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GLN3 encodes a global regulator of nitrogen metabolism and virulence of *C. albicans*

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Abstract

The function of *GLN3*, a GATA factor encoding gene, in nitrogen metabolism of *C. albicans* was examined. *GLN3* null mutants had reduced growth rates on multiple nitrogen sources. More severe growth defects were observed in mutants lacking both *GLN3* and *GAT1*, a second GATA factor gene. *GLN3* was an activator of two genes involved in ammonium assimilation, *GDH3*, encoding NADP-dependent glutamate dehydrogenase, and *MEP2*, which encodes an ammonium permease. *GAT1* contributed to *MEP2* expression, but not that of *GDH3*. A putative general amino acid permease gene, *GAP2*, was also activated by both *GLN3* and *GAT1*, but activation by *GLN3* was nitrogen source dependent. *GLN3* was constitutively expressed, but *GAT1* expression varied with nitrogen source and was reduced 2–3 fold in *gln3* mutants. Both *gln3* and *gat1* mutants exhibited reduced sensitivity to rapamycin, suggesting they function downstream of TOR kinase. Hyphae formation by *gln3* and *gat1* mutants differed in relation to nitrogen source. The *gln3* mutants formed hyphae on several nitrogen sources, but not ammonium or urea, suggesting a defect in ammonium assimilation. Virulence of *gln3* mutants was reduced in a murine model of disseminated disease. We conclude that *GLN3* has a broad role in nitrogen metabolism, partially overlapping, but distinct from that of *GAT1*, and that its function is important for the ability of *C. albicans* to survive within the host environment.

Keywords

Candida albicans; nitrogen metabolism; GATA factors; GLN3; GAT1; virulence

1. Introduction

Candida albicans is a commensal inhabitant of the alimentary canal of warm-blooded animals, but capable of opportunistic infections in a debilitated or compromised host (Odds, 1988). As a pathogen, it can cause disease in most host tissues and organs (Odds, 1988). Essential to its survival as either commensal or pathogen is the ability to adapt to the various and dynamic nutritive environments presented by the host. For instance, within the alimentary tract nutrient availability ranges widely in association with periods of feeding and fasting and, in addition, *C. albicans* must compete within a large and complex resident microbial community. Invasion

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of internal tissues poses similar adaptive challenges, such as transitions between the amino acid rich plasma and the nutrient limited environment of immune cell phagosomes (Lorenz et al., 2004; Lorenz and Fink, 2002; Rubin-Bejerano et al., 2003).

Several regulatory systems have been implicated in the response of *C. albicans* to nitrogen source availability. The external amino acid sensing system detects the presence of at least seven different amino acids and induces the expression of several permeases that facilitate uptake of amino acids from the medium (Brega et al., 2004). *CSY1* encodes a component of the putative sensor complex and *csy1* mutants fail to activate permease expression and exhibit reduced filamentation (Brega et al., 2004). Enhanced expression of permeases is mediated by the transcription factors Stp1p and Stp2p, which are proteolytically activated in response to external amino acids (Martinez and Ljungdahl, 2005). In addition to *CSY1*, activation of these proteins is dependent upon the chaperone protein Csh3p (Martinez and Ljungdahl, 2004). Mutants lacking *CSH3* have reduced virulence in a murine disseminated disease model suggesting that acquisition of amino acids from the host is important for pathogenesis. In accord with this idea is the observation that *C. albicans* strains auxotrophic for histidine, leucine, arginine, or methionine are virulent in this animal model (Kirsch and Whitney, 1991; Manning et al., 1984; Noble and Johnson, 2005).

Responses to internal amino acid pools are regulated by the general amino acid control system (Tripathi et al., 2002). Starvation for a single amino acid induces multiple amino acid biosynthetic pathways and induces hyphae formation (Pereira and Livi, 1995; Tripathi et al., 2002). Both responses require the transcription activator encoded by *GCN4*, a functional homolog of *S. cerevisiae GCN4* (Tripathi et al., 2002).

Additional control over nitrogen metabolism is provided by *GAT1*, which encodes a member of the GATA factor family of transcription factors (Limjindaporn et al., 2003). Members of this transcription factor family contain a zinc-finger domain that binds the nucleotide sequence 5'-GATA-3', for which they are named (Wilson and Arst, 1998). These factors are conserved in fungi and regulate expression of core enzymes of nitrogen metabolism as well as genes required for the utilization of secondary nitrogen sources (Coffman et al., 1997; Magasanik and Kaiser, 2002; Marzluf, 1997). When preferred nitrogen sources such as ammonium or glutamine are limiting or absent, the genes necessary for metabolism of secondary sources are activated, a response commonly called nitrogen catabolite repression (NCR) (Coffman et al., 1997; Magasanik and Kaiser, 2002; Marzluf, 1997). Nitrogen metabolism in fungi is typically regulated by multiple GATA factors, some activate transcription while others negatively regulate expression (Coffman et al., 1997; Magasanik and Kaiser, 2002; Marzluf, 1997). *C. albicans gat1* null mutants have reduced capacity to metabolize certain secondary nitrogen sources and induction of permeases in response to secondary nitrogen sources is impaired. Furthermore, the mutants were avirulent in a disseminated disease model demonstrating the importance of *GAT1* and nitrogen metabolism to virulence (Limjindaporn et al., 2003).

In a recent study a second *C. albicans* GATA factor was described, *GLN3*, which was found to activate expression of *MEP2* (Dabas and Morschhauser, 2007). *MEP2* has dual roles, acting as an ammonium permease and as a sensor of nitrogen starvation. In its latter role *MEP2* provides a nitrogen starvation signal that induces differentiation from the yeast to the hyphal growth form (Biswas and Morschhauser, 2005). Here we show that *GLN3* has a more global role in nitrogen metabolism. It influences metabolism of various nitrogen sources and activates expression of genes of central nitrogen metabolism as well as various permeases. Its function partially overlaps that of *GAT1*, yet it has separate and distinct roles. Expression of *GLN3* was independent of nitrogen source and *GAT1*, but *GAT1* requires *GLN3* for full expression. Sensitivity to the drug rapamycin is decreased in *gln3* and *gat1* mutants suggesting they lie downstream of the nutrient sensing TOR kinase pathway. *GLN3* was also required for full

virulence further demonstrating that *C. albicans* must adapt its nitrogen metabolic capacity to constraints imposed by the host.

2. Materials and methods

2.1 Strains and culture conditions

C. albicans strains used in this study are listed in Table 1. Strains were routinely cultured in YPD medium (2% glucose, 1% yeast extract, 2% Bacto peptone) or in YNB medium (2% glucose, 0.17% Difco yeast nitrogen base without amino acids and ammonium sulfate, 0.5% ammonium sulfate). Uridine was added at a concentration of 25 µg/ml as needed.

To evaluate utilization of nitrogenous compounds, test strains were pre-cultured 24 h in YNB medium at 30°C. Cells were collected by centrifugation, washed with sterile water, suspended in an equal volume of nitrogen depletion medium (NDM) consisting of 100 mM glucose, 0.17% yeast nitrogen base without amino acids and ammonium sulfate (Difco), and incubated 24 h at 30°C. The depleted cells were diluted 1:1000 into 100 µl of fresh medium consisting of 100 mM glucose, 0.17% yeast nitrogen base without amino acids and ammonium sulfate (Difco), 100 mM MES buffer, pH 5.0, and containing 10 mM nitrogenous test compound, except for ammonium sulfate which was added at 5 mM. OD₅₉₅ of the cultures was monitored in a Tecan GENios plate reader at 30°C for 24 to 48 h. Growth in fetal calf serum was assessed by measuring changes in cell dry weight as previously described (Limjindaporn et al., 2003).

Hyphae formation was assessed by spotting 2 µl of a suspension of nitrogen-depleted cells (5×10^8 cells/ml) onto medium consisting of 1 mM nitrogen source, 100 mM glucose, 0.17% yeast nitrogen base without amino acids and ammonium sulfate (Difco), 100 mM MOPS buffer, pH 7.0, 1.5% washed agar. Spots were imaged after 4 days incubation at 37°C. To assess growth rate differences on these media, 5 µl of serial 10-fold dilutions of the cell suspensions were spotted on the plates and incubated 48 h at 37°C.

2.2 Rapamycin sensitivity

Sensitivity to rapamycin was assessed on YPD agar medium supplemented with 30 ng/ml or 100 ng/ml of rapamycin. Stationary phase cells cultured in YPD were washed and suspended to a density of 2×10^8 cells per ml. Five µl of cells from serial 10-fold dilutions were spotted on the plates, which were imaged after 5 days incubation at 30°C.

2.3 Construction of GLN3 mutants

A 3 kb DNA fragment encompassing *GLN3* and 500 bp of upstream and downstream sequence was amplified by PCR from genomic DNA of strain CAI-12 (Porta et al., 1999). Extend High Fidelity PCR System (Roche Diagnostic Corporation) and the primers CaGLN3-F and CaGLN3-R (Table 2), which incorporated non-template *XhoI* and *PstI* restriction sites respectively, were used. Thermal cycles were 95°C for 3 min, 20 cycles of 94°C for 1 min, 48°C for 1 min, and 68°C for 3 min, and a final extension at 68°C for 7 min. PCR products were precipitated, digested with *XhoI* and *PstI*, and the purified *XhoI-PstI* digested DNA was cloned into the like sites of plasmid LITMUS28i (New England Biolabs) to create plasmid pLGLN1.

GLN3 was deleted by homologous recombination and marker recycling using a *hisG-URA3-hisG* cassette (Fonzi and Irwin, 1993). To construct a deleted allele, plasmid pLGLN1 was digested with *MfeI*, made blunt with Klenow fragment, and digested with *SalI*. This removed a 1712 bp *SalI-MfeI* fragment encompassing nucleotides +52 to +1763 relative to the AUG start codon, which was replaced with a 3.9 kb *SalI-Ecl136II* fragment from plasmid pMB-7 containing the *hisG-URA3-hisG* cassette (Fonzi and Irwin, 1993). The resulting plasmid was designated pLGLN2.

Plasmid pLGLN2 was digested with *XhoI* and *PstI* to release a 5.2kb fragment containing the deletion allele, which was transformed into strain CAI-4 as described by Walther and Wendland (Walther and Wendland, 2003). The CAI-4 parental isolate was verified to be disomic for chromosome#1 prior to use (Chen et al., 2004). Uri⁺ transformants were selected on YNB agar at 30°C. Strain CWL-1A was a representative heterozygous mutant. Spontaneous Uri⁻ derivatives of CWL-1A were obtained by selection on 5-fluoroorotic acid (5-FOA) medium (Boeke et al., 1984). A Uri⁻ isolate heterozygous for the deletion, CWL-2A, was transformed with the *XhoI-PstI* fragment as above to delete the remaining wild-type allele, generating strain CWL-3A. A Uri⁻ derivative of CWL-3A was selected on 5-FOA and designated CWL-4A. Three independent lineages of mutants were constructed, as designated by the suffix A, B, or C, e.g. CWL-1A, CWL-1B, and CWL-1C.

Restoration of a functional *GLN3* allele in the *gln3* null mutants was achieved by integrative transformation with plasmid pLGLN1-URA3. Plasmid pLGLN1-URA3 was constructed by subcloning a 2.1 kb *Ecl136II-XbaI* fragment of *URA3* from plasmid pSMS44 (Saporito-Irwin et al., 1995) into the *EcoRV-XbaI* sites of pLGLN1. Plasmid pLGLN1-URA3 DNA was linearized with *BsmF1*, which cuts 7 bp upstream of the *GLN3* coding region, to target integration to the *GLN3* locus. The resulting rescued strains derived from the three mutant lineages were designated GLR-11, GLR-21, and GLR-31.

Mutants lacking both *GLN3* and *GAT1* were produced by deletion of *GAT1* from two lineages of *gln3Δ* mutants. *GAT1* was deleted as previously described (Limjindaporn et al., 2003). The Uri⁻ double mutants were designated GLG-1111, and GLG-2111.

The *iro1-ura3Δ* mutation each lineage was reverted (Garcia et al., 2001) by transformation with a 4.9 kb *BglIII-PstI* fragment of *URA3* from plasmid pLUBP. Integration events were verified by Southern blot analysis in all strains. The strains and their genotypes are listed in Table 1. (Ramon and Fonzi, 2003).

2.4 Northern blot analysis

Cultures for RNA extraction were inoculated to an OD₅₉₅ of 0.07 and incubated at 30°C with vigorous aeration until the OD₅₉₅ reached 0.6 to 0.7. The medium consisted of 100 mM glucose, 0.17% Difco yeast nitrogen base without amino acids and ammonium sulfate, and 5 mM ammonium sulfate or 10 mM of alternate nitrogen sources. Inoculum was prepared from cells cultured approximately 24 h at 30°C in YNB, harvested, and washed twice with sterile dH₂O. After achieving the desired OD, cultures were harvested by centrifugation, immediately frozen in dry ice-ethanol, and stored at -70°C. RNA isolation and northern blotting were conducted as previously described (Ramon et al., 1999). Hybridization probes were prepared from DNA amplified by PCR using primers listed in Table 2. Hybridization bands were detected with a STORM 840 PhosphorImager, quantified using ImageJ (NIH, <http://rsb.info.nih.gov/ij/>), and normalized to that of *HHT1*.

2.5 Virulence assay

Virulence in a murine model of disseminated disease was tested as described by Sharkey *et al.* (Sharkey et al., 2005), except that groups of 8–10 male BALB/c mice (Harlan), 8–10 weeks old, were used. Two independent trials were performed. Survival data were compared using the generalized Wilcoxon and log-rank tests as implemented in the NCSS software package. P values < 0.05 were considered statistically significant.

3. Results

3.1 Structural conservation of GLN3 proteins

GATA-factors are a family of zinc-finger containing transcription factors that regulate diverse processes in fungi including nitrogen metabolism (Cooper, 2002; Marzluf, 1997). The *C. albicans* genome contains multiple ORFs encoding putative GATA-factors (Jones et al., 2004), one of which, *GAT1*, was previously shown to participate in nitrogen metabolism (Limjindaporn et al., 2003). A recent study demonstrated that another GATA factor of *C. albicans*, designated *GLN3* (orf19.3912), regulated expression of ammonium permease *MEP2* (Dabas and Morschhauser, 2007). The designation *GLN3* reflects the fact that the most closely related *S. cerevisiae* protein is encoded by *GLN3* (Braun et al., 2005). However, global alignment of CaGln3p and ScGln3p shows little overall sequence conservation (approximately 21% identity) and only two notable blocks of conservation, the zinc-finger domain and the carboxyl terminus (Fig. 1A). The single type IVa zinc-finger motif, C-X₂-C-X₁₇-C-X₂-C, (Teakle and Gilmartin, 1998) characteristic of GATA-family transcriptional factors was approximately 75% identical between the proteins and this included the nuclear export signal, LHGTMRPLSL (Carvalho and Zheng, 2003). The ten C-terminal amino acids were conserved between the *GLN3* proteins and though distinct, this sequence block was clearly related to a conserved C-terminal domain present in a broad range of fungal GATA-factors (Platt et al., 1996)(Fig. 1B). The functional significance of this region has been demonstrated for the GATA factors NIT2 of *N. crassa* and AreA of *A. nidulan* (Pan et al., 1997; Platt et al., 1996).

3.2 GLN3 influences metabolism of multiple nitrogen sources

Given the limited homology, it was not clear that CaGln3p has a global role in nitrogen metabolism, as does ScGln3p (Magasanik and Kaiser, 2002). To assess whether *GLN3* has such a global role, a set of deletion mutants lacking *GLN3*, *GAT1*, or both was constructed and their ability to grow on a variety of nitrogen sources was evaluated. Mutational inactivation of fungal GATA factors that regulate nitrogen metabolism generally restricts the spectrum of nitrogen sources that can be used for growth, since expression of the enzymes needed to metabolize secondary nitrogen sources is impaired (Fu and Marzluf, 1987; Limjindaporn et al., 2003; Stanbrough et al., 1995; Tanzer et al., 2003). The parental control strain, CAI-12, grew equally well with 10 mM glutamine, ammonium, arginine, or urea as nitrogen source, but progressively slower with γ -aminobutyric acid (GABA), glutamate, proline, isoleucine, or tryptophan (Table 3). In comparison, the *gln3* mutant exhibited a reduction in growth rate of 25 to 40% on the preferred nitrogen sources, glutamine, ammonium, arginine, and urea, as well as the secondary source isoleucine (Table 3). The growth rate was reduced 2-fold on tryptophan, but identical to the control strain with GABA, glutamate, or proline as nitrogen sources (Table 3). These growth rate differences were observed in three independent *gln3* mutants and were restored to the control rate in rescued strains, verifying their association with *GLN3*.

The phenotype of the *gln3* mutants was distinct from that of a *gat1* mutant. Deletion of *GAT1* had no measurable effect on utilization of any of the tested nitrogen sources except for isoleucine and tryptophan (Table 3). In agreement with our previous report (Limjindaporn et al., 2003), a *gat1* mutation resulted in a 5 to 6-fold reduction in growth rate when isoleucine or tryptophan was provided as the sole nitrogen source.

The observation that metabolism of isoleucine and tryptophan was impaired by mutation of either gene suggested that they have partially redundant functions, as seen for Gln3p and Gat1p of *S. cerevisiae* (Magasanik and Kaiser, 2002). To explore the extent of overlap, a mutant lacking both genes was examined. With glutamine, ammonium, or arginine as nitrogen source, the growth rate of the double mutant was the same as that of the *gln3* mutant, indicating that

GLN3 and *GAT1* do not overlap in controlling the metabolism of these compounds (Table 3). The growth rate of the *gln3 gat1* mutant on isoleucine or tryptophan was comparable to that of the *gat1* mutant, demonstrating that the *gat1* mutation is epistatic to the *gln3* mutation (Table 3). Interestingly, the double mutant had a more severe phenotype with either urea or GABA as nitrogen source. Although the *gat1* mutant was unaffected in urea metabolism and the *gln3* mutant had only a 30% reduced growth rate, the growth rate of the double mutant was reduced nearly 30-fold (Table 3). Similarly, neither single mutation influenced growth on GABA, yet the double mutant grew approximately 3 times slower. Thus, *GLN3* and *GAT1* have largely redundant functions in controlling metabolism of these compounds. Growth of the *gln3 gat1* mutant on glutamate or proline was comparable to the parental control. These phenotypes were confirmed in an independent double mutant. Thus, *GLN3* appears to have a global role in nitrogen metabolism and its role, while overlapping, is also distinct from that of *GAT1*.

3.3 *GLN3* activates expression of core nitrogen metabolic genes and participates in NCR

The role of *GLN3* in nitrogen metabolism was further explored by examining its ability to influence transcription of genes involved in nitrogen metabolism. As potential targets, several genes were chosen based on their homology with *S. cerevisiae* genes that are directly activated by *S. cerevisiae* Gln3p. *ScGDH1* and *ScGDH3* encode isozymes of NADP-dependent glutamate dehydrogenase, a key enzyme in ammonium assimilation, converting ammonium and α -ketoglutarate to glutamate (Magasanik and Kaiser, 2002; ter Schure et al., 2000). *GDH1* encodes the major isozyme, which is ~87% identical to Gdh3p. The *C. albicans* genome contains a single homolog designated *GDH3* (orf19.4716). Expression of this gene, like *ScGDH1* (Daugherty et al., 1993; Riego et al., 2002), varied little in cells cultured on a range of nitrogen sources (data not shown). However, its expression was highly *GLN3*-dependent. The abundance of *GDH3* mRNA was reduced about 5-fold in mutants lacking *GLN3* and genetic rescue of the mutant restored normal expression (Fig. 2A). Several nitrogen sources were tested including ammonium, glutamine, and proline and *GDH3* expression was *GLN3*-dependent in each instance (Fig. 2A and data not shown). In contrast, deletion of *GAT1* was without measurable effect and a *gln3 gat1* double mutant was comparable to the *gln3* mutant (Fig. 2A). Thus, *GLN3* positively regulates *GDH3* expression independent of *GAT1* and irrespective of nitrogen source.

A second enzyme in ammonium assimilation, glutamine synthetase, combines ammonia and glutamate to yield glutamine and is encoded by *GLN1*. In *S. cerevisiae* *GLN1* expression is 100-fold higher in glutamate versus glutamine grown cells and this activation is dependent upon *GLN3* (Minehart and Magasanik, 1992). In contrast, *C. albicans* *GLN1* varied little in relation to nitrogen source and was only about 2-fold higher in glutamate versus glutamine grown cells. Furthermore, neither *gln3* nor *gat1* mutations, singly or in combination, significantly influenced *GLN1* expression in cells cultured with glutamine or proline (data not shown).

Neither *GDH3* nor *GLN1* were sensitive to nitrogen catabolite repression, a phenomenon that in *S. cerevisiae* relies on *GLN3* and *GAT1* (Coffman et al., 1997). To assess whether *C. albicans* *GLN3* participates in nitrogen catabolite repression, expression of *MEP2* and *GAP2* was examined. *MEP2* encodes a high affinity ammonium transporter (Biswas and Morschhauser, 2005; Marini et al., 1997) and its expression in *S. cerevisiae* is sensitive to nitrogen catabolite repression in a *GLN3* and *GAT1*-dependent manner (Marini et al., 1997). Analogous to *S. cerevisiae*, expression of *C. albicans* *MEP2* varied with the quality of the nitrogen source, characteristic of genes sensitive to nitrogen catabolite repression (Fig. 2B). Expression was lowest in cells cultured with glutamine, increased about 5-fold in ammonium or glutamate cultured cells, and at least 15-fold higher when the nitrogen source was proline, urea or

isoleucine. Expression of *MEP2* was influenced by both *GLN3* and *GAT1*. In *gln3* mutants expression was reduced >20-fold (Fig. 2C). The absence of *GAT1* had a less pronounced effect and reduced *MEP2* expression about 5-fold (Fig. 2C). In both instances, genetic rescue of the mutants restored *MEP2* expression, though only to about half of the wild-type level, suggesting a gene dosage effect. No *MEP2* mRNA was detected in the double mutant (Fig. 2C). These results were obtained with proline as the nitrogen source. Because of the low level of expression with glutamine, the effect of the mutations could not be reliably measured, although expression appeared to be reduced in both the *gln3* and *gat1* mutants (data not shown).

Expression of another permease, *GAP2* (orf19.6993), was also examined. *GAP2*, previously referred to as *GAP1* (Limjindaporn et al., 2003), encodes a protein similar to the general amino acid permease of *S. cerevisiae*. In cells cultured with glutamine, *GAP2* expression was strongly *GLN3*-dependent, being reduced to undetectable levels in a *gln3* null mutant (Fig. 2D). Genetic rescue of the mutant restored *GAP2* expression, but not to control levels, suggestive of a gene dosage effect. As previously reported (Limjindaporn et al., 2003), a *gat1* null mutant exhibited a 10-fold reduction in *GAP2* expression (data not shown). However, a different expression pattern was observed with proline. Under these conditions loss of *GLN3* had no measurable effect on *GAP2* expression (Fig. 2D). However, *GAT1* was still required since *GAP2* expression was significantly reduced in the *gat1* null mutant. Mutants lacking both *GLN3* and *GAT1* expressed no detectable *GAP2* (data not shown). This suggests that *GLN3* and *GAT1* both contribute to *GAP2* expression, even with proline as the nitrogen source.

3.4 Expression of *GLN3* and *GAT1*

In *S. cerevisiae* *GLN3* is constitutively expressed and, in response to secondary nitrogen sources, activates expression of *GAT1* (Magasanik and Kaiser, 2002). Thus the expression and interrelationship of *C. albicans* *GLN3* and *GAT1* was examined. Northern blot analysis of RNA from the parental control strain cultured on several nitrogen sources showed no reproducible variation in expression of *GLN3* (data not shown). *GAT1* expression, however, was about 3-fold less with glutamine than with any of the other nitrogen sources tested (data not shown). Comparison of *GLN3* expression in the parental strain and *GAT1* null mutant showed similar amounts of *GLN3* mRNA in both strains (data not shown). In contrast, with proline as nitrogen source, *GAT1* mRNA abundance was reduced approximately 3-fold in the *GLN3* null mutant relative to the parental control or the genetically rescued *Gln3*⁺ strain (Fig 2E). Thus, *GLN3* is constitutively expressed independent of nitrogen source, but *GAT1* is moderately influenced by nitrogen source and dependent on *GLN3* for induction.

3.5 *GLN3* and *GAT1* contribute to rapamycin sensitivity

In *S. cerevisiae* the TOR (target of rapamycin) kinase pathway controls cell growth in response to nutrient availability (Beck and Hall, 1999; Cardenas et al., 1999). Cell growth is inhibited in the presence of rapamycin, an inhibitor of TOR kinases, and this inhibition is relieved in *GLN3* and *GAT1* mutants, indicating that the TOR kinase pathway functions, in part, through these GATA factors (Beck and Hall, 1999; Bertram et al., 2000; Cardenas et al., 1999). To determine if a similar signaling relationship exists in *C. albicans*, the rapamycin sensitivity of *gln3* and *gat1* mutants was tested. As shown in Fig. 7, deletion of either *GLN3* or *GAT1* imparted resistance to rapamycin and to a similar degree. Sensitivity was restored by genetic rescue of the mutants (Fig. 7). Resistance was further enhanced in mutants lacking both genes (Fig. 7), which was even more evident at higher concentrations of rapamycin (data not shown). This suggested that the TOR kinase signaling pathway acts through *GLN3* and *GAT1* and that they have overlapping function in this regard.

3.6 Effect of GLN3 and GAT1 on filamentation

It has long been known that the transition of *C. albicans* from yeast to hyphal morphology is sensitive to the quality and quantity of nitrogen source available (Dabrowa et al., 1976; Holmes and Shepherd, 1987; Hrmova and Drobnica, 1981; Land et al., 1975) and *GLN3* is necessary for nitrogen starvation induced filamentation (Dabas and Morschhauser, 2007). When filamentation of *gln3* and *gat1* mutants was tested under nitrogen sufficient conditions, each mutant responded differently. The ability of colonies to form laterally spreading hyphae was marginally affected by *gln3* or *gat1* mutations when either 1 mM glutamine or proline was provided as the nitrogen source (Fig. 8). Even a double *gln3 gat1* mutant filamented well, indicating that filamentation of the single mutants was not the result of the redundant function of these genes. The results also show that under these conditions nitrogen quality did not have a significant effect, since cells filamented as well with the primary nitrogen source glutamine as with the secondary source proline.

A different response was seen with arginine and glutamate. On these nitrogen sources, the *gat1* mutant was notably impaired. Though abundant hyphae were produced, they were substantially shorter than the parental control or the rescued control (Fig. 8). In contrast, the *gln3* mutation had little effect and the phenotype of the double mutant was similar to a *gat1* mutant (Fig. 8). Filamentation of *gat1* mutants was similarly impaired on media containing ammonium or urea. However, on these media *gln3* mutants were grossly affected and essentially devoid of hyphae (Fig. 8). The double mutant was also non-filamentous, behaving as a *gln3*, rather than a *gat1*, mutant (Fig. 8). Except for the *gln3 gat1* double mutant cultured on urea, none of the strains had substantial growth defects on these media as tested in spot plate assays (data not shown). Thus, *GLN3* and *GAT1* are not essential for hyphae formation per se, but influence the process depending upon the nitrogen sources available.

Interestingly, *gln3* mutants were non-filamentous when the nitrogen source was either ammonium or urea. Urea is degraded to ammonium and then assimilated in like manner. This suggested that the mutants could be deficient in ammonium incorporation under hyphal inducing conditions. Increasing the concentration of ammonium from 1 mM to 25 mM, a concentration that supports growth of mutants lacking ammonium transporters (Biswas and Morschhauser, 2005; Marini et al., 1997), did not promote filamentation of *gln3* mutants and was inhibitory to the parental strain (Fig. 9). In contrast, reducing the glucose concentration from 100 mM to 10 mM enhanced hyphae formation by the parental strain and allowed a low, but significant level of filamentation by the mutant (Fig. 9). When both low glucose and high ammonium concentrations were supplied, filamentation of the mutant was further enhanced, approaching control levels (Fig. 9). Analogous experiments with urea showed the same pattern of enhancement, although filamentation of the mutant was still reduced relative to the control (data not shown). As elaborated in the discussion, these results are consistent with a defect in ammonium assimilation.

3.7 Effect of *gln3* mutations on virulence

The importance of nitrogen regulation in pathogenesis was demonstrated previously with the observation that mutants lacking *GAT1* were avirulent in a murine model of hematogenously disseminated infection (Limjindaporn et al., 2003). Since *GLN3* participates in nitrogen regulation, virulence of *gln3* mutants was assessed. As a prelude to virulence testing, the growth rate of a *gln3* mutant was assessed in 100% fetal calf serum at 37°C. The mean doubling time of the control strain CAI-12 was 2.8 ± 0.5 h versus 3.3 ± 0.3 h for the *gln3* null mutant GUI-11. The growth yield at 24 h was also comparable.

To simulate a disseminated infection, male BALB/c mice were injected via the lateral tail vein with 1×10^6 cells of the test strains. All mice infected with the control strain rapidly succumbed

to the infection and mortality was 100% by day 8. Survival of mice infected with a heterozygous *gln3* mutant was significantly prolonged relative to the control strain ($P < 0.005$, generalized Wilcoxon test) (Fig. 10). Survival time was further increased in mice infected with the *gln3* null mutant and 50% of these mice survived the duration of the experiment (Fig. 10). The rescued strain in which a functional *GLN3* allele was introduced into the null mutant yielded an intermediate survival curve comparable to the heterozygous *gln3* mutant. Statistically indistinguishable results were obtained in a second independent trial (data not shown) demonstrating that *GLN3*, like *GAT1*, is needed within the host environment and that a full complement of alleles is required for normal cell function.

4. Discussion

The foregoing results demonstrated that the *C. albicans* GATA-factor encoded by *GLN3* plays a global regulatory role and is integrated into multiple aspects of nitrogen metabolism. It was required for optimal growth on a variety of nitrogen sources and was a positive regulator of several genes that contribute to nitrogen metabolism. At the core of nitrogen metabolism is the synthesis and interconversion of glutamate and glutamine, which are the principal nitrogen donors for all other biosynthetic reactions of the cell (Magasanik and Kaiser, 2002). One of the core metabolic reactions is under *GLN3* control, the conversion of ammonium and α -ketoglutarate to glutamate catalyzed by NADP-dependent glutamate dehydrogenase. This enzyme is presumably encoded by *GDH3* and expression of this gene was reduced 5-fold in a *gln3* null mutant.

GLN3 also controlled expression of NCR sensitive genes. The NCR response promotes efficient utilization of available nitrogen sources by preventing expression of genes involved in assimilation of less preferred compounds when preferred nitrogen sources are present (Coffman et al., 1997). *MEP2* exhibited a characteristic NCR-sensitive expression pattern, exhibiting low expression in the presence of glutamine and enhanced expression with secondary nitrogen sources such as proline. Expression of *MEP2* in proline grown cells was *GLN3*, as well as *GAT1*, dependent. However, *GLN3* and *GAT1* cannot be conclusively linked to induction by secondary nitrogen sources, since expression with a preferred nitrogen source, glutamine, appeared also to be reduced. It's possible that *GLN3* and *GAT1* are strictly required for expression, regardless of nitrogen source, and that an alternate factor(s) controls expression in relation to nitrogen quality. In this regard, previous studies showed that under conditions of nitrogen limitation *MEP2* expression was independent of nitrogen source and *GLN3* and *GAT1*-dependent on all nitrogen sources tested (Biswas and Morschhauser, 2005; Dabas and Morschhauser, 2007). Nitrogen sources were supplied at a concentration of 100 μ M in those studies versus the 10 mM concentration used here. Thus *MEP2* expression is responsive to both nitrogen quantity and quality and may be regulated differently in response to these variables.

GAP2 expression was also regulated by *GLN3* and the results suggest a possible interaction between *GLN3* and the external amino acid sensing pathway. Expression of *GAP2* is induced by the presence of external amino acids, even when the preferred nitrogen source ammonium is abundant (Brega et al., 2004). This induction requires the amino acid sensor Csy1p and the downstream transcription factor Stp2p (Brega et al., 2004; Martinez and Ljungdahl, 2005). The presence of glutamine causes proteolytic activation of Stp2 and subsequent activation of *GAP2* (Martinez and Ljungdahl, 2005). Since a *gln3* mutation blocked *GAP2* expression in the presence of glutamine, this indicates that Gln3p directly or indirectly interacts with the external amino acid sensing pathway. The observation that *GLN3* was not required for *GAP2* expression in the presence of proline, an amino acid that does not activate the external amino acid sensing system (Brega et al., 2004), is also consistent with this idea. *GAT1*, in contrast, activated expression independent of nitrogen source. This control arrangement has similarities to that of

AGP1 permease of *S. cerevisiae* wherein Gln3p strongly enhances, but is not required for, induction of *AGP1* by the amino acid sensing pathway (Abdel-Sater et al., 2004). However, induction does depend on the GATA-factor Uga35p/Dal81p (Abdel-Sater et al., 2004).

Another nutrient sensing pathway, the TOR kinase pathway, also relied on *GLN3*. The TOR pathway is a global nutrient sensing pathway and is the target of inhibition by the drug rapamycin in *C. albicans* and other fungi (Cruz et al., 2001). In *C. albicans* rapamycin binds Rbp1p, a homolog of the rapamycin target protein FKBP12, and this complex interacts with and inhibits the kinase Tor1p (Cruz et al., 2001). The sensitivity of *C. albicans* to rapamycin was greatly decreased by deletion of either *GLN3* or *GAT1*. Sensitivity was further reduced in *gln3 gat1* double mutants. This suggests that Gln3p and Gat1p are subject to TOR kinase control and function downstream of TOR kinase. An analogous arrangement is seen in *S. cerevisiae* (Beck and Hall, 1999; Bertram et al., 2000; Cardenas et al., 1999), in contrast to *A. nidulans* (Fitzgibbon et al., 2005).

From the foregoing it is evident that *GLN3* and *GAT1* both contribute to the regulation of nitrogen metabolism through partially overlapping as well as distinct roles. Expression of *GLN3* mRNA, however, was not nitrogen regulated and was not altered in a *gat1* null mutant. In contrast, *GAT1* mRNA abundance was enhanced several fold with nitrogen sources other than glutamine and enhanced expression was dependent on *GLN3*. This again is similar to the situation in *S. cerevisiae* wherein *GAT1/NIL1* is nitrogen regulated and partially controlled by *GLN3* (Coffman et al., 1996; Rowen et al., 1997).

One of the phenotypic consequences of null mutations in *GLN3* and *GAT1* was reduced growth rates on certain nitrogen sources. Loss of either gene altered growth on isoleucine or tryptophan, but a substantial growth rate reduction on urea or GABA was observed only in a double mutant. Interestingly, even the double mutant grew well with a range of nitrogen sources, including arginine, glutamate, proline, ammonium and glutamine. This contrasts with *S. cerevisiae gln3 gat1* deletion mutants, which grow poorly with nitrogen sources other than glutamine (Stanbrough et al., 1995). *GLN3* and *GAT1* also contributed to morphogenesis. Neither gene was essential to hyphae formation per se, rather, null mutations in these genes conferred a nitrogen source-dependent phenotype. Deletion of either or both genes caused only a small diminution of filamentation with glutamine or proline as nitrogen sources. When arginine or glutamate were provided, filamentation of the *gat1* null mutant was substantially reduced, though not eliminated. Deletion of *gln3* had little effect on these nitrogen sources, while the *gln3 gat1* mutant was indistinguishable from mutants lacking *GAT1* alone. Conversely, mutation of *gln3* eliminated filamentation on ammonium or urea containing medium. The *gat1* mutant was partially compromised and the double mutant mirrored the *gln3* mutant. Interestingly, the mutants all grew well as yeast under these conditions, suggesting that hyphae have metabolic needs or constraints distinct from the yeast form.

These results have similarities and differences with those of Dabas and Morschhauser (Dabas and Morschhauser, 2007). They found *gln3* mutants to be more compromised for filamentation on glutamine or proline than observed here (Dabas and Morschhauser, 2007) and reported that *gat1* mutants filament normally on ammonium and urea (Dabas and Morschhauser, 2007), whereas we observed a noticeable reduction. These differences may be attributable to the nitrogen limiting conditions used in those studies, 100 μ M versus 1 mM in the present studies, other methodological differences, or possibly the genetic background of the strains (Dabas and Morschhauser, 2007). Both studies agree, however, that loss of *GLN3* profoundly diminishes the ability to filament with ammonium or urea as nitrogen source. Under nitrogen limiting conditions, this defect was traced to the reduction in *MEP2* expression in the *gln3* mutant (Dabas and Morschhauser, 2007). *MEP2* was previously shown to provide a nitrogen starvation signal that induces hyphae formation (Biswas and Morschhauser, 2005).

In addition to the effect of reduced *MEP2* expression, hyphae formation may also be influenced by the ability of the *gln3* null mutant to metabolize ammonium, at least under the conditions used here. Filamentation of the *gln3* mutant was abolished on both ammonium and urea, but not other nitrogen sources. Urea is degraded to ammonium then assimilated in the same manner as ammonium. An important reaction in ammonium assimilation is reductive amination of α -ketoglutarate to yield glutamate, a reaction catalyzed by NADP-dependent glutamate dehydrogenase. Expression of the presumptive gene for this enzyme, *GDH3*, is reduced several fold in the *gln3* mutant. This reaction lies at the intersection of carbon and nitrogen metabolism and the filamentation defect of the *gln3* mutant was partially alleviated by reducing the concentration of glucose and further mitigated by increasing the ammonium concentration. The effect of ammonium concentration is consistent with an impairment of the cell's ability to assimilate ammonium. Reduction of the glucose concentration to 10 mM would be expected to relieve glucose repression (Meijer et al., 1998) and conceivably enhance mitochondrial production of α -ketoglutarate. Increasing the concentration of either or both substrates would increase the forward reaction and enhance assimilation.

Previous work demonstrated the importance of nitrogen regulation in disseminated disease (Limjindaporn et al., 2003) and this conclusion was further substantiated in these studies. In a mouse model of disseminated candidiasis, a *gln3* null mutant was significantly reduced in virulence compared to the control or heterozygous mutants. Strains containing a single functional copy of *GLN3* were also attenuated, but to a lesser extent, suggestive of a gene dosage effect. While the *gln3* null mutant retained some measure of virulence, a *gat1* mutant is avirulent (Limjindaporn et al., 2003), indicating that each factor makes distinct contributions to the ability of *C. albicans* to survive in the host niche. The relationship between GATA factors and virulence may be a common theme in fungal pathogens as *areA* null mutations attenuate virulence of *Aspergillus fumigatus* in pulmonary disease model (Hensel et al., 1998). While the significance of *GLN3* and *GAT1* to virulence is clearly demonstrated, full appreciation of their role awaits a more complete analysis of the gene targets and processes regulated by these factors.

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Fig. 1. Sequence relatedness of CaGln3p

A ClustalX alignment (Jeanmougin et al., 1998) of CaGln3p and ScGln3p is shown in panel A. Identical residues are marked with an asterisk. The zinc-finger and carboxy-terminal domains are boxed. Panel B shows an alignment of the carboxy-termini of CaGln3p and GATA factors of other fungi; Sc, *S. cerevisiae*; Mg, *Magnaporthe grisea* Nut1 (Acc.# Q01168), An, *A. nidulans* Area (Acc.# CAA36731); Gf, *Gibberella fujikuroi* Area (Acc.# P78688); Cf, *Cladosporium fulvum* Nrf1 (Acc.# AAG48616); Nc, *Neurospora crassa* Nit-2 (Acc.# A34755). The thin lines enclose residues that are identical in Ca_Gln3p and Sc_Gln3p. The thick lines enclose amino acids conserved in >50% of the sequences shown.

Fig. 2. Effect of nitrogen source and GATA-factor mutations on gene expression

Northern blot analysis of RNA hybridized with *GDH3* (A.), *MEP2* (B. and C.), *GAP2* (D.) or *GAT1* (E.). RNA was extracted from strains CAI-12 (Parent), GUI-11 (*Gln3*⁻), the rescued *gln3* mutant GLR-11 (*Gln3*⁺-R), CTL-7 (*Gat1*⁻), the rescued *gat1* mutant CTL-5 (*Gat1*⁺-R), or GGU-11 (*Gln3*⁻ *Gat1*⁻) cultured with 10 mM proline (A., C., D., and E.) or glutamine (D.). RNA samples used in B. were prepared from strain CAI-12 cultured with 10 mM of the indicated nitrogen source. As a control, all blots were hybridized with *HHT1*.

Fig. 3. Effect of GATA-factor mutations on rapamycin sensitivity

Serial dilutions of strains CAI-12 (Parent), GUI-11 (*Gln3*⁻), the rescued *gln3* mutant GLR-11 (*Gln3*⁺-R), CTL-7 (*Gat1*⁻), the rescued *gat1* mutant CTL-5 (*Gat1*⁺-R), or GGU-11 (*Gln3*⁻ *Gat1*⁻) were spotted on YPD or YPD + 30 ng/ml rapamycin and imaged after incubation 5 days at 30°C.

Fig. 4. Filamentation phenotype of GATA-factor mutants

Strains CAI-12 (parental), GUI-11 (Gln3⁻), GLR-11 (Gln3⁺ rescued), CTL-7 (Gat1⁻), CTL-5 (Gat1⁺ rescued), or GGU-11 (Gln3⁻ Gat1⁻) were nitrogen starved and 2 μ l of each, containing 1×10^6 cells, was spotted on medium containing 1 mM of the indicated nitrogen source. Colonies were imaged after incubated 4 d at 37°C. A section of the colony from each strain is shown.

Fig. 5. Effect of nutrient conditions on hyphae formation by *gln3* mutant

Strains CAI-12 (parental), GUI-11 (*Gln3*⁻) were nitrogen starved and 2 μ l of each, containing 1×10^6 cells, was spotted on medium containing the indicated concentration of ammonium and glucose. Colonies were imaged after incubated 4 d at 37°C. A section of the colony from each strain is shown.

Fig. 6. Virulence phenotype of *gln3* mutants

Ten BALB/c mice were infected with 1×10^6 cells of strain CAI-12 (*GLN3/GLN3*), a heterozygous mutant CWL-5A (*gln3/GLN3*), a null mutant, GUI-11 (*gln3/gln3*) or a rescued mutant, GLR-11 (*GLN3/gln3*) and monitored for 19 days post infection.

Table 1

C. albicans

strains used in this study

Strain	Parental strain	Genotype ^a	Source
CAI-4			(Fonzi and Irwin, 1993)
CAI-12	CAI-4	<i>iro1-ura3Δ::λimm434/IRO1-URA3</i>	(Porta et al., 1999)
CTL-7		<i>gat1-Δ1::hisG/gat1-Δ1::hisG iro1-ura3Δ::λimm434/IRO1-URA3</i>	(Limjindaporn et al., 2003)
CTL-5		<i>GAT1-pUC18-URA3-gat1Δ::hisG/gat1Δ::hisG</i>	(Limjindaporn et al., 2003)
CWL-1A	CAI-4	<i>gln3Δ::hisG-URA3-hisG/GLN3</i>	This work
CWL-1B	CAI-4	<i>gln3Δ::hisG-URA3-hisG/GLN3</i>	This work
CWL-1C	CAI-4	<i>gln3Δ::hisG-URA3-hisG/GLN3</i>	This work
CWL-2A	CWL-1A	<i>gln3Δ::hisG/GLN3</i>	This work
CWL-2B	CWL-1B	<i>gln3Δ::hisG/GLN3</i>	This work
CWL-2C	CWL-1C	<i>gln3Δ::hisG/GLN3</i>	This work
CWL-3A	CWL-2A	<i>gln3Δ::hisG/gln3Δ::hisG-URA3-hisG</i>	This work
CWL-3B	CWL-2B	<i>gln3Δ::hisG/gln3Δ::hisG-URA3-hisG</i>	This work
CWL-3C	CWL-2C	<i>gln3Δ::hisG/gln3Δ::hisG-URA3-hisG</i>	This work
CWL-4A	CWL-3A	<i>gln3Δ::hisG/gln3Δ::hisG</i>	This work
CWL-4B	CWL-3B	<i>gln3Δ::hisG/gln3Δ::hisG</i>	This work
CWL-4C	CWL-3C	<i>gln3Δ::hisG/gln3Δ::hisG</i>	This work
CWL-5A	CWL-2A	<i>gln3Δ::hisG/GLN3 iro1-ura3Δ::λimm434/IRO1-URA3</i>	This work
GUI-11	CWL-4A	<i>gln3Δ::hisG/gln3Δ::hisG iro1-ura3Δ::λimm434/IRO1-URA3</i>	This work
GUI-21	CWL-4B	<i>gln3Δ::hisG/gln3Δ::hisG iro1-ura3Δ::λimm434/IRO1-URA3</i>	This work
GUI-31	CWL-4C	<i>gln3Δ::hisG/gln3Δ::hisG iro1-ura3Δ::λimm434/IRO1-URA3</i>	This work
GLR-11	CWL-4A	<i>GLN3-URA3-gln3Δ::hisG/gln3Δ::hisG</i>	This work
GLR-21	CWL-4B	<i>GLN3-URA3-gln3Δ::hisG/gln3Δ::hisG</i>	This work
GLR-31	CWL-4C	<i>GLN3-URA3-gln3Δ::hisG/gln3Δ::hisG</i>	This work
GLG-1	CWL-4A	<i>gat1Δ::hisG-URA3-hisG/GAT1 gln3Δ::hisG/gln3Δ::hisG</i>	This work
GLG-2	CWL-4B	<i>gat1Δ::hisG-URA3-hisG/GAT1 gln3Δ::hisG/gln3Δ::hisG</i>	This work
GLG-11	GLG-1	<i>gat1Δ::hisG/GAT1 gln3Δ::hisG/gln3Δ::hisG</i>	This work
GLG-21	GLG-2	<i>gat1Δ::hisG/GAT1 gln3Δ::hisG/gln3Δ::hisG</i>	This work
GLG-111	GLG-11	<i>gat1Δ::hisG/gat1Δ::hisG-URA3-hisG gln3Δ::hisG/gln3Δ::hisG</i>	This work
GLG-211	GLG-21	<i>gat1Δ::hisG/gat1Δ::hisG-URA3-hisG gln3Δ::hisG/gln3Δ::hisG</i>	This work
GLG-1111	GLG-111	<i>gat1Δ::hisG/gat1Δ::hisG gln3Δ::hisG/gln3Δ::hisG</i>	This work
GLG-2111	GLG-211	<i>gat1Δ::hisG/gat1Δ::hisG gln3Δ::hisG/gln3Δ::hisG</i>	This work
GGU-11	GLG-1111	<i>gat1Δ::hisG/gat1Δ::hisG gln3Δ::hisG/gln3Δ::hisG iro1-ura3::λimm434/IRO1-URA3</i>	This work
GGU-21	GLG-2111	<i>gat1Δ::hisG/gat1Δ::hisG gln3Δ::hisG/gln3Δ::hisG iro1-ura3::λimm434/IRO1-URA3</i>	This work

^aUnless indicated otherwise, the background genotype is *iro1-ura3Δ::λimm434/iro1-ura3Δ::λimm434*.

Table 2

Oligonucleotide primers used for PCR

Target gene	Primer name	Primer sequence
<i>GAT1</i>	GAT1-F	5'-ATCATTTTAATACTTCGTTATCTGTG -3'
	GAT1-R	5'-ATCCCAATCTTGATTATTAGCAG -3'
<i>GLN3</i>	GLN3-F	5'-AATGCACCTTCTCCTGATGG -3'
	GLN3-R	5'-AATGTCAAACCTCAACCAATCC -3
<i>GLN3</i>	CaGLN3-F	5'-GGCTCGAGGGTCTTTTTTCATTTTGTGTGTCG -3'
	CaGLN3-R	5'-GGCTGCAGCAGCTAATGAAATATCGGCAAC -3'
<i>GLN1</i>	GLN1-F	5'-ATGACTACTTCCCTTACAGA-3'
	GLN1-R	5'-TTAGTTATCATCACTGCTTTC-3'
<i>GDH3</i>	GDH1-F	5'-CATGAGACAATTGGCCAGATAC-3'
	GDH1-R	5'-TCAAACATGGCATCAGCAAC-3'
<i>MEP2</i>	MEP2-F	5'-TTATCTGGTTGACTGTTGTTTACTG -3'
	MEP2-R	5'-TACTAGACCCATCAGCAACACC -3'
<i>GAP2</i>	GAP2-F	5'-AAAACCTGGTATATTGAAGGTGCTC-3'
	GAP2-R	5'-TCAACACCAAGCATTGTAGACTC-3'
<i>HHT1</i>	HHT1-F	5'-CAAACAGCAAGAAAATCTACTGGTGG-3'
	HHT2-R	5'-CTTTCACCTCTTAATCTTCTAGCTAATTGC-3'

Table 3
Effect of GATA factor mutation on growth rate with various nitrogen sources.

Nitrogen source	Growth rate (h) ^a					
	CAL-12 (parental)	GUL-11 (Gln3 [−])	GLR-11 (Gln3 ⁺ rescued)	CTL-7 (Gat1 [−])	CTL-5 (Gat1 ⁺ rescued)	GGU-11 (Gln3 [−] Gat1 [−])
Glutamine	1.6 ± 0.1	2.0 ± 0.2 ^c	1.8 ± 0.2	1.6 ± 0.2	1.6 ± 0.1	1.9 ± 0.2 ^c
Ammonium	1.6 ± 0.1	2.3 ± 0.4 ^c	1.6 ± 0.1	1.6 ± 0.1	1.6 ± 0.1	2.3 ± 0.2 ^c
Arginine	1.5 ± 0.1	2.0 ± 0.2 ^c	1.6 ± 0.2	1.7 ± 0.2	1.5 ± 0.2	2.1 ± 0.2 ^c
Urea	1.7 ± 0.1	2.3 ± 0.2 ^c	1.8 ± 0.2	1.7 ± 0.1	1.7 ± 0.2	47 ± 6 ^c
GABA	1.9 ± 0.1 ^b	2.1 ± 0.2	2.0 ± 0.1	1.9 ± 0.1	1.9 ± 0.1	7.5 ± 2.0 ^c
Glutamate	2.4 ± 0.2 ^b	2.3 ± 0.2	2.3 ± 0.2	2.3 ± 0.1	2.2 ± 0.1	2.4 ± 0.2
Proline	2.3 ± 0.1 ^b	2.5 ± 0.1	2.4 ± 0.1	2.1 ± 0.1	2.5 ± 0.2	2.5 ± 0.3
Isoleucine	4.2 ± 0.5 ^b	5.6 ± 1.2 ^c	4.2 ± 0.4	20 ± 3 ^c	4.5 ± 0.5	22 ± 4 ^c
Tryptophan	5.0 ± 0.6 ^b	10.2 ± 2.3 ^c	5.4 ± 0.9	29 ± 7	5.2 ± 0.9	33 ± 7 ^c

^a Values are mean doubling time in hours of at least four independent determinations ± standard deviation.

^b Growth rate significantly different from strain CAI-12 cultured with glutamine (P<0.05 in 2-tailed t-test).

^c Growth rate significantly different from strain CAI-12 cultured with the respective nitrogen source (P<0.05 in 2-tailed t-test).