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Nitrogen metabolism and virulence of *Candida albicans* require the GATA-type transcriptional activator encoded by *GAT1*

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Summary

Nitrogen acquisition and metabolism is central to microbial growth. A conserved family of zinc-finger containing transcriptional regulators known as GATA-factors ensures efficient utilization of available nitrogen sources by fungi. GATA factors activate expression of nitrogen catabolic pathways when preferred nitrogen sources are absent or limiting, a phenomenon known as nitrogen catabolite repression. *GAT1* of *Candida albicans* encodes a GATA-factor homologous to the AREA protein of *Aspergillus nidulans* and related transcription factors involved in nitrogen regulation. Two observations implicated *GAT1* in nitrogen regulation. The growth of mutants lacking *GAT1* was reduced when isoleucine, tyrosine or tryptophan were the sole source of nitrogen. Secondly, when cultured on a secondary nitrogen source, *gat1*Δ mutants were unable to activate expression of *GAP1*, *UGA4* or *DAL5*, which were shown to be nitrogen regulated in *C. albicans*. This regulatory defect did not prevent filamentation of *gat1*Δ mutants in nitrogen repressing or non-repressing conditions, demonstrating that nitrogen catabolite repression does not influence dimorphism. The mutants were, however, highly attenuated in a murine model of disseminated candidiasis. Attenuation was not associated with any diminution of growth in serum or ability to utilize serum amino acids. The results indicate an important role for nitrogen regulation in the virulence of *C. albicans*.

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Introduction

Nitrogen availability has profound significance in fungal biology. In response to nitrogen limitation, fungi initiate morphological changes, sexual and asexual sporulation and expression of virulence determinants (Marzluf, 1997). For instance, haploid MATα cells of *Cryptococcus neoformans*, which typically grow as yeast, will develop hyphae and fruiting bodies in nitrogen deficient media and these processes are inhibited by ammonium (Wickes *et al*., 1996). The fruiting bodies form haploid basidiospores, which are suggested to be the infectious propagule (Wickes *et al*., 1996). Virulence of another pulmonary pathogen, *Aspergillus fumigatus*, is dependent upon its ability to respond to nitrogen limitation *in vivo*, mutants lacking the nitrogen response regulator encoded by *areA* are attenuated in a murine model of pulmonary aspergillosis (Hensel *et al*., 1998). Phytopathogens are similarly influenced. Nitrogen starvation signals the development of rice blast disease symptoms by *Magnaporthe grisea* (Talbot *et al*., 1997) and induces expression of *Avr9*, an avirulence gene of *Cladosporium fulvum* (Perez-Garcia *et al*., 2001).

Nitrogen catabolite repression, the overriding control phenomenon in relation to nitrogen availability, has been demonstrated in numerous fungi (Marzluf, 1997). Though somewhat of a misnomer (Magasanik, 1992), this global control mechanism prevents expression of the many genes required to utilize various secondary nitrogen sources when an adequate supply of preferred nitrogen sources is available, typically ammonium or glutamine (Marzluf, 1997). Over 100 genes are nitrogen regulated in *A. nidulans* and at least 30 in *S. cerevisiae* (Platt *et al*., 1996; Cox *et al*., 1999). Nitrogen regulation is controlled by global positive and negative transcription factors, as well as pathway-specific transcription factors (Coffman *et al*., 1997; Marzluf, 1997). The global factors are members of the GATA-family of transcription factors, the name reflecting the core DNA sequence motif recognized by the defining zinc-finger domain (Teakle and Gilmartin, 1998). The principal transcriptional activator is highly conserved amongst fungi and typified by the AREA protein of *Aspergillus* spp., NIT-2 of *Neurospora crassa* and, in *S. cerevisiae*, the dual factors encoded by *GLN3* and *GAT1* (Marzluf, 1997). In the absence of, or limitation of, a pri-

mary nitrogen source, these factors activate expression of genes involved in secondary nitrogen metabolism either alone or in conjunction with pathway-specific factors (Marzluf, 1997).

Evidence of nitrogen catabolite repression in the opportunistic fungal pathogen *Candida albicans* includes the observations that ammonium ion inhibits expression of peptide transporters (Payne *et al*., 1991; Basrai *et al*., 1992), induction of proline uptake (Holmes and Shepherd, 1987) and expression of secreted aspartyl proteinase (Ross *et al*., 1990; Banerjee *et al*., 1991; Hube *et al*., 1994; White and Agabian, 1995). Nitrogen sources have long been known to influence dimorphism, the ability of *C. albicans* to reversibly transit between yeast and hyphal morphologies (Land *et al*., 1975; Dabrowa *et al*., 1976). Some amino acids, particularly proline, arginine and alanine, promote conversion of *C. albicans* to the hyphal form (Land *et al*., 1975). Recent work has implicated *GCN4*, which regulates the general amino acid starvation response, in dimorphism (Tripathi *et al*., 2002). Furthermore, the yeast to pseudohyphal transition of *S. cerevisiae* is under nitrogen catabolite repression (Gimeno *et al*., 1992; Lorenz and Heitman, 1998). The potential importance of nitrogen regulation in dimorphism and virulence of *C. albicans* prompted the present studies. Neither the molecular basis of nitrogen catabolite repression in *C. albicans* nor its biological role have been reported.

This study demonstrates that *GAT1* of *C. albicans* encodes a zinc-finger containing transcriptional activator homologous to *areA* and related GATA factors. Nitrogen catabolite repression was shown for several genes encoding permeases of nitrogenous compounds and their activation was found to be *GAT1*-dependent. Mutants lacking *GAT1*, though deficient in nitrogen catabolite repression, were unaffected in dimorphic capacity or chlamydospore formation. They were, however, grossly attenuated in a murine model of disseminated infection despite their ability to grow in serum and utilize serum amino acids. This indicates a significant role of nitrogen regulation in the infectious process.

Results

Identification and cloning of GAT1

The *areA* gene encodes a transcriptional activator that mediates nitrogen catabolite repression in *A. nidulans* (Kudla *et al*., 1990). A BLASTN comparison of *areA* (Kudla *et al*., 1990) with the *C. albicans* genome sequence database identified several sequence reads annotated as *GAT99* and encoding a peptide similar to the *A. nidulans* protein (Kudla *et al*., 1990). The corresponding genomic DNA was cloned from *C. albicans* strain SC5314 as a 5.5 kb *Sac*I fragment and partially sequenced. A 2268 bp open reading frame was identified which contained the *GAT99* sequence reads. The gene was designated *GAT1* in recognition of its homology with *GAT1* of *S. cerevisiae*. Comparison of this sequence with subsequent assemblies of the genome sequence showed that *GAT1* was nearly identical to orf6.2898 on Contig6–2178. Five nucleotide differences were noted, C versus T at position −96 relative to the start codon of orf6.2898, G versus A at +121, A versus G at +1003, C versus A at +1494 and A versus T at +1516. The change at −96 eliminated a putative stop codon and extended orf6.2898 an additional 201 bp upstream. Three of the four remaining differences resulted in minor amino acid changes.

The most notable feature of Gat1p, the predicted 755 amino acid protein, was the presence of a single type IVa zinc-finger motif, C-x_2-C-x_{17}-C-x_2-C (Teakle and Gilmartin, 1998). Sequence comparisons showed Gat1p was most similar to AREA and the closely related orthologues of other filamentous fungi (Fig. 1 and data not shown). The most similar yeast homologue was Gat1p, which like AREA is a transcriptional activator involved in nitrogen catabolite repression (Stanbrough *et al*., 1995; Coffman *et al*., 1996). Except for the zinc finger domain, there was no significant similarity with fungal GATA factors that regulate other cell processes such as iron acquisition (Voisard *et al*., 1993), response to light (Ballario *et al*., 1996; Linden and Macino, 1997), mating type switching and pseudohypal growth (Long *et al*., 1997; Chandarlapaty and Errede, 1998), or premRNA processing (Hess *et al*., 1994) (data not shown).

Similarity between Gat1p and related fungal proteins was confined to three sequence blocks (Fig. 1). The amino terminal block, located between residues Asn64 and Met90, corresponded to a region of AREA that is

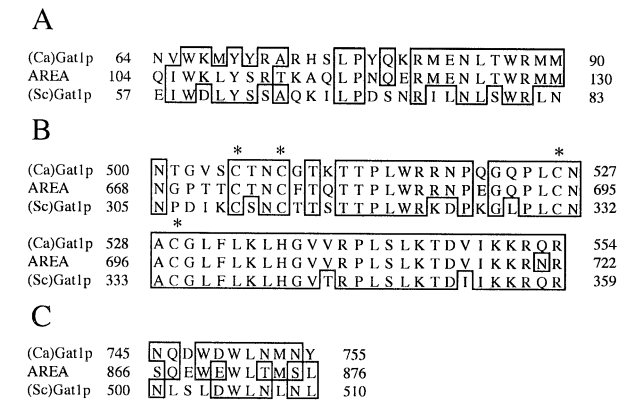

Fig. 1. Blocks of conserved sequence in the amino terminus (A), zinc-finger region (B) and carboxy terminus (C) of Gat1p. Amino acids conserved in at least two of the three sequences are boxed. Asterisks indicate the cysteine residues of the zinc-finger. Sequences used for comparison were AreA (Accession No. S70168) and Gat1p (Accession No. NP_116632).

dispensable for function and only subtle phenotypes are associated with mutations in this region (Langdon *et al*., 1995). The most significant block of homology, having 88% identity, encompassed amino acids Asn500 through Arg554 and corresponded to the DNA binding domain of AREA and *N. crassa* NIT2 (Fu and Marzluf, 1990; Wilson and Arst, 1998) (Fig. 1). This region contains the zinc-finger, an adjacent alpha-helical and extended loop region followed by a block of basic amino acids (Marzluf, 1997; Wilson and Arst, 1998). The DNA binding domain is essential and mutations within this region affect both the specificity of DNA recognition and modulation of activity in response to nitrogen (Marzluf, 1997; Wilson and Arst, 1998). All of the DNA contact residues in this domain are conserved in the *C. albicans* protein except for Gln553, corresponding to Asn721 of AREA (Wilson and Arst, 1998), suggesting similar binding specificity for Gat1p.

The third sequence similarity block encompassed the 12 C-terminal amino acids (Fig. 1). These residues are not required for transcriptional activation by AREA or NIT2, but are necessary for proper modulation of AREA activity and nitrogen repression of transcription (Platt *et al*., 1996; Marzluf, 1997).

GAT1 influences nitrogen source utilization

Despite limited sequence conservation, the clear relationship with AREA and other GATA factors that regulate nitrogen metabolism suggested a similar role for Gat1p. Evidence of this role was obtained by assaying *gat1* null mutants for their ability to utilize various nitrogen sources and to regulate expression of nitrogen catabolite repressed genes. Targeted gene deletion was used to sequentially delete both alleles of *GAT1* (Fig. 2A). A rescued strain, in which a functional copy of *GAT1* was introduced into the homozygous null mutant, was constructed as a control (Fig. 2B). The presence and integrity of the mutated alleles was verified by Southern blot analysis (Fig. 2) and Northern blot hybridization verified the loss of *GAT1* mRNA in the homozygous mutant and its restoration in the rescued strain (data not shown).

The growth rate of the homozygous *gat1*Δ mutant was comparable to the control strain in YCB medium when 2 mM ammonium sulphate, glutamine, proline, urea, γ-aminobutyric acid or allantoin were added as the sole nitrogen source (data not shown). However, the mutant exhibited a >fourfold increase in mean generation time, from 3.5 ± 0.4 h to 16.5 ± 1.9 h, with isoleucine as the nitrogen source. Growth was restored to the control rate by re-introduction of a functional *GAT1* allele indicating that the *gat1*Δ mutation results in a diminished capacity to utilize isoleucine as a nitrogen source. Notable growth rate reductions were also observed with 2 mM tyrosine or tryptophan as nitrogen source, from 7.6 ± 0.6 to 25.2 ± 2.1 h and from 6.3 ± 0.8 to 26.8 ± 3.5 h respectively.

GAT1 is required for nitrogen catabolite repression

In many fungi the presence of a preferred nitrogen source prevents activation of genes required to utilize secondary nitrogen sources, a phenomenon known as nitrogen catabolite repression (Marzluf, 1997). Nitrogen catabolite repression in *C. albicans* was GAT1-dependent, providing further evidence of its role in nitrogen metabolism. Homologues of *GAP1*, *UGA4* and *DAL5*, which are sensitive to nitrogen catabolite repression in *S. cerevisiae* (Coffman *et al*., 1996), were cloned and their expression in repressing and non-repressing nitrogen sources was examined (Fig. 3). Both *GAP1* and *UGA4*, which encode a putative

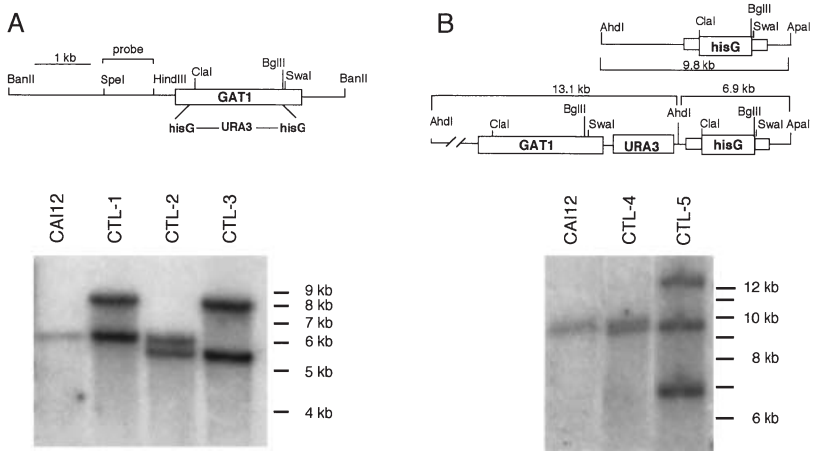

Fig. 2. Design and validation of *GAT1* mutations.
A. Illustrates the design of the *GAT1* deletion-substitution mutation and Southern blot validation of the replacement events. The labelled box in the diagram indicates the *GAT1* coding region and the location of relevant restriction sites is marked. The region used as a hybridization probe is indicated by the inverted bracket. The lower portion shows a Southern blot of *Ban*II digested genomic DNA from the indicated strains. The size and electrophoretic position of DNA size markers is indicated to the right.
B. Shows the predicted structure of the GAT1 locus in the rescued strain and Southern blot validation of the insertion event. Relevant coding regions are boxed and labelled accordingly. Expected restriction fragments are bracketed and their sizes indicated. The lower portion shows a Southern blot of *Ahd*I-*Apa*I digested genomic DNA from the indicated strains. The hybridization probe was the same as in A.

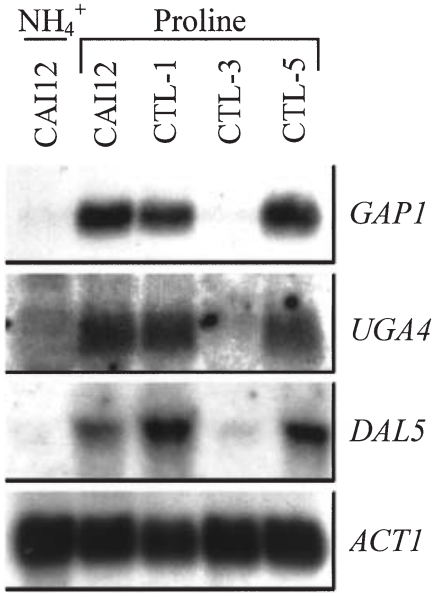

Fig. 3. *GAT1* activates expression of nitrogen catabolite repressed genes. Strain CAI12 (*GAT1/GAT1*) was cultured in YCB with 10 mM ammonium sulphate or 10 mM proline as the sole nitrogen source. Strains CTL-1 (*gat1*Δ/*GAT1*), CTL-3 (*gat1*Δ/*gat1*Δ) and CTL-5 (*GAT1-gat1*Δ/*gat1*Δ) were cultured with 10 mM proline. Total RNA was examined by Northern blot hybridization with *GAP1*, *UGA4*, *DAL5* or *ACT1* DNA. The strains and nitrogen source are indicated along the top of the figure. Hybridization probes are indicated on the right.

general amino acid permease and γ-aminobutyric acid permease, respectively, were sensitive to nitrogen catabolite repression as demonstrated by their reduced expression in cells cultured with ammonium and 10–20-fold induction with proline as nitrogen source (Fig. 3). Induction in the presence of proline was *GAT1*-dependent as demonstrated by the near absence of *GAP1* and *UGA4* mRNA in strain CTL-3, homozygous for the *gat1*Δ allele, and its restoration by re-introduction of a wild-type allele, as shown for strain CTL-5 (Fig. 3). Deletion of a single copy of *GAT1* had no discernable effect on the pattern or level of expression of these genes, as shown for strain CTL-1 (Fig. 3). Quantitative phosphorimage analyses indicated that expression in the null mutant was independent of the nitrogen source and equivalent to the parental control strain cultured in ammonium. Essentially identical results were obtained with a third gene, *DAL5* (Fig. 3) encoding a putative allantoate permease and when *UGA4* expression was examined in cells cultured at pH 7.0, 37°C (data not shown). Therefore, *GAT1*, like *areA*, *nit-2* and yeast *GAT1*, is required for transcriptional activation of genes under nitrogen catabolite control.

Morphological transitions do not require GAT1

Candida albicans, like many fungi, can reversibly alter its morphology between yeast and hyphae. *GAT1* was not required for morphological development. When cells were cultured in Lee's medium or Media 199 under conditions that promote germ tube formation, the initial stage of this morphological switch, >90% of *gat1*Δ cells formed germ tubes, a frequency comparable to the parental control strain CAI12. The only notable difference was a slight temporal lag in germ tube emergence, which was ameliorated in the rescued strain. However, given the abundance of preferred nitrogen sources, the cells are likely to be nitrogen catabolite repressed in these media. Therefore, *GAT1* would not be required for gene activation and no effect would be anticipated for *GAT1* mutations. Germ tube formation was tested in 10% serum and buffered proline as well. These media arguably represent nitrogen derepressing conditions and any effects of *GAT1* loss might be evident in these media. But again, the mutant was comparable to the control (data not shown).

To definitively evaluate the potential role of *GAT1* in hyphae formation, conditions were sought in which cell morphology was sensitive, at least in part, to the nitrogen catabolite repression. Germ tube formation was tested in a YNB-based medium buffered to pH 7.0. With 10 mM proline as the sole nitrogen source, CAI12 cells grew predominantly as yeast and short pseudohyphae, but 10–15% of the population formed germ tubes and progressed to highly elongated psuedohyphae and a few true hyphae with morphological indexes ranging from 2.8 to 3.9 (Merson-Davies and Odds, 1989) (Fig. 4). When 10 mM ammonium or 10 mM glutamine served as the nitrogen source no germ tubes were observed and the cells remained as yeast (Fig. 4). Inclusion of ammonium or glutamine with proline suppressed the formation of filamentous cells (Fig. 4). Despite the sensitivity of cell morphology under these conditions to nitrogen catabolite repressing versus non-repressing nitrogen sources, the *gat1*Δ mutant behaved identically to the control (Fig. 4).

Because some mutations differentially influence filamentation in broth culture versus agar solidified media (Liu *et al*., 1994; Sharkey *et al*., 1999), *gat1*Δ mutants were also tested on agar plates of YNB-based medium. Because the plates are incubated for 24 h or more, assessing nitrogen catabolite repression in this format is complicated by progressive changes in the nutrient content of the medium as a result of the metabolic activity of the cells. Therefore, the glucose concentration was reduced to 10 mM to ensure that the carbon source, not nitrogen, would become limiting with growth. Also, glutamine, 10 or 50 mM, was used as the repressive nitrogen source. The use of glutamine instead of ammonium is expected to prevent the release of nitrogen catabolite repression that occurs upon carbon source starvation, the lack of carbon prevents synthesis from ammonium of the true repressor, glutamine (Cox *et al*., 2002). As seen in Fig. 4, strain CAI12 formed abundant lateral, agar-

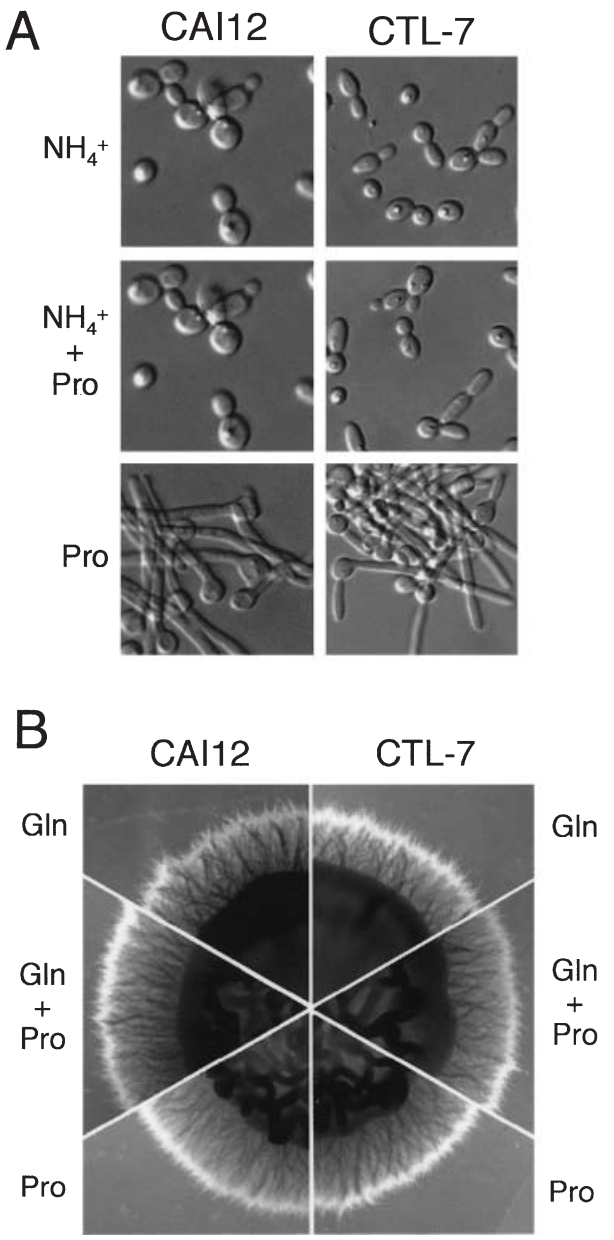

Fig. 4. Filamentation is not nitrogen repressed and is independent of *GAT1*.
A. Shows photomicrographs of strain CAI12 (*GAT1/GAT1*) or CTL-7 (*gat1*Δ/*gat1*Δ) cultured 4 h at 37°C in YNB-based medium containing 10 mM proline, 5 mM ammonium sulphate, or both as the sole nitrogen source.
B. Displays the colony morphology of strain CAI12 and CTL-7 after 72 h at 37°C on YNB-based medium with 10 mM glutamine, 10 mM proline or 10 mM of each as nitrogen source.

invasive hyphae with either glutamine or proline as sole nitrogen source indicating that filamentation was not nitrogen catabolite repressed under these conditions. Furthermore, the colony morphology of the *gat1*Δ mutant was indistinguishable from the control strain demonstrating that *GAT1* is not required for filamentations under these conditions. When ammonium was used as the nitrogen source, the mutant and control showed abundant filamentation with concentrations ranging from 76 mM to 100 μM, the latter limiting conditions promote pseudohypha formation in *S. cerevisiae* (Gimeno *et al*., 1992). The mutant also formed filamentous colonies on agar solidified Lee's medium, Media 199, 10% serum and Spider medium (data not shown).

The *GAT1* null mutation also had no effect on chlamydospore production (data not shown). Thus, *GAT1* had no discernable role in the morphological processes of *C. albicans*.

GAT1 is required for disseminated infection

Despite the modest *in vitro* phenotypes of the *gat1*Δ mutant, *GAT1* was critical *in vivo* when tested in a murine model of disseminated candidiasis. Balb/c mice were infected intravenously via the tail vein with the *GAT1* mutants and survival was monitored for 20 days. Mice challenged with the parental control, CAI12, or the heterozygous *gat1*Δ mutant, CTL-6, succumbed to infection within one week (Fig. 5). In contrast, mice challenged with the homozygous *gat1*Δ mutant survived the entire test period (Fig. 5). Restoration of a single functional allele, as in the rescued strain CTL-5, fully restored virulence (Fig. 5) demonstrating that attenuation could be attributed directly to the loss of *GAT1*. Statistical comparison of all four groups by Kruskal–Wallis analysis of variance demonstrated significant variance between the populations ($P < 0.001$). Individual comparisons using the Mann–

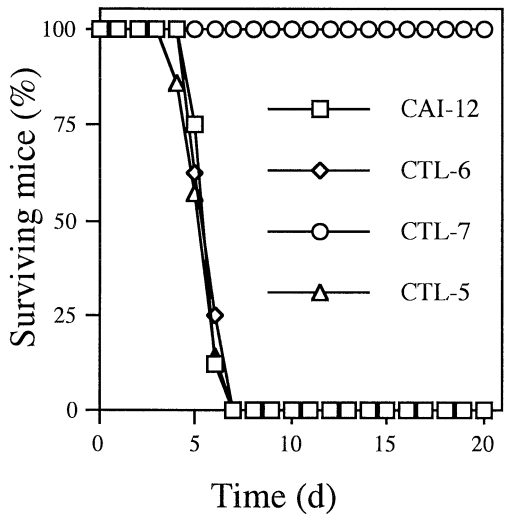

Fig. 5. Effect of *gat1*Δ mutations on virulence in disseminated infection. Mice ($n = 8$) were injected intravenously with 1×10^6 cells of strain CAI12 (*GAT1*/*GAT1*), CTL-6 (*gat1*Δ/*GAT1*), CTL-7 (*gat1*Δ/*gat1*Δ) or CTL-8 (*GAT1- gat1*Δ/*gat1*Δ). Moribund mice were euthanized and the number of surviving mice was recorded each day.

Whitney *U*-test, Gehan's generalization of the Wilcoxon rank sum test, or logranks showed a significant ($P < 0.001$) difference between mice injected with control strains versus the null mutant. These results were confirmed in a second independent repetition of the infection study (data not shown).

GAT1 is not required for growth in serum

One explanation for the gross attenuation of the *gat1*Δ mutant would be a specific growth defect in serum, related perhaps to the inability to derepress permeases needed to metabolize amino acids and other nitrogen sources present in serum. This possibility was assessed by culturing the control strain CAI12 and the *gat1*Δ mutant CTL-7 in 100% serum at 37°C in shake flasks. Under these conditions, the growth rate of the mutant was essentially identical to that of the control. The mean doubling time, based on the first four mass doublings, was 2.6 ± 0.5 h ($n = 6$) for the control and 2.8 ± 0.4 h ($n = 6$) for the mutant. The mutant had no overt deficiency in serum amino acid utilization. The serum content of 27 amino compounds was measured before and after growth of the control or mutant strains (Table 1). Utilization of only five of these was significantly reduced in the mutant relative to the control. Interestingly, isoleucine was not one of these, but tryptophan and tyrosine were (Table 1). However, even in these instances the mutant had utilized ≥67% as much as the control strain indicating that the *gat1*Δ mutation did not prevent metabolism of these amino acids. Serum urea concentrations were also measured, but there was no significant utilization by either strain (data not shown). Thus, the attenuated virulence of the mutant was not attributable to an inability to grow in serum.

Table 1. Utilization of serum amino acids.

Amino acid	Initial conc. (uM)	% Utilized	
		CAI-12	CTL-7
Alanine	951 ± 16	99.7 ± 0.5	99.3 ± 0.6
Glutamate	757 ± 31	98.0 ± 0.9	98.9 ± 0.4
Glycine	507 ± 18	89.9 ± 2.1	76.9 ± 4.1[a]
Glutamine	385 ± 14	100 ± 0	100 ± 0
Valine	324 ± 3	99.1 ± 1.5	100 ± 0
Serine	240 ± 7	96.2 ± 0.8	96.2 ± 0.3
Ornithine	237 ± 2	98.8 ± 1.0	99.2 ± 1.4
Leucine	224 ± 2	98.4 ± 0.6	99.5 ± 0.4
Lysine	206 ± 2	98.1 ± 0.5	98.5 ± 1.3
Proline	200 ± 2	100 ± 0	100 ± 0
Hydroxyproline	152 ± 4	36.5 ± 4.2	24.4 ± 5.3[a]
Threonine	151 ± 2	97.5 ± 4.3	100 ± 0
Isoleucine	133 ± 2	93.5 ± 1.0	95.0 ± 0.3
Taurine	133 ± 3	6.88 ± 1.3	7.72 ± 1.0
Phenylalanine	133 ± 4	100 ± 0	100 ± 0
Aspartate	95.0 ± 1.6	78.5 ± 2.9	79.9 ± 1.0
Tyrosine	91.2 ± 2.7	98.9 ± 1.9	94.0 ± 1.4[a]
Citrulline	78.8 ± 3.3	32.9 ± 5.9	32.4 ± 5.9
Histidine	69.7 ± 4.4	100 ± 0	100 ± 0
Tryptophan	45.5 ± 0.2	94.1 ± 10	72.9 ± 1.5[a]
3-Methyl-Histidine	45.3 ± 0.1	94.5 ± 9.4	70.8 ± 3.3[a]
α-Aminobutryic acid	41.5 ± 1.2	100 ± 0	100 ± 0
β-Alanine	40.8 ± 0.7	100 ± 0	100 ± 0
α-Aminoadipic	35.1 ± 1.6	41.6 ± 7.3	29.3 ± 13
Methionine	31.5 ± 0.3	85.6 ± 1.0	86.8 ± 1.9
Asparagine	20.6 ± 1.3	98.7 ± 2.1	97.5 ± 2.1
Cystathione	9.96 ± 0.21	99.3 ± 1.2	95.6 ± 5.9

a. Probability ≤0.05 by two-tailed Student's *t*-test.

Discussion

Candida albicans GAT1 was identified based on its homology with other fungal GATA factors involved in nitrogen regulation. As expected of a nitrogen regulatory factor, a null mutation in *GAT1* altered the nitrogen utilization capacity of the cells. Growth of *gat1*Δ mutants was normal with a number of nitrogen sources, but was compromised on isoleucine, tyrosine and tryptophan. This provides some limited insight into the catabolic pathways under *GAT1* control and demonstrates some of the divergence of targets of nitrogen regulation in *C. albicans* compared to other fungal species. For instance, *A. nidulans areA* mutants are more severely affected, retaining the ability to catabolise only ammonium and glutamine (Langdon *et al*., 1995). Similarly, *N. crassa nit-2* mutants are unable to utilize proline, histidine, phenylalanine or nitrate and have diminished growth on a number of amino acids including branched chain amino acids, tryptophan and phenylalanine, but not tyrosine (Facklam and Marzluf, 1978). The modest phenotype of the *gat1*Δ mutant may indicate redundant uptake or utilization pathways or overlapping transcriptional control as seen for *GLN3* and *GAT1/NIL1* of *S. cerevisiae* (Coffman *et al*., 1996). Deletion of yeast *GAT1/NIL1* did not affect growth with proline, ammonium or citrulline as sole nitrogen sources, but did reduce growth on allantoin (Coffman *et al*., 1996).

GAT1, like the homologous GATA factors of other fungi, activated expression of genes sensitive to nitrogen catabolite repression. *GAP1*, *UGA4* and *DAL5* are three of at least 30 nitrogen catabolite repressed genes in *S. cerevisiae* (Cox *et al*., 1999). Each of these three genes encodes a transporter of nitrogenous compounds. Expression of the homologous genes in *C. albicans* was similarly under nitrogen regulation and was *GAT1*-dependent. Despite their sensitivity to nitrogen catabolite repression, the regulation of these genes differed between *C. albicans* and *S. cerevisiae*, most notably with regard to *GAP1*. In *S. cerevisiae GAP1* expression is under dual control of *GAT1* and *GLN3* (Stanbrough *et al*., 1995; Coffman *et al*., 1996). Mutation of *GAT1* or *GLN3* individually reduces *GAP1* expression approximately 50%, expression is completely eliminated in the double mutant (Stanbrough *et al*., 1995;Coffman *et al*., 1996). In contrast,

GAT1 alone appears largely or completely responsible for *C. albicans GAP1* activation, even though a homologue of *GLN3* appears to be present in *C. albicans* genome (unpubl. results). *UGA4* and *DAL5* of yeast are also regulated by both *GAT1* and *GLN3*, but expression is strongly influenced by *GAT1* similar to the effects seen here (Coffman *et al*., 1996). It is of interest to note that *areA* and *nit-2* appear to be the only global nitrogen activators in *A nidulans* and *N. crassa* respectively (Marzluf, 1997).

GAT1 had no discernable influence on cell morphology. The null mutant formed normal chlamydospores and filamented as well as the control strain. The absence of filamentation defects in the mutant is not surprising since filamentation is not sensitive to nitrogen catabolite repression. This statement is supported by a number of observations. Filamentation proceeds readily in medium containing abundant, preferred nitrogen sources. Examples include Lee's medium, which contains 76 mM ammonium (Lee *et al*., 1975), peptone containing Spider medium and, in this work, agar invasive filamentation was shown to occur equally well in medium with repressing and non-repressing nitrogen sources. Furthermore, hyphae contain elevated internal pools of glutamine (Torosantucci *et al*., 1984), which signals nitrogen repression (Marzluf, 1997; Cox *et al*., 2002) and a brief period of nitrogen deprivation, which should release nitrogen catabolite repression, inhibits, rather than stimulates, germ tube formation (Holmes and Shepherd, 1987). Thus, as nitrogen catabolite repression is not involved, a requirement for *GAT1* is not anticipated. Conversely, it can be argued that the absence of morphological defects in the *gat1*Δ mutant indicates that dimorphism is not subject to nitrogen catabolite repression. This statement is tempered by the observation that multiple GATA factors regulate nitrogen catabolite repression in *S. cerevisiae* (Coffman *et al*., 1997) and homologous genes are present in *C. albicans* (unpubl. results). The absence of nitrogen repression distinguishes *C. albicans* filamentation from pseudohyphae formation in *S. cerevisiae*, which is under nitrogen control (Gimeno *et al*., 1992; Lorenz and Heitman, 1998).

The virulence of a strain lacking *GAT1* was grossly attenuated in a murine model of disseminated infection. This was surprising for two reasons. Dimorphic ability, a strong virulence determinant (Braun and Johnson, 1997; Lo *et al*., 1997), was unaltered *in vitro* and, based on preliminary experiments, *in vivo* as well (unpublished results). Secondly, the growth rate of the mutant in serum at 37°C was comparable to the control and it utilized the full range of amino acids present in the serum. The utilization of a few amino acids was reduced, but by only 30% or less. This may, nonetheless, be sufficient to marginalize growth *in vivo*.

One factor likely to contribute to attenuation of the *gat1*Δ mutant is the lack of, or reduction in, secreted aspartyl proteinase expression. These are encoded by SAP genes, a multigene family in *C. albicans*, and some members are sensitive to nitrogen catabolite repression (Ross *et al*., 1990; Banerjee *et al*., 1991; Hube *et al*., 1994; White and Agabian, 1995). Preliminary experiments indicate that the *gat1*Δ is deficient in utilization of bovine serum albumin as a nitrogen source, indicative of an inability to express secreted proteinases. However, mutants lacking SAPs do not appear to be as grossly attenuated as the *gat1*Δ mutant (Hube *et al*., 1997; Sanglard *et al*., 1997) suggesting that *GAT1* controls expression of additional genes relevant to pathogenesis.

In conclusion, *GAT1 has* been identified as a mediator of nitrogen regulation in *C. albicans* and its function is important for virulence. Further analysis of this mutant may provide insight into the *in vivo* growth of *C. albicans* and factors relevant to its ability to cause disease.

Experimental procedures

Strains and culture conditions

The strains used in this study are listed in Table 2. Strains were routinely cultured in either YPD (2% glucose, 2% Bactopeptone, 1% yeast extract) or YNB (2% glucose, 0.67% Difco yeast nitrogen base without amino acids). Media were supplemented with uridine, 25 μg ml^{-1}, as needed. Uridine auxotrophs were selected on medium containing 5-fluoroorotic acid (5FOA) (Boeke *et al*., 1984).

Nitrogen source utilization was assessed in YCB (Difco yeast carbon base). Strains were precultured in YPD medium at 25°C overnight to a cell density of approximately 2×10^8 cells ml^{-1}. The cells were collected by centrifugation, washed three times with sterile dH_2O, suspended in YCB at the initial cell density and incubated 12 h at 25°C to deplete nitrogen reserves. The depleted cells were inoculated to a density of 5×10^6 cells ml^{-1} in YCB medium containing 2 mM or 10 mM nitrogen source. The cultures were incubated at 37°C with vigorous aeration. Growth was monitored spectrophotometrically at 600 nm.

Growth in serum was assessed by measuring the dry weight of cells. Strains were precultured and washed as above. Washed cells were inoculated into heat inactivated fetal bovine serum (Life Technologies) at a density of 1–5×10^6 cells ml^{-1}. Ten ml aliquots of cell suspension, one for each time point, were placed in 125 ml flasks and incubated at 37°C with vigorous shaking. The culture was apportioned at the start of the experiment to avoid sampling errors cause by the extensive cell clumping that occurs under these growth conditions. Cells were harvested by filtration on preweighed glass fibre filters and washed with 5 ml of sterile dH_2O. Samples were dried *in vacuo* and weighed. Growth rate values are the result of three or more independent experiments.

Germ tube formation was assessed in the medium of Lee *et al*. (1975), 199 medium (Gibco-BRL) buffered with 150 mM Hepes, pH 7.0, Spider medium (Liu *et al*., 1994), 10% fetal calf serum, or a defined medium consisting of 100 mM glucose, 0.17% Difco yeast nitrogen base without ammonium sulphate or amino acids, 150 mM MOPS, pH 7.0, and 10 mM

Table 2. Strains of *Candida albicans* used in this work.

Strain	Parent	Genotype	Source
SC5314		Clinical isolate	Gillum *et al*. (1984)
CAI4		*iro1-ura3*Δ::λimm434/*iro1-ura3*Δ::λimm434	Fonzi *et al*. (1993); Garcia *et al*. (2001)
CAI12	CAI4	*iro1-ura3*Δ::λimm434/*IRO1 URA3*	Fonzi and Irwin (1993)
CTL-1	CAI4	*gat1*Δ::*hisG-URA3-hisG*/*GAT1* *iro1-ura3*Δ::λimm434/*iro1-ura3*Δ::λimm434	This work
CTL-2	CTL-1	*gat1*Δ::*hisG*/*GAT1* *iro1-ura3*Δ::λimm434/*iro1-ura3*Δ::λimm434	This work
CTL-3	CTL-2	*gat1*Δ::*hisG*/*gat1*Δ::*hisG-URA3-hisG* *iro1-ura3*Δ::λimm434/*iro1-ura3*Δ::λimm434	This work
CTL-4	CTL-3	*gat1*Δ::*hisG*/*gat1*Δ::*hisG* *iro1-ura3*Δ::λimm434/*iro1-ura3*Δ::λimm434	This work
CTL-5	CTL-4	*GAT1*-pUC18-*URA3-gat1*Δ::*hisG*/*gat1*Δ::*hisG* *iro1-ura3*Δ::λimm434/*iro1-ura3*Δ::λimm434	This work
CTL-6	CTL-2	*gat1*Δ::*hisG*/*GAT1* *iro1-ura3*Δ::λimm434/*IRO1 URA3*	This work
CTL-7	CTL-4	*gat1*Δ::*hisG*/*gat1*Δ::*hisG* *iro1-ura3*Δ::λimm434/*IRO1 URA3*	This work

proline or glutamine or 5 mM $(NH_4)_2SO_4$. Strains were precultured in YPD at 30°C for 24 h and inoculated into the desired medium at a density of 2–5 × 10^6 cells ml^{-1}. When using YNB-based medium, the cells were washed once with one volume of sterile dH_2O before inoculation. Cultures were incubated at 37°C with vigorous agitation on a rotary shaker and assessed microscopically for the presence of germ tubes.

Colony filamentation was assessed on the preceding media solidified with 2% agar. However, the YNB-based medium was solidified with washed agar and the concentration of glucose was reduced to 10 mM. Five microlitres of cell suspension, approximately 1 × 10^6 cells, was spotted on the plates and the plates were incubated at 37°C for 2–5 days (Sharkey *et al*., 1999).

GAT1 identification and cloning

Sequence data for *Candida albicans* was obtained from the Stanford Genome Technology Center website at http://www-sequence.stanford.edu/group/candida. Sequencing of *Candida albicans* was accomplished with the support of the NIDR and the Burroughs Wellcome Fund. A BLASTN comparison of the *Aspergillus nidulans areA* sequence (Kudla *et al*., 1990) with a *C. albicans* genome sequence database consisting of individual sequence reads with 1.5× annotated coverage (http://www-sequence.stanford.edu:8080/ybc.html) identified several overlapping sequence reads annotated as *GAT99* and encoding a protein similar to the zinc finger region of the *A. nidulans* protein (Kudla *et al*., 1990). A corresponding 706 bp region was amplified from strain SC5314 genomic DNA by PCR using primers 5′-GCACCATTCTCG CATAG-3′ and 5′-TTAAGAGCACTGTTACTGTTG-3′. Thermal cycles were 95°C for 5 min, 33 cycles of 94°C for 45 s, 49°C for 1 minute and 72°C for 45 s, and a final extension at 72°C for 10 min. The PCR product was used for hybridization screening of a GEM-12 genomic library (Birse *et al*., 1993). Positive plaques were purified and phage DNA was isolated using Promega's Wizard Preps (Promega, Madison, WI) following the manufacturers recommendations. Restriction mapping of phage inserts identified a 5.5 kb *Sac*I fragment that hybridized with the PCR product. This fragment was subcloned into pUC18 to create plasmid pTH-1. The nucleotide sequence was determined for approximately 3 kb of the pTH-1 insert using custom-synthesized oligonucleotide primers and the ABI Prism dRhodamine Terminator Cycle Sequencing Kit and AmpliTaq DNA polymerase (Applied Biosystems). Nucleotide and protein sequence analyses were performed with DNA Strider (Marck, 1988). Homology searches of the non-redundant Genebank Database were done using the BLAST algorithm (Altschul *et al*., 1990; Altschul *et al*., 1997). Multiple sequence alignments were constructed using CLUSTALX 1.8 (Thompson *et al*., 1997). The sequenced gene was designated *GAT1* in recognition of its similarity to *S. cerevisisae GAT1* and the sequence was entered in GenBank with Accession # AY293736.

Mutant strain construction

Mutants containing heterozygous or homozygous deletions of *GAT1* were constructed by targeted recombination and marker recycling (Fonzi and Irwin, 1993). A mutant allele was produced *in vitro* by digesting plasmid pTH-1 with *Cla*I and *Bgl*II. This removed a 1705 bp region encompassing bases +285 to +1990 of the coding region. The deleted region was replaced with a 3.9 kb *Acc*I-*Bgl*II fragment from plasmid pMB-7 containing the *hisG-URA3-hisG* cassette (Fonzi and Irwin, 1993). The resulting plasmid, pTH-2, was digested with *Spe*I and *Swa*I, which cut at positions −1320 and +2024 of the *GAT1* coding region respectively. *Candida albicans* strain CAI4 was transformed by the method of Geitz *et al*. (Gietz *et al*., 1992). Uri^+ transformants were selected on YNB. A representative heterozygous mutant was designated CTL-1.

Homozygous null mutants were generated following selection of spontaneous Uri^- derivatives of CTL-1 on medium containing 5-fluoroorotic acid (5FOA) (Boeke *et al*., 1984). The Uri^- strain, CTL-2, was transformed with the *Spe*I-*Swa*I fragment as above. Strain CTL-3 was a representative

homozygous mutant obtained from this transformation. A spontaneous Uri$^-$ derivative of CTL-3 was selected on 5FOA and designated strain CTL-4.

Restoration of a functional allele was achieved by transformation of strain CTL-4 with plasmid pTH-3. Plasmid pTH-3 consisted of the 5.5 kb *Sac*I fragment from pTH-1 cloned into the *Sac*I site of plasmid pSMS-44. Plasmid pSMS-44 is a pUC18 derivative containing a 2 kb *Eco*RV-*Xba*I fragment of *URA3* (Porta *et al*., 1999). Plasmid pTH-3 DNA was digested with *Spe*I, 1320 nucleotides 5′ of the coding region, before transformation in order to target integration to the *GAT1* locus. The resulting strain was named CTL-5.

The *IRO1- URA3* locus of *gat1* mutants was reconstituted by transformation with a 5 kb *Bgl*II-*Pst*I fragment of pUR3 (Kelly *et al*., 1987). Transformation of the heterozygous mutant CTL-2 yielded strain CTL-6. Strain CTL-7 was generated from the homozygous mutant CTL-4. All integration events were verified by Southern blot analysis.

Southern and Northern blots

Extraction of genomic DNA and RNA, Southern and Northern blot preparation and hybridization were described previously (Saporito-Irwin *et al*., 1995). Southern blots were hybridized with a 0.9 kb *Spe*I-*Hin*dIII fragment of plasmid pTH-1 or a 1.3 kb *Sca*I-*Xba*I fragment of *URA3* labelled by random priming with the DNA Ready-to-Go Labelling Beads (–dCTP) Kit and [α-^{32}P]-dCTP (Amersham Pharmacia Biotech). Hybridizing DNA was visualized by autoradiography.

Cells for RNA samples were precultured 24 h in YPD medium at 25°C. Cells were washed three times with sterile dH_2O and inoculated to a density of 6×10^6 cells ml^{-1} in 200 ml of YNB or YCB containing 10 mM proline. The cells were harvested after 6 h incubation with vigorous agitation at 37°C. Blots were hybridized with a 1705-bp *Cla*I-*Bgl*II fragment of *GAT1* or the PCR amplified DNA of *GAP1, UGA4* or *DAL5*, described below. Blots were subsequently stripped and hybridized with *ACT1* DNA as a control. Hybridized RNA was detected by autoradiography or using a PhosphoImager 445SI (Molecular Dynamics). Images were quantified with ImageQuant software or on a Macintosh computer using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/). All Northern data was confirmed with at least two independent RNA preparations. Transcript sizes were approximated by comparison with rRNA.

The orthologue of *S. cerevisiae GAP1*, orf6.7739 of Assembly 6 of the *C. albicans* genome sequence, was amplified by PCR using primers GAP1-F, 5′-ATGCCAGAAAAG GAATTAGATTAC-3′, and GAP1-R, 5′-TCAACACCAAGCAT TGTAGACTC-3′. A portion of *UGA4*, orf6.6161, was amplified with primers UGA4-F and UGA4-R, 5′-ATGTTTTG GAGTGGCATTCT-3′ and 5′-TCAAGACTTTTCACTCAAC TCTG-3′ respectively. *DAL5*, orf6.4447, was amplified with primers DAL5-F, 5′-ATGACCGAAAAAAAAGAAT-3′, and DAL5-R, 5′-TCAAATGTAATAAGAAACATAGATT-3′. The amplification products were cloned into plasmid pCR2.1 TOPO using the TOPO cloning kit (Invitrogen).

Systemic infection of mice

The Gat1$^+$ control strain, CAI12, heterozygous mutant, CTL-6, *gat1*Δ mutant, CTL-7, and rescued strain, CTL-5, were cultured in YNB medium at 25°C for 24 h. Cells were harvested by centrifugation, washed with sterile dH_2O, suspended in saline, and enumerated with a haemacytometer. Cell suspensions were adjusted to a density of 5×10^6 cells ml^{-1} and 200 μl were injected into the tail veins of 9- to 11-week-old male BALB/C mice weighing 20–25 g (Charles River Laboratories, Kingston, N.Y). Groups of seven or eight mice were inoculated with each strain. Moribund mice were sacrificed by asphyxiation in CO_2 according to AVMA guidelines (AVMA, 2000). The experimental end-point was overall mortality at 21 days following challenge, expressed as percent survival. Kruskal–Wallis analysis of variance was conducted for statistical comparison of all animals in the experiment and the Mann–Whitney *U*-test and Gehan's generalization of the Wilcoxon rank sum test were used for comparisons between groups (Campbell, 1989; Dawson and Trapp, 2001).

The genotype of *C. albicans* in infected tissue was verified by Southern blot analysis. Kidneys were aseptically removed from sacrificed mice, weighed and homogenized in 1 ml of sterile saline. The homogenates were serially diluted and plated on YPD agar. After 2 days of incubation at 30°C, random colonies were picked and DNA from these isolates was analyzed by Southern blot analysis as described above.

Serum nitrogen source utilization

To compare the utilization of serum components, 10 ml of heat inactivated fetal bovine serum was inoculated to 5×10^6 cells ml^{-1} of either the Gat1$^+$ strain, CAI12 or the Gat1$^-$ strain, CLT-7. The cells were precultured at 28°C in YPD and washed three times in sterile dH_2O prior to inoculation. After 24 h incubation at 200 r.p.m., 37°C, cells were removed by centrifugation for 10 min at 10 000 *g*. The supernatant was passed through a 0.45 μ pore filter and frozen at −70°C until analysis. Quantitative amino acid analysis was performed Dr M. Tuchman, Department Laboratory Medicine, Children's National Medical Center, Washington, DC. Serum urea analysis was performed by American Medical Laboratories, Chantilly, VA. Three independent culture supernatants were collected for each analysis.

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