INTRODUCTION

Species of the genus Candida are common commensals of mammalian mucous membranes (Wrobel et al., 2008). However, only a limited number of species are commonly associated with humans as colonizers and opportunistic pathogens: Candida albicans, Candida glabrata, Candida parapsilosis, Candida tropicalis, Candida lusitaniae and Candida krusei (Köhler et al., 2015). The yeast C. glabrata ranks number two as an aetiologic agent of candidemia and is the most frequently encountered non-C. albicans species in patients with invasive candidiasis. Transcriptome analysis in C. albicans, C. glabrata and Cryptococcus neoformans has revealed that, when engulfed by macrophages, these yeasts upregulate genes involved in nutrient acquisition, including nitrogen transporters such as the general amino acid permease Gap1, the dicarboxylic amino acid permease Dip5, the basic amino acid permease Can1 and the ammonium permeases Mep1 and Mep2. Nitrogen assimilation has been well studied in model species of fungi, such as Aspergillus nidulans, Neurospora crassa and Saccharomyces cerevisiae. However, little is known about nitrogen assimilation in C. glabrata. In the present study, we report a major role for Gln3 in the assimilation of glutamine, ammonium and proline. Ure2 also has a role in nitrogen assimilation, but it is only observable in ammonium and glutamine. In addition, Gat1 has a minor role, which is only observable in the absence of Ure2 and Gln3. Gln3 is absolutely necessary for full ammonium uptake from media. We have also shown that MEP2 gene expression in C. glabrata is completely dependent on Gln3, whereas GAP1 regulation is mainly exerted by Gln3, with the exception of proline where Gat1 has a minor role. In addition, in C. glabrata Ure2 appears to be a negative regulator of these NCR-sensitive genes, similarly to what has been described in S. cerevisiae. Our data place Gln3 as a key regulator of nitrogen assimilation.

After Candida albicans, the yeast Candida glabrata ranks second as an aetiologic agent of candidaemia and is the most frequently encountered non-C. albicans species in patients with invasive candidiasis. Transcriptome analysis in C. albicans, C. glabrata and Cryptococcus neoformans has revealed that, when engulfed by macrophages, these yeasts upregulate genes involved in nutrient acquisition, including nitrogen transporters such as the general amino acid permease Gap1, the dicarboxylic amino acid permease Dip5, the basic amino acid permease Can1 and the ammonium permeases Mep1 and Mep2. Nitrogen assimilation has been well studied in model species of fungi, such as Aspergillus nidulans, Neurospora crassa and Saccharomyces cerevisiae. However, little is known about nitrogen assimilation in C. glabrata. In the present study, we report a major role for Gln3 in the assimilation of glutamine, ammonium and proline. Ure2 also has a role in nitrogen assimilation, but it is only observable in ammonium and glutamine. In addition, Gat1 has a minor role, which is only observable in the absence of Ure2 and Gln3. Gln3 is absolutely necessary for full ammonium uptake from media. We have also shown that MEP2 gene expression in C. glabrata is completely dependent on Gln3, whereas GAP1 regulation is mainly exerted by Gln3, with the exception of proline where Gat1 has a minor role. In addition, in C. glabrata Ure2 appears to be a negative regulator of these NCR-sensitive genes, similarly to what has been described in S. cerevisiae. Our data place Gln3 as a key regulator of nitrogen assimilation.

Gln3 is a main regulator of nitrogen assimilation in Candida glabrata

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After Candida albicans, the yeast Candida glabrata ranks second as an aetiologic agent of candidaemia and is the most frequently encountered non-C. albicans species in patients with invasive candidiasis. Transcriptome analysis in C. albicans, C. glabrata and Cryptococcus neoformans has revealed that, when engulfed by macrophages, these yeasts upregulate genes involved in nutrient acquisition, including nitrogen transporters such as the general amino acid permease Gap1, the dicarboxylic amino acid permease Dip5, the basic amino acid permease Can1 and the ammonium permeases Mep1 and Mep2. Nitrogen assimilation has been well studied in model species of fungi, such as Aspergillus nidulans, Neurospora crassa and Saccharomyces cerevisiae. However, little is known about nitrogen assimilation in C. glabrata. In the present study, we report a major role for Gln3 in the assimilation of glutamine, ammonium and proline. Ure2 also has a role in nitrogen assimilation, but it is only observable in ammonium and glutamine. In addition, Gat1 has a minor role, which is only observable in the absence of Ure2 and Gln3. Gln3 is absolutely necessary for full ammonium uptake from media. We have also shown that MEP2 gene expression in C. glabrata is completely dependent on Gln3, whereas GAP1 regulation is mainly exerted by Gln3, with the exception of proline where Gat1 has a minor role. In addition, in C. glabrata Ure2 appears to be a negative regulator of these NCR-sensitive genes, similarly to what has been described in S. cerevisiae. Our data place Gln3 as a key regulator of nitrogen assimilation.

INTRODUCTION

Species of the genus Candida are common commensals of mammalian mucous membranes (Wrobel et al., 2008). However, only a limited number of species are commonly associated with humans as colonizers and opportunistic pathogens: Candida albicans, Candida glabrata, Candida parapsilosis, Candida tropicalis, Candida lusitaniae and Candida krusei (Köhler et al., 2015). The yeast C. glabrata ranks number two as an aetiologic agent of candidemia in adults in North America and Europe, second to C. albicans (Arendrup et al., 2008; Horn et al., 2009). In addition, C. glabrata is the most frequently encountered species that is not C. albicans in patients with invasive candidiasis in North America (Pfaller et al., 2014). Pathogenic fungi are ubiquitous organisms, which inhabit different niches within the host and, in order to ensure cell survival, they must adapt rapidly to these challenging conditions (Ene et al., 2014).

Recently, transcriptome studies have revealed that C. albicans, C. glabrata and Cryptococcus neoformans, when engulfed by macrophages, upregulate genes involved in nutrient acquisition including those required for the utilization of alternative carbon sources and several permeases, including nitrogen transporters, such as the general amino acid permease Gap1, the dicarboxylic amino acid permease Dip5, the basic amino acid permease Can1, and the ammonium permeases Mep1 and Mep2 (Fan et al., 2005; Kaur et al., 2007; Lorenz et al., 2004).

In Saccharomyces cerevisiae, control over nitrogen assimilation is provided by two positive (Gat1 and Gln3) and two negative (Dal80 and Gzf3) transcription factors, which contain a zinc-finger domain that binds to the core sequence 5'-GATAAG-3' in the promoter of their target genes. An additional layer of regulation is provided by the negative regulator of Gat1 and Gln3, Ure2 (Cooper, 2002; Magasanik & Kaiser, 2002). GATA factors are conserved in fungi and regulate expression of genes coding for enzymes and permeases, which are required for nitrogen assimilation (Marzluf, 1997). Nitrogen assimilation has been well

Abbreviations: Cyt, Cytoplasm; FRT, Flp1 recognition sequence; NCR, nitrogen catabolite repression; NMR, nitrogen metabolism repression; Nuc, nucleus; WT, parental strain.

One supplementary figure and two supplementary tables are available with the online Supplementary Material.
studied in diverse fungi such as Aspergillus nidulans, Neurospora crassa and S. cerevisiae (Wong et al., 2008). These organisms selectively use readily assimilated nitrogen sources (e.g. ammonium or glutamine) in preference to those that are not easily assimilated (e.g. GABA or proline). This preferential use of the nitrogen sources is achieved through a similar mechanism in these organisms: nitrogen metabolite repression (NMR) in A. nidulans and N. crassa, and nitrogen catabolite repression (NCR) in S. cerevisiae (Cooper, 2002; Magasanik & Kaiser, 2002; Marzluf, 1997).

In the present study, we have investigated the role of the GATA factors (Gat1 and Gln3) and the Ure2 protein in C. glabrata when yeast cells were grown in glutamine, ammonium or proline as the sole nitrogen sources. We found that in the absence of Gln3, the growth rate is significantly decreased in all the nitrogen sources tested. In addition, Gln3 mutants were unable to efficiently transport ammonium from media. Furthermore, transcription of the genes encoding the ammonium permease (MEP2) and the general amino acid permease (GAPI) were dependent on Gln3. However, Gat1 has a minor role in GAPI regulation, which is only observed in the absence of Ure2 and Gln3. Our data indicate that Gln3 is a key regulator of nitrogen assimilation in the pathogenic fungus C. glabrata.

METHODS

Strains and growth conditions. All strains used in this study were generated in the BG14 background (parental strain) (Cormack & Falkow, 1999) and are described in Table S1 (available in the online Supplementary Material). Yeast cultures were routinely incubated at 30°C with constant shaking. Yeast liquid medium was prepared as previously described (Sherman et al., 1986) and 2% (w/v) agar (Sigma-Aldrich) was added if plates were needed. YPD medium contained 10 g l⁻¹ yeast extract (BD; Bionox), 20 g l⁻¹ peptone (BD; Bionox) and 2% (w/v) glucose (J. T. Baker). Synthetic complete medium (SC) contained 1.7 g l⁻¹ yeast nitrogen base (without amino acids and (NH₄)₂SO₄) (BD; Difco), 5 g l⁻¹ (NH₄)₂SO₄ (J.T. Baker) and 2% (w/v) glucose (J.T. Baker), supplemented with 0.6% (w/v) casamino acids (Fisher Scientific).

For the solid media assay, the parental strain was grown overnight in liquid YPD media (30°C with constant spinning in a roller drum) then, the cells were washed twice with sterile milliQ water and the OD₆₀₀ nm was adjusted to 0.6. Ten-fold serial dilutions were made and 5 μl were spotted onto minimal media (MM) containing 1.7 g l⁻¹ yeast nitrogen base (without amino acids and (NH₄)₂SO₄) (BD; Difco), 2% (w/v) glucose (J.T. Baker), 1 mg ml⁻¹ of the indicated nitrogen source and supplemented with 30 μg ml⁻¹ uracil. For the liquid media assay, the C. glabrata parental strain as well as gat1Δ, gln3Δ, ure2Δ, ure2Δ gat1Δ, ure2Δ gln3Δ, gat1Δ gln3Δ and ure2Δ gat1Δ gln3Δ mutant strains were grown in MM supplemented with the nitrogen source indicated (1 mg ml⁻¹) in a Bioscreen C MBR machine and incubated at 30°C with continuous maximal shaking. Optical density (OD₆₀₀ nm) measurements were taken every 30 min. Doubling time was calculated using data from the mid-log phase of exponential growth.

Construction of deletion strains. We generated fusion PCR products for gene disruption using the URA3 gene to construct the knockout mutant strains as the selection marker, as previously described (Gutiérrez-Escobedo et al., 2015). All primers used in this study are listed in Table S2. Three PCR products for each gene deletion were amplified separately: (1) the URA3 gene marker flanked by the Flp1 recognition sequence (FRT) amplified from the pGE80 vector (De Las Peñas laboratory collection), (2) the corresponding 5’-upstream fragment of the GAT1 ORF or GLN3 ORF, and (3) the corresponding 3’-downstream fragment of the GAT1 ORF or GLN3 ORF. For each deletion cassette, fragments were linked using two independent PCR reactions as follows: first, a 15 cycle primer-free reaction where the corresponding three fragments were added; second, a 30 cycle reaction where 4 μl of the previous reaction were used as a template, with specific primers for the 5’-upstream and 3’-downstream of GAT1 or GLN3. Fusion PCR products were purified using the Wizard SV Gel and PCR Clean-Up System (Promega).

The parental strain of C. glabrata and ure2Δ strain were transformed as previously described (Castatto et al., 2003) with GAT1-deletion and GLN3-deletion cassettes to obtain gat1Δ::URA3, gln3Δ::URA3, ure2Δ gat1Δ::URA3 and ure2Δ gln3Δ::URA3. To obtain unmarked deletion strains, gat1Δ::URA3, gln3Δ::URA3, ure2Δ gat1Δ::URA3 and ure2Δ gln3Δ::URA3 strains (containing the URA3 marker gene flanked by F RT sites) were transformed with pLS9 plasmid, as described previously (Juárez-Reyes et al., 2012). To generate gat1Δ gln3Δ and ure2Δ gat1Δ gln3Δ strains, gat1Δ and ure2Δ gat1Δ strains were transformed with GLN3-deletion cassettes as described above. Strains gat1Δ gln3Δ::URA3 and ure2Δ gat1Δ gln3Δ::URA3 were later transformed with pLS9 plasmid to obtain unmarked mutant strains (Table S1).

Homologous recombinations and gene deletions were verified by PCR analysis using primers that anneal in the sequences external to GAT1- or GLN3- deletion cassettes and a primer annealing within the URA3 marker. We also verified the absence of each gene deleted by the inability to PCR amplify an internal fragment from each deleted gene (Table S2).

Sequence analysis. C. glabrata orthologues of S. cerevisiae genes GAT1 (CAGL0K07634g), GLN3 (CAGL0C02277g), URE2 (CAGL007392g), GAPI (CAGL0I03267g), MEP2 (CAGL0I06028g) and GLN1 (CAGL0I05357g) were identified using the Yeast Gene Order Browser (http://ygob.ucd.ie) (Byrne & Wolfe, 2005). Protein sequences of S. cerevisiae were downloaded from the Saccharomyces Genome Database (http://www.yeastgenome.org) (Cherry et al., 2012) and C. glabrata protein sequences were downloaded from the Candida Genome Database (http://www.candidagenome.org) (Inglis et al., 2012). Pairwise sequence alignments were made with the Needle software from EMBOSS (Rice et al., 2000).

qRT-PCR. Total RNA extraction was performed according to Schmitt et al. (1990). Yeast cultures of the wild-type and mutant strains were grown overnight in liquid YPD medium (30°C with constant spinning in a roller drum); afterwards, cells were washed twice with sterile milliQ water and resuspended in 1 ml of water. OD₆₀₀ nm was adjusted to 0.1 in 50 ml MM with (NH₄)₂SO₄. Flasks were incubated at 30°C with constant shaking (220 rpm) until an OD₆₀₀ nm of 0.8 was reached. Cells were then pelleted and total RNA was extracted. For the shift to proline, cultures were grown to an OD₆₀₀ nm of 0.8, and then cells were pelleted and transferred to MM with proline (1 mg ml⁻¹) for 60 min, after which total RNA was extracted. RNA was treated with Turbo DNase (Ambion) and cDNA synthesis was made with the Superscript II (Invitrogen) reverse transcriptase kit with oligo(DT)₁₆ primers by following the manufacturer’s recommended protocol. Quantitative PCR was carried out using the Fast SYBR Green Master Mix (Applied Biosystems) in the 7500 Fast Real-Time PCR System (Applied Biosystems). ACT1 was used as a control for normalization; all primers used are described in Table S2.
RESULTS

*C. glabrata* can use a variety of nitrogen sources

As yeast cells do not assimilate all nitrogen-containing compounds with equal efficiency, we decided to analyse the growth phenotype of the parental strain of *C. glabrata* on solid media with different compounds available as the sole nitrogen source (Fig. 1). After 48 h of incubation, the parental strain grew equally well in almost all the nitrogen sources tested. However, growth was diminished in adenine and residual growth only was observed when lysine or uracil was used as the nitrogen source (Fig. 1a). As the solid media assay did not allow us to observe significant growth differences between the nitrogen sources tested, parental strain doubling times were calculated based on the mid-log phase of exponential growth in liquid media (Methods). The shortest doubling times of all of the nitrogen sources tested were obtained when ammonium or glutamine were used as the sole nitrogen source (1.1 and 1.4 h, respectively).

Additionally, the doubling time was increased with respect to ammonium when proline (1.8 h), glycine (1.8 h), asparagine (1.9 h), leucine (1.9 h), valine (2.0 h) and isoleucine (3.0 h) were used as the sole nitrogen source. Moreover, the doubling time was even higher using adenine (7.3 h) as the sole nitrogen source (Fig. 1b). These observations allow us to group the nitrogen sources into four categories: (1) a fast growing category (ammonium and glutamine), (2) a medium growing category (proline, glycine, asparagine, leucine, valine and isoleucine), (3) a slow growing category (adenine), and (4) a non-growing category (lysine and uracil).

Gln3 regulates nitrogen assimilation in *C. glabrata*

In *S. cerevisiae* the GATA-type transcription factors, Gat1 and Gln3, activate gene expression in response to the nitrogen source available in media through a mechanism known as NCR. We sought to investigate the role of the GATA factors, Gat1 and Gln3, and the Ure2 repressor in nitrogen assimilation in *C. glabrata*. We identified the syntenic *GAT1, GLN3* and *URE2* genes of *C. glabrata* using the Yeast Gene Order Browser (http://ygob.ucd.ie) and orthologous protein sequences were downloaded from the *Candida* Genome Database (http://www.candidagenome.org) (Byrne & Wolfe, 2005; Inglis et al., 2012). The *C. glabrata* *GAT1* (CAGL0K07634g) gene encodes a putative protein of 460 amino acids, which exhibits 37% similarity to *S. cerevisiae* Gat1, across the entire length of the protein (511 amino acids). The putative *C. glabrata* Gln3 (CAGL0C02277g; 824 amino acids) has 46% similarity with *S. cerevisiae* Gln3 (731 amino acids). The putative *C. glabrata* Ure2 repressor (CAGL0J07392g, 356 amino acids) exhibits 86% similarity with *S. cerevisiae* Ure2 (355 amino acids). Thus, based on sequence and synteny conservation, we identified the putative Gat1, Gln3 and Ure2 orthologues in *C. glabrata*.

*C. glabrata* is able to use glutamine, ammonium or proline as the sole nitrogen source (Fig. 1); so next, we wanted to determine the role of the *C. glabrata* GATA factors (Gat1 and Gln3) and the Ure2 protein in the assimilation of these nitrogen compounds. We evaluated the growth phenotype of the parental strain, single *gat1Δ, gln3Δ, ure2Δ*, double *ure2Δ gat1Δ, ure2Δ gln3Δ, gat1Δ gln3Δ* and triple *ure2Δ gat1Δ gln3Δ* mutants in MM containing glutamine, ammonium or proline (1 mg ml⁻¹) as the sole nitrogen source and their respective doubling times are shown in Table 1. In YPD, all yeast strains grew with a similar doubling time; although, all the *gln3Δ* strains showed a slight increase in the doubling time compared to the parental strain. The doubling times obtained for parental and *gat1Δ* strains were similar in all the nitrogen sources used. In glutamine, *gln3Δ, ure2Δ, ure2Δ gat1Δ, ure2Δ gln3Δ, gat1Δ gln3Δ* and *ure2Δ gat1Δ gln3Δ* showed a mild increase in the doubling times compared to the parental strain (Table 1). When ammonium was used as the nitrogen source, *ure2Δ* (1.3 ± 0.02 h) and *ure2Δ gat1Δ* (1.4±0.04 h) growth were slightly

![Fig. 1](https://example.com/fig1.png)

**Fig. 1.** The parental strain of *C. glabrata* exhibits different growth phenotypes depending on the nitrogen source provided. The parental strain was grown on minimal media with the nitrogen source indicated (all at 1 mg ml⁻¹). Plates and cell cultures were incubated at 30 C. Minimal medium without a nitrogen source was used as a negative control (YNB). (a) Solid media assay. Tenfold serial dilutions (5 μl of each) were spotted, incubated and recorded at 48 h. (b) Liquid media assay. Cell cultures were grown in a Bioscreen C MBR. Doubling time was calculated based on the mid-log phase. Amm, ammonium; Gln, glutamine; Pro, proline; Gly, glycine; Asn, asparagine; Leu, leucine; Val, valine; Ile, isoleucine; Ade, adenine; Lys, lysine; Ura, uracil; YNB, yeast nitrogen base without nitrogen source. NG: non-growing culture. The reported doubling times (gray bars) represent the mean of three independent experiments ± standard deviations.

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Doubling time was statistically different from the parental strain (WT) grown in the same media. GAP1 (CAGL0L03267g; 597 amino acids) of C. glabrata exhibits 86% similarity to Gap1 (602 amino acids) of S. cerevisiae. Finally, the putative glutamine synthetase Gln1 (CAGL0K05357g; 372 amino acids) of C. glabrata showed 94% similarity to Gln1 (370 amino acids) of S. cerevisiae. Thus, given the synteny and the high overall sequence conservation of the Mep2, Gap1 and Gln1 proteins of C. glabrata and S. cerevisiae, we propose that these are the true orthologues in C. glabrata. Next, we decided to evaluate the effect of the nitrogen source on the gene expression of MEP2, GAP1 and GLN1 in C. glabrata. qRT-PCR experiments were performed in the parental strain grown in ammonium (1 mg ml⁻¹) as the sole nitrogen source, or after a 60 min shift from ammonium to proline (1 mg ml⁻¹) (Methods). As illustrated in Fig. 2, MEP2 and GAP1 gene expression was low in the presence of ammonium and increased after moving to proline (15-fold, approximately, for both genes). Additionally, GLN1 gene expression exhibits approximately a six fold increase after a shift to proline.

### Table 1. Doubling times of parental strain (WT), GLN1, GAT1 and URE2 mutant strains grown in different nitrogen sources

<table>
<thead>
<tr>
<th>Media</th>
<th>WT</th>
<th>gat1Δ</th>
<th>gln3Δ</th>
<th>ure2Δ</th>
<th>ure2Δ gat1Δ</th>
<th>ure2Δ gln3Δ</th>
<th>gat1Δ gln3Δ</th>
<th>ure2Δ gat1Δ gln3Δ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamine</td>
<td>1.3±0.09</td>
<td>1.3±0.07</td>
<td>1.6±0.05*</td>
<td>1.5±0.06*</td>
<td>1.6±0.04*</td>
<td>1.6±0.06*</td>
<td>1.5±0.02*</td>
<td>1.7±0.15*</td>
</tr>
<tr>
<td>Ammonium</td>
<td>1.1±0.06</td>
<td>1.1±0.04</td>
<td>3.0±0.09*</td>
<td>1.3±0.02*</td>
<td>1.4±0.04*</td>
<td>3.0±0.09*</td>
<td>3.1±0.14*</td>
<td>3.6±0.08*</td>
</tr>
<tr>
<td>Proline</td>
<td>1.8±0.18</td>
<td>1.7±0.07</td>
<td>2.5±0.12*</td>
<td>1.9±0.14</td>
<td>2.2±0.10*</td>
<td>2.7±0.16*</td>
<td>2.5±0.09*</td>
<td>3.2±0.09*</td>
</tr>
<tr>
<td>YPD</td>
<td>0.9±0.07</td>
<td>0.9±0.02</td>
<td>1.2±0.04*</td>
<td>0.9±0.03</td>
<td>1.0±0.03*</td>
<td>1.2±0.04*</td>
<td>1.3±0.06*</td>
<td>1.4±0.04*</td>
</tr>
</tbody>
</table>

*Doubling time was statistically different from the parental strain (WT) grown in the same media (P<0.05 in two-tailed t-test).
compared to the expression in the presence of ammonium. These results show that MEP2, GAP1 and GLN1 transcription levels are NCR regulated (low in ammonium and increase in proline).

We then determined whether Gat1, Gln3 and Ure2 have a role in MEP2, GAP1 and GLN1 gene expression. Fig. 3 shows that when cells were grown in ammonium, MEP2 and GAP1 expression was similar in a gat1Δ mutant compared to the parental strain. In ure2Δ and ure2Δ gat1Δ strains, the expression observed was not significantly different between strains, but there was a 3-fold increase in MEP2 and a 10-fold increase in GAP1 expression compared to the parental strain. MEP2 and GAP1 expression was also abolished in all the mutant strains that lacked Gln3 (Fig. 3a, c). These results show that even in ammonium where the relative expression of MEP2 and GAP1 is low, it

Fig. 3. Gln3 is a key regulator of MEP2, GAP1 and GLN1 gene expression. Gene expression was determined in total RNA from the C. glabrata cultures of the indicated strains, which were harvested during the mid-log phase on ammonium (gray bars) or after a 60 min shift from ammonium to proline (black bars). (a, b) MEP2 gene expression. (c, d) GAP1 gene expression. (e, f) GLN1 gene expression. Relative levels of gene expression were quantified by qRT-PCR. The ACT1 gene was used as a control for normalization. Values reported are the means of three independent experiments ± standard deviation. *Gene expression significantly different (P<0.05 in a two-tailed t-test) from that calculated for the parental strain (WT).
C. glabrata is still dependent on Gln3. Also, we observed that the absence of Ure2 increases the expression of these genes, and this increment is also completely dependent on the presence of Gln3.

After a shift to proline, MEP2 expression in the gat1Δ mutant strain was similar to that in the parental strain. A repression in MEP2 expression was observed in ure2Δ and ure2Δ gat1Δ mutant strains. These results suggest that Ure2 could retain a repressor of MEP2 in the cytoplasm. As observed in ammonium, in mutant strains that lacked Gln3, MEP2 expression was completely ablated after a shift to proline (Fig. 3b). In addition, transcription of GAP1 was similar in parental, gat1Δ, ure2Δ and ure2Δ gat1Δ strains (Fig. 3d). As observed for MEP2 expression, in all gln3Δ mutants tested, transcriptional regulation of GAP1 was decreased, indicating that Gln3 also has a main role for this gene in proline. Furthermore, when Gat1 was also deleted, GAP1 expression decreased even more (strain ure2Δ gat1Δ gln3Δ), suggesting that Gat1 has a minor role in GAP1 expression, only evident in the absence of Gln3 and with proline as the sole nitrogen source.

In ammonium, GLN1 gene expression was similar between the parental strain, gat1Δ and gln3Δ mutants. In the ure2Δ and ure2Δ gat1Δ mutant strains the transcription level was approximately 10-fold higher than in the parental strain (Fig. 3e). This level of expression was abolished in the absence of Gln3, suggesting that Ure2 prevents Gln3 from activating GLN1 gene transcription. In addition, an unknown transcriptional regulator could be acting on this gene, given that there is residual expression in ure2Δ gat1Δ gln3Δ, similar to the expression observed in the parental strain. After a shift to proline, the level of transcription of GLN1 was similar in the parental, gat1Δ, ure2Δ and ure2Δ gat1Δ strains and a decrease, which was statistically not significant, was observed in ure2Δ gln3Δ and ure2Δ gat1Δ gln3Δ (Fig. 3f). However, a small, but statistically significant, decrease was observed in the gln3Δ and gat1Δ gln3Δ strains suggesting that Gln3 has a role and, as observed in ammonium, is not the only transcriptional regulator of GLN1 gene expression when cells grow with proline as the sole nitrogen source.

**DISCUSSION**

In *S. cerevisiae*, ammonium, glutamine and asparagine are easily used as nitrogen sources (Messenguy *et al.*, 2006). *C. glabrata* can also grow rapidly in the presence of ammonium or glutamine. In contrast, we found that growth of *C. glabrata* in asparagine resembles its growth in proline, glycine, leucine and valine (Fig. 1b). Remarkably, *C. glabrata* is able to use glycine and adenine, while *S. cerevisiae* is unable to grow when these are the sole nitrogen sources. Finally, neither yeast can grow in uracil or lysine as the sole source of nitrogen (Fig. 1) (Andersen *et al.*, 2006; Brunke *et al.*, 2014; Gojković *et al.*, 1998).

*C. glabrata* possesses orthologous genes for the *S. cerevisiae* *GAT1*, *GLN3* and *URE2* genes. At the level of protein sequences, Gat1 and Gln3 share little identity with their orthologues in *S. cerevisiae*. In the case of Gat1, this identity is limited to the zinc finger. Gat1 has been described in many different species in the kingdom fungi, such as *N. crassa* (Nit2), *A. nidulans* (AreA), *C. neoformans* (Gat1) and *C. albicans* (Gat1) (Kmetzsch *et al.*, 2011; Lee *et al.*, 2011; Limjindaporn *et al.*, 2003; Marzluf, 1997). However, Gln3 appears to be present only in the class hemiascomycetes (Wong *et al.*, 2008). At the protein sequence level, Gln3 from *C. glabrata* shows conserved regions important for the function of the *S. cerevisiae* orthologue. These conserved regions are the activation domain, the GATA zinc finger, the nuclear export signal and the nuclear localization signal (Carvalho & Zheng, 2003; Kulkarni, 2001). Finally, the Ure2 protein has been found in diverse species of yeast, such as *Saccharomyces paradoxus*, *Saccharomyces bayanus*, *Kluyveromyces lactis*, *Ashbya gossypii* and *C. albicans*. Strikingly, Ure2 from these species of yeast can complement the absence of Ure2 in *S. cerevisiae* (Edskes & Wickner, 2002).

In *S. cerevisiae*, Ure2 retains Gat1 and Gln3 in the cytoplasm of the cell when glutamine or ammonium is available as a source of nitrogen (Cooper, 2002; Magasanik & Kaiser, 2002). A role for Ure2 as a regulator of amino acid transport and metabolic genes has been observed in *C. albicans* (Edskes *et al.*, 2011). Given the observed growth phenotype in the ure2Δ mutant strain when glutamine or ammonium are used as nitrogen sources (Table 1), we propose that *C. glabrata* Ure2 plays a regulatory role, similar to that observed in *S. cerevisiae*, in nitrogen assimilation. In contrast, the role of Gat1 in nitrogen assimilation is only evident in the absence of Ure2 and Gln3. In addition, a major role of Gln3 is evident as its absence increases doubling time independently of the nitrogen source provided in the media. In *S. cerevisiae*, the absence of either Gat1 or Gln3 increases the doubling time in different nitrogen sources and it is more evident in the double mutant gat1Δ gln3Δ (Stanbrough *et al.*, 1995). The role of Gat1 has been studied in the fungi *A. nidulans* and *N. crassa*, where mutant strains in Gat1 (AreA and Nit2, respectively) can only grow in media with glutamine or ammonium (Arst & Cove, 1973; Marzluf, 1997). In *C. albicans*, the absence of Gln3 affects cell growth when glutamine or ammonium, but not proline, are used as nitrogen sources (Liao *et al.*, 2008). Remarkably, in *C. glabrata* the regulation exerted mainly by Gln3 in all the nitrogen sources tested differs significantly from what has been observed in *S. cerevisiae* (absence of Gln3, Gat1 or both GATA factors affect growth), *C. albicans* (only absence of Gln3 affects growth in glutamine or ammonium), *A. nidulans* and *N. crassa* (Gat1 mutant is only able to grow in glutamine or ammonium).

In *S. cerevisiae*, ammonium transport is given by a family of transporters encoded by *MEP1*, *MEP2* and *MEP3* (Marini *et al.*, 1997). In addition, most of the 14-amino acids are transported through the general amino acid permease,
encoded by GAP1 (Jauniaux & Grenson, 1990; Regenberg et al., 1999). Glutamine synthetase (EC 6.3.1.2) encoded by GLN1, catalyzes the condensation of ammonium and glutamate to yield glutamine (Mitchell, 1985). As we have shown, C. glabrata possesses orthologous genes to MEP2, GAP1 and GLN1. Orthologous genes to two of the three ammonium transporters (MEP1 and MEP2) and the general amino acid permease (GAP1) have also been found in C. albicans (Biswas & Morschhäuser, 2005; Kraidlova et al., 2011). In addition, C. albicans possesses a putative GLN1
orthologue gene; its function has not yet been demonstrated.

Here, we have shown that as in S. cerevisiae, MEP2, GAP1 and GLN1 gene expression in C. glabrata is under nitrogen repression (i.e. low in ammonium and induced in proline) (Figs 2 and 3) (Janiaux & Grenson, 1990; Marini et al., 1997; Stanbrough et al., 1995). Ammonium repression of MEP2 and GAP1 has also been observed in C. albicans (Liao et al., 2008; Limjindaporn et al., 2003). In agreement with the low expression of genes related to transporters and nitrogen metabolism observed in ammonium, absence of Gln3 appeared to affect ammonium uptake slightly at a low ammonium concentration (0.5 mg ml$^{-1}$) and at early time points (1 and 2 h), when the cell mass in the parental and gln3 strains did not change greatly (Fig. S1). However, more experimentation is needed to demonstrate fully whether Gln3 has a role in the regulation of ammonium transport in C. glabrata.

C. glabrata MEP2 gene expression in ammonium and proline was dependent on Gln3, but not Gat1 (Fig. 3a, b), which is different to what has been observed in S. cerevisiae and in C. albicans, where MEP2 expression completely depends on both GATA factors (Dabas & Morschhäuser, 2007; Marini et al., 1997).

Our results for GAP1 gene expression in ammonium and proline place Gln3 as the main regulator, with a modest role for Gat1 in proline (Fig. 3c, d). This regulation is different to that previously observed in S. cerevisiae where both transcription factors are necessary for complete GAP1 gene expression in both nitrogen sources (Soussi-Boudekou & Andre, 1999; Stanbrough et al., 1995). To our knowledge, the roles of Gat1 and Gln3 in GAP1 gene expression in ammonium for C. albicans have not been assessed, but in proline both GATA factors, Gat1 and Gln3, are necessary for full GAP1 gene expression (Liao et al., 2008).

In S. cerevisiae, GLN1 gene expression is dependent on Gat1 and Gln3 when cells are grown in both ammonium and proline (Stanbrough et al., 1995). In contrast, here we show that in C. glabrata, GLN1 regulation was Gat1 independent, Gln3 dependent and driven by an additional unknown transcription factor (Fig. 3e, f).

The overexpression of MEP2, GAP1 and GLN1 in ammonium observed in the absence of Ure2 suggests that in C. glabrata Ure2 acts as a negative regulator (Fig. 3a, c, e), similarly to what has been observed in S. cerevisiae. Overexpression of these genes in the absence of Ure2 has been observed previously in S. cerevisiae and C. albicans (Edskes et al., 2011). However in C. glabrata, a negative effect was not found of Ure2 on MEP2 expression (Edskes et al., 2011). This result contrasts with our own observations, which could be due to differences in the growth media used; we used minimal media with ammonium as the sole nitrogen source and the experiments performed by Edskes et al. (2011) were performed in YPD. Another possibility is that differences were possibly due to strain differences.

We identified C. glabrata orthologous genes encoding the GATA factors, GAT1 and GLN3, and URE2 of S. cerevisiae and showed that, as in S. cerevisiae, the ammonium assimilation related genes, MEP2, GAP1 and GLN1, are under NCR. However, important differences should be highlighted with respect to the role of GATA factors in C. glabrata in nitrogen assimilation, compared to what is known in S. cerevisiae: (i) Gln3 is absolutely necessary and Gat1 is dispensable for MEP2 gene expression in ammonium and proline, (ii) GAP1 transcriptional regulation is largely dependent of Gln3 with a modest role of Gat1 in proline and (iii) GLN1 expression is Gln3 dependent, Gat1 independent and appeared to be regulated by an unknown transcriptional factor (Fig. 4).

Hence, the understanding of the molecular mechanisms governing nitrogen assimilation in C. glabrata, and the identification of the set of genes regulated by Gat1 and Gln3 in different nitrogen conditions, could lead to the identification of species-specific regulatory networks controlling nitrogen sensing and uptake in this pathogenic yeast.

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REFERENCES


Cooper, T. G. (2002). Transmitting the signal of excess nitrogen in Saccharomyces cerevisiae from the Tor proteins to the GATA factors: connecting the dots. FEMS Microbiol Rev 26, 223–238.


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