrations used.

Transcriptional responses to urea exposure. To examine the direct effects of urea on C. neoformans gene expression, we carried out DNA microarray analysis on WT strain H99 grown with either proline alone or urea-supplemented proline medium. Identifying significant modifications to the cryptococcal transcriptome may better inform us of the mechanism by which urea directly induces capsule. Proline was selected as the control nitrogen source because it appears to be a poor inducer of capsule on its own (9).

Following growth in proline medium, C. neoformans was resuspended in either proline medium or biologically significant concentrations of urea plus proline for 1 h. Following this 1-h exposure, 626 genes in strain H99 showed significantly different expression in urea-supplemented medium than in proline medium lacking urea (P < 0.000024434; see Table S2 in the supplemental material) (Table 3).

GO enrichment analysis indicated that oxidoreductase activity, various nitrogen transmembrane activities, and flavin mononucleotide binding were molecular functions all significantly enriched in the response to the presence of urea. Induced oxidoreductase activity indicates nonpreferred nitrogen assimilation, as nonpreferred nitrogen sources are generally reduced to ammonia (35). Biological processes enriched by the presence of urea included defense and organismal responses, ribosome-related biogenesis, and transport of carboxylic and amino acids.

Gene transcription altered by the presence of urea covered a variety of functional categories, though many genes were specifi-
cally related to nitrogen catabolism and the stress response (see Table S2 in the supplemental material). In general, genes highly upregulated in urea-supplemented medium were uncharacterized, whereas downregulated genes were enriched for those related to nitrogen transport (see Table S2 in the supplemental material). Several genes either directly or indirectly related to capsule regulation were upregulated in the presence of urea. For example, the CNAG_03395, CNAG_00815, and CNAG_02083 genes were related to lipid glycosylation and amino acid transporter activity. An increased extracellular urea environment appears to alter the transcription of cellular processes known to result in increased capsule and further supports our observation that urea leads to induction of capsule.

The ure1Δ urea pathway mutant affects the C. neoformans transcriptome. In order to gain further insight into how urea affects capsule induction on the transcriptional level, we evaluated the gene expression differences of urea catabolism pathway mutants under urea-supplemented conditions. Since the ure1Δ mutant showed a further increased capsule size when grown in defined medium with urea and a low CO2 concentration compared to the size for both the WT and the amt1/2Δ mutant, we reasoned that the ure1Δ mutant could provide an even more specific subset of genes involved in capsule production in response to urea. The inability of the ure1Δ mutant to degrade urea to ammonia and carbon dioxide should enhance urea levels in the direct extracellular environment relative to those for the WT and amt1/2Δ strains. Among the 10,601 gene probes on the array, those for 1,623 genes showed significantly different expression. After excluding genes differentially expressed in the WT versus the amt1/2Δ mutant, 504 genes in C. neoformans displayed significantly different expression in the ure1Δ mutant than in the two other strains (Fig. 7).

In comparing the transcriptional responses of the 3 strains, the response to urea was more similar in the WT and the amt1/2Δ mutant compared with that in the ure1Δ mutant. As expected, there were more differentially expressed genes in the ure1Δ mutant strain (which showed the highest level of capsule induction) than in the WT and the amt1/2Δ mutant (which showed lower levels of capsule induction). Intuitively, the strains that displayed the least capsule induction in urea-supplemented medium, the WT and the amt1/2Δ mutant, exhibited the most similar transcriptional responses and displayed the smallest number of differentially expressed genes (Fig. 7).

GO enrichment analysis more specifically revealed differences in trends among strains in response to urea medium (Fig. 8). While WT genes differentially expressed from ure1Δ genes were enriched for genes for defense-related responses and ion binding, genes of the amt1/2Δ mutant differentially expressed from genes of the ure1Δ mutant were related to lipid glycosylation and amino acid transporter activity. An increased extracellular urea environment appears to alter the transcription of cellular processes known to result in increased capsule and further supports our observation that urea leads to induction of capsule.

The ure1Δ urea pathway mutant and urea-supplemented conditions provide a capsule-specific gene list. Both our urea-supplemented microarray and ure1Δ urea pathway mutant microarray experiments provided lists of potential genes that might explain the level of transcription of the large capsule phenotype that we observed when Cryptococcus species were exposed to urea. In order to obtain a subset of genes linking capsule with urea-specific gene expression (rather than urea metabolism or other ure1Δ mutant functions), we compared these two lists and looked for common genes.

### Table 3: Urea-dependent gene expression in WT strain H99

| Gene identification | Functional annotation | Fold change | P value |
|---------------------|-----------------------|-------------|---------|
| CNAG_02777          | Phosphate transporter | 0.08        | 1.64E-07|
| CNAG_06932          | Sugar transporter     | 0.09        | 1.05E-09|
| CNAG_00539          | Membrane transport protein | 0.10 | 9.10E-11|
| CNAG_05160          | Predicted protein     | 0.11        | 8.43E-09|
| CNAG_06346          | Conserved hypothetical protein | 0.16 | 1.03E-08|
| CNAG_02083          | Siderochrome-iron transporter | 0.16 | 4.01E-08|
| CNAG_05201          | Conserved hypothetical protein | 0.18 | 4.39E-11|
| CNAG_04325          | Conserved hypothetical protein | 0.24 | 3.05E-07|
| CNAG_06242          | Conserved hypothetical protein | 0.24 | 1.16E-09|
| CNAG_04675          | Conserved hypothetical protein | 0.25 | 4.68E-06|
| CND02430            | Amino acid transporter | 9.21        | 2.22E-13|
| CNAG_02851          | Threonine aldolase    | 9.21        | 5.01E-14|
| CNAG_06329          | Hypothetical protein  | 9.44        | 1.89E-07|
| CNAG_07448          | Urea transporter      | 10.85       | 3.34E-22|
| CNAG_07902          | Amino acid transporter | 11.15      | 5.55E-16|
| CNAG_06374          | Malate dehydrogenase  | 15.32       | 5.32E-15|
| CNAG_07448.2        | Urea transporter      | 16.30       | 5.64E-13|
| CNAG_04758          | Ammonium transporter  | 16.93       | 1.47E-13|
| CNAG_02049          | Proline dehydrogenase | 24.09       | 3.10E-13|
| CNAG_01118          | Amino acid transporter | 27.06      | 1.63E-02|

a Genes with the greatest fold change for WT strain H99 in urea-supplemented proline medium. The gene identification and functional annotation were obtained from the Broad Institute database (http://www.broadinstitute.org/annotation/genome/cryptococcus_neoformans/) or the NCBI database (http://www.ncbi.nlm.nih.gov/) with hand editing.

b Fold change represents the level of expression on proline minus the level of expression on proline plus urea.
The comparisons of the transcription profiles from the two microarray experiments did show similarities. For example, genes for several sugar transporters were significantly upregulated when strains were grown in the presence of urea (see Table S2 in the supplemental material). The genes for these sugar transporters were also significantly upregulated in the ureA strain compared to the levels of expression in both the WT and the ammonia-supplemented conditions. This is consistent with our observations of increased capsule under urea-supplemented conditions (Fig. 2) and in the ureA strain (Fig. 6). Additionally, genes involved in vesicle transport were also upregulated in the ureA background and WT in the presence of urea. Lastly, the gluconeoxygenate gate-way gene PCK1, encoding phosphoenolpyruvate carboxykinase, exhibited significant induction in the ureA mutant background as well as in the WT under urea-supplemented conditions. Pck1 presumably supports increased pentose and hexose anabolism from 2- and 3-carbon precursors, which would be important for making the building blocks of capsule. As these genes were differentially expressed in both microarray experiments, they provide a specific subset of genes linking urea exposure to an increased capsule phenotype.

Urea induces unique genes, in addition to known capsule-associated genes, compared to those induced under other capsule-inducing conditions. In a comprehensive study, Haynes and
colleagues made a step toward defining the transcriptional signature of capsule induction using microarray data from *C. neoformans* exposed to various capsule-inducing conditions (32). This composite included 826 genes in which gene transcript levels were significantly higher. These genes can be further defined as either positively or negatively correlating with capsule size, and these data for patterns of transcriptional regulators are consistent with the previously demonstrated roles of the genes in capsule regulation. We compared our differentially expressed gene lists from our two microarray studies to this transcriptional signature.

Our microarray experiment examining wild-type strain H99 under urea-supplemented conditions provided us with a general list of genes differentially expressed in the presence of urea. In order to gain insight into genes specifically involved in capsule induction, rather than urea metabolism, we compared our gene list to the list of capsule transcriptional signature genes of Haynes et al. (32). A total of 78 unique genes appeared on both lists and were differentially expressed under both urea-supplemented conditions and the conditions of Haynes et al. (32). Twenty-eight of the genes appearing on both lists were upregulated under urea-supplemented conditions (see Table S3 in the supplemental material). Notably, 4 genes upregulated under urea-supplemented conditions were also positively correlated with capsule size, according to Haynes et al. (32). Two of these genes are conserved hypothetical proteins (CNAG_05057, CNAG_02041) and may be important in inducing capsule in the presence of urea in novel ways.

In addition, we compared these two lists (i.e., the capsule transcriptional signature genes and genes differentially expressed in urea-supplemented medium) to data from our second microarray experiment where we further examined urea exposure. We noted genes differentially expressed in the *ure1Δ* mutant strain compared to either the WT or the *amt1Δ/2Δ* mutant within the context of urea-supplemented medium and compiled them together (Fig. 9). There were 99 genes displaying differential expression in the *ure1Δ* mutant strain under urea-supplemented conditions that also appeared on the capsule transcriptional signature list. From these 99 genes, we compiled a list of 26 genes, shown in Fig. 8, differentially expressed at significant levels linking our studies of capsule induction using microarray data from 3 microarray experiments were compared for overlapping genes. Differential expression of WT strain H99 in proline plus urea medium, differential expression of *ure1Δ* genes in proline plus urea medium, and the capsule transcriptional signature from Haynes et al. (32) were examined for overlapping genes.

**FIG 9** Gene expression under capsule formation–implicated conditions. Lists of genes from 3 microarray experiments were compared for overlapping genes. Differential expression of WT strain H99 in proline plus urea medium, differential expression of *ure1Δ* genes in proline plus urea medium, and the capsule transcriptional signature from Haynes et al. (32) were examined for overlapping genes.

**Discussion**

As a cosmopolitan, pathogenic fungus found in a wide variety of environments, nutrient acquisition and nitrogen assimilation are vital for *Cryptococcus*. One recent study illustrated the differences in utilization of 23 amino acids, such as phenylalanine, tryptophan, d-alanine, and d-proline, between 67% strains of *C. neoformans* and *C. gattii* (10). In a more expansive survey of 42 nitrogen sources, 16 strains of *Cryptococcus* species were revealed to utilize large macromolecules, including amino acids and purines (9). For our study, we assessed an ecologically and biologically relevant subset of these nitrogen sources in a larger pool of 817 strains collected from clinical and environmental locations throughout North America and Africa. These clinical, veterinary, and environmental strains represent all 8 currently recognized molecular types. Confirming the previous studies, Casamino Acids were almost universally assimilated as the sole nitrogen source, indicating the fundamental utility of amino acids for nitrogen assimilation; the same was also true for uric acid. Since uric acid is present in high quantities in pigeon guano, the environmental reservoir most typically associated with *C. neoformans* var. *neofor manis* and *C. neoformans* var. *grubii* strains (37), the ubiquitous assimilation of uric acid by *C. gattii* strains is more surprising. *C. gattii* is typically associated with a variety of trees, especially those in tropical to subtropical climates and, more recently, in temperate and high mountain climates (38–42). It is tempting to speculate that *C. gattii* is associated with the guano of bird species associated with these trees, as no link between *C. gattii* and pigeons currently exists.

Our global survey of nitrogen utilization among *Cryptococcus* strains also confirmed the paradigm-shifting observation previously made in the characterization of *TAR1* (13): a significant number of isolates, particularly *C. gattii* strains, appear to utilize nitrate as the sole nitrogen source. This ability to use nitrate is less frequent in strains of the *C. neoformans* species. While these results conflict with the prevailing understanding of nitrate nonutilization by all pathogenic *Cryptococcus* species, they do confirm the published results of Jiang and colleagues showing a latent ability of pathogenic cryptococci to utilize nitrate as the sole nitrogen source (13). Furthermore, we confirmed the accepted paradigm that nonpathogenic species, such as *Cryptococcus albidos*, display robust growth on nitrate (data not shown). Our results suggest
that the loss of nitrate utilization may have correlated with the pathogenic phenotype in \textit{C. neoformans} and \textit{C. gattii} and that possible selection is occurring in the environment to reestablish this phenotype in some of these strains. Further work needs to be done to establish the genetic basis for this reversal in phenotype.

Nutrient acquisition within the central nervous system (CNS) is essential for the fungus at this site and has significant clinical implications. We have clearly described how critical carbon metabolism is in this sequestered sanctuary (2), and in this study, we have begun to identify the nitrogen products' impact on \textit{Cryptococcus} physiology. Uric acid is present in cerebrospinal fluid (CSF), but only in small amounts. Urea, however, comprises the most abundant nitrogen metabolite in the CSF, surpassing even glucose in concentration. Impressively, the sum of all average amino acid concentrations does not equal a quarter of the concentration of urea (16). While we demonstrated with our capsule induction assay that uric acid can increase capsule size, particularly in \textit{C. gattii} strains, and we confirmed the observation of Lee et al. (9) that creatinine as the sole nitrogen source induces the largest capsule in certain serotype A strains, we found that urea produced the largest capsule sizes in all other serotypes, especially the Vancouver outbreak VGIIa strain R265. We have identified this abundant nitrogenous product as a signaling molecule for induction of cryptococcal capsule, and this mechanism is generalizable across all species and genotypes.

Furthermore, we observed that this induction of capsule by urea is dose dependent. At the concentrations selected, capsule size increased with increasing urea concentration; this effect was observed more prominently in \textit{C. gattii} strains. As urea is highly soluble in water, practically nontoxic, and slightly alkaline in aqueous solutions, this induction effect could result in a substantial, high upper limit. While these values may exceed the established biological concentrations in CSF, urea is frequently used at very high concentrations as a soil fertilizer, and this exposure may have selected for prominent capsule induction in response to elevated urea in certain strains. As fertilizer lacks typical reagents known to induce capsule production, including high CO$_2$ and serum, nitrogen sources such as urea may help this fungus survive environmental predatory scavengers, such as amoebae or worms that phagocytose the fungus, and killing by inducing the same large protective capsule that we observed during persistence in human CSF.

Our phenotypic observations that the presence of urea increases capsule size were further reinforced by our microarray results. Analysis of the WT under urea-supplemented conditions revealed enrichment for a variety of molecular functions related to capsule production. In the presence of various extracellular urea concentrations, the urea pathway mutants yielded further differences in their transcriptional responses to the presence of urea; in the presence of higher local urea concentrations, the \textit{ure1}$\Delta$ strain was indeed enriched for various molecular functions related to capsule production. Numerous sugar transporters and vesicle transport-related genes were upregulated in the \textit{ure1}$\Delta$ mutant. Taken together, the increased transcription of these genes indicates cellular conditions favorable for increased capsule production in response to intact urea and corroborates our phenotypic observation of increased capsule production in the \textit{ure1}$\Delta$ strain in the presence of urea compared to that for the WT. The genes differentially expressed in both of these microarray experiments with increased urea concentrations provide a specific group of genes linking capsule production to urea exposure.

From our microarray studies, we also found that the mechanism of urea catabolism may be concentration dependent in \textit{C. neoformans}. In \textit{Arabidopsis} species, urea degradation to CO$_2$ and NH$_3$ by urease occurs extracellularly at high concentrations. At low concentrations, though, urea is imported via \textit{Arabidopsis thaliana} DUR3, a high-affinity urea/H$^+$ symporter (43). \textit{Saccharomyces cerevisiae} also follows a dual transport mechanism for urea transport. The first mode of uptake involves active transport, is subject to nitrogen catabolite repression, and makes use of the two-step urea carboxylase/allophanate hydrolase mechanism, while the second mode occurs via passive or facilitated diffusion at concentrations greater than 0.5 mM (44). The results from our microarray experiments support a similar regulated mechanism in \textit{C. neoformans}. The urea transporter is upregulated under low-urea conditions or conditions without urea relative to the regulation under urea-supplemented conditions in all strains tested (~16-fold increase). This finding suggests the importance of urea exposure for \textit{Cryptococcus} species and that urea catabolism is probably controlled in a concentration-dependent manner, as observed in other model organisms.

The work by Haynes and colleagues established a capsule transcriptional signature and assembled a comprehensive list of genes that may be directly vital to capsule induction (32). Their selection of 8 media for performing microarray analysis included low-iron medium (LIM) with and without the chelating agent EDTA, phosphate-buffered saline (PBS) with and without fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM) in room air (RA) or in 5% CO$_2$, and Littman's medium (LIT) with two concentrations of thiamine (LO-THI and HI-THI). These conditions are well established as being variably capsule inducing. However, several other nutrient sources, including urea, have also been shown to increase capsule size and, in fact, may not do so by the same mechanisms that these environments exploit (45). Comparison of their transcriptional signature genes with our lists of differentially expressed genes determined from the use of urea-supplemented conditions and urea pathway mutants did reveal a number of genes differing between the two lists. As the phenotypes were comparable among the various conditions, this could indicate that urea may induce a unique pathway(s) for capsule production in \textit{Cryptococcus} species.

On the other hand, there were some transcriptional similarities between the lists of differentially expressed genes determined from the use of urea-supplemented conditions and capsule-inducing conditions that included 26 genes. This finding likely identifies common responses among the transcriptional controls of capsule induction pathways. These comparisons may help us not only determine which genes are uniquely required for urea-specific capsule induction but also identify common genetic control over capsule induction. The consensus 26 differentially expressed genes that appeared at significant levels under all urea-supplemented conditions that we studied and that were on the signature list of Haynes et al. (32) may provide the best opportunity for further direct investigation into the ability of urea to induce capsule production in \textit{C. neoformans}. While many of these genes are hypothetical proteins (CNAG_01751, CNAG_04475) and may present novel pathways linking urea with capsule induction, other genes on this list have already been clearly implicated in capsule production. For instance, the sugar transporter CNAG_03140...
may transport vital raw sugar materials for synthesizing capsule in the presence of urea, and a nitrogen compound metabolic process gene (CNAG_06067) may function in regulation of the large capsule phenotype observed in the presence of urea. We have now identified a list of genes with which to dissect networks and the linkage of genes to phenotypes.

The pathobiological consequences of urea at the site of CNS infection are less apparent. As noted in our studies with the physiological CO₂ concentrations, further induction of capsule with urea is not detected in the ureIΔ mutant, and in the model of acute rabbit meningitis, this mutant appears to survive as well as the wild-type strain in the CNS (17). However, the impact on long-term yeast survival in the leptomeninges and in CNS cryptococcomas has not been addressed, and cryptococcal urease functions and urea exposure in the lung have been shown to have influences on host immunity (46). Furthermore, there are many known positive and negative effects of the capsular polysaccharide on local immune functions that could be impacted by its induction. As we have shown, there is a dose effect of urea concentrations on capsular polysaccharide production, and increased urea in the host is dynamic and can occur in patients with renal dysfunction from an underlying disease and/or can be caused by polycy therapy, which could directly affect capsule production in the host to the benefit of the yeast at the CNS site. On another note, the urea effect on reducing expression of the gene for uracil phosphoribosyltransferase (CNAG_02337) might even have a deleterious effect on the fungidical activity of flucytosine treatment at the site of infection. This observation will need further examination. Clearly, urea in the mammalian host can be co-opted by this encapsulated yeast as a signal to help it survive the hostility of the host environment and, potentially, its treatment.

In summary, we have demonstrated that urea, as a signal, is a significant inducer of capsule at biologically relevant concentrations in the model fungal pathogen C. neoformans or C. gattii. Our results indicate that this increase in capsule size is not due to growth deficiency in the presence of urea but is due to urea signaling. Furthermore, these results may explain why pathogenic Cryptococcus species have such a pronounced capsule size following persistence within the CSF. As one of the most abundant nitrogen sources in the CSF, urea may prove significant in inducing the prominent capsular phenotype within the CNS.

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