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Glutathione biosynthesis in the yeast pathogens *Candida glabrata* and *Candida albicans*: essential in *C. glabrata*, and essential for virulence in *C. albicans*

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Redox pathways play a key role in pathogenesis. Glutathione, a central molecule in redox homeostasis in yeasts, is an essential metabolite, but its requirements can be met either from endogenous biosynthesis or from the extracellular milieu. In this report we have examined the importance of glutathione biosynthesis in two major human opportunistic fungal pathogens, *Candida albicans* and *Candida glabrata*. As the genome sequence of *C. glabrata* had suggested the absence of glutathione transporters, we initially investigated exogenous glutathione utilization in *C. glabrata* by disruption of the *MET15* gene, involved in methionine biosynthesis. We observed an organic sulphur auxotrophy in a *C. glabrata met15*Δ strain; however, unlike its *Saccharomyces cerevisiae* counterpart, the *C. glabrata met15*Δ strain was unable to grow on exogenous glutathione. This inability to grow on exogenous glutathione was demonstrated to be due to the lack of a functional glutathione transporter, despite the presence of a functional glutathione degradation machinery (the Dug pathway). In the absence of the ability to obtain glutathione from the extracellular medium, we examined and could demonstrate that γ-glutamyl cysteine synthase, the first enzyme of glutathione biosynthesis, was essential in *C. glabrata*. Further, although γ-glutamyl cysteine synthase has been reported to be non-essential in *C. albicans*, we report here for what is believed to be the first time that the enzyme is required for survival in human macrophages *in vitro*, as well as for virulence in a murine model of disseminated candidiasis. The essentiality of γ-glutamyl cysteine synthase in *C. glabrata*, and its essentiality for virulence in *C. albicans*, make the enzyme a strong candidate for antifungal development.

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INTRODUCTION

Glutathione, γ-Glu-Cys-Gly, is an essential metabolite in almost all eukaryotic organisms (Fahey & Sundquist, 1991; Meister & Anderson, 1983), and plays a key role in redox homeostasis and in the cellular response to oxidative stress (Meister & Anderson, 1983; Penninckx, 2002; Sipos *et al.*, 2002). The importance of oxidative stress responses in the virulence and survival of pathogens in their natural environment has been suggested by many studies, but the relative importance of the glutathione-dependent redox pathway, as opposed to other pathways for redox homeostasis and oxidative stress, has not been rigorously evaluated. In parasitic protozoans such as *Leishmania infantum* and *Trypansoma brucei*, however, glutathione biosynthesis (which leads to trypanothione synthesis), is essential (Huynh *et al.*, 2003; Mukherjee *et al.*, 2009), while in the malarial parasite *Plasmodium berghei*, glutathione biosynthesis has been shown to be essential for the survival of the protozoan during its passage through insects (Vega-Rodríguez *et al.*, 2009). Further, in *L. infantum*, even the deletion of one of the two copies of the *GSH1* gene is not tolerated by the organism. These findings describing the essentiality of glutathione biosynthesis in these pathogens prompted us to investigate the importance, if any, of

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Abbreviations: FOA, 5-fluoroorotic acid; ROS, reactive oxygen species; UTR, untranslated region.

A supplementary figure, showing the growth of *C. glabrata* wild-type (ABG2367) and *met15*Δ (ABG2370) strains on inorganic sulphate and methionine, and a supplementary table, listing the primers used in this study, are available with the online version of this paper.

glutathione biosynthesis in the survival of yeast pathogens in their mammalian host. *Candida albicans* and *Candida glabrata* are the two most important yeast pathogens that cause bloodstream infections (Fidel *et al.*, 1999; Pfaller *et al.*, 2010; Wingard, 1995). Although *C. glabrata* is phylogenetically much closer to *Saccharomyces cerevisiae* than to *C. albicans*, both are human commensals and are rarely seen in the environment outside the human host, where their ecological niche is the vaginal mucosa, skin and blood (Fidel *et al.*, 1999; Kaur *et al.*, 2005).

Work with the yeasts *S. cerevisiae* and *Schizosaccharomyces pombe* have shown that yeasts have the ability to obtain their glutathione requirements from both endogenous biosynthesis and the extracellular medium (Bourbouloux *et al.*, 2000; Thakur *et al.*, 2008). Disruption of glutathione biosynthesis in the yeasts *Schizosaccharomyces pombe* and *S. cerevisiae*, carried out by knocking out the first enzyme of glutathione biosynthesis, γ-glutamyl cysteine synthase (*GSH1 or GCS1*), leads to glutathione auxotrophy, in which the cells become dependent for growth on exogenous glutathione, the uptake of which is mediated by high-affinity glutathione transporters. Furthermore, in *S. cerevisiae*, the exogenous glutathione can also be utilized as a sulphur source, and this utilization depends on the presence of the glutathione transporter as well as on an alternative pathway of glutathione degradation that involves the 'Dug pathway complex', comprising the three proteins Dug1p, Dug2p and Dug3p (Ganguli *et al.*, 2007).

In *C. albicans*, disruption of glutathione biosynthesis has been shown to cause glutathione auxotrophy similar to that observed in *S. cerevisiae* and *Schizosaccharomyces pombe*, and the disruptant displays higher reactive oxygen species (ROS) levels and undergoes apoptosis (Baek *et al.*, 2004). In *C. glabrata*, the genome sequence has revealed that a large number of ORFs have been lost in comparison with its close relative *S. cerevisiae* (De Hertogh *et al.*, 2006; Dujon *et al.*, 2004), and among the ORFs that appear to be absent are homologues of members of the oligopeptide transporter family, to which the yeast high-affinity glutathione transporters (*HGT1* of *S. cerevisiae* and *PGT1* of *Schizosaccharomyces pombe* and others) belong. It was relevant, therefore, first to investigate the importance of glutathione biosynthesis and assimilation in *C. glabrata*. We demonstrate in this study that *C. glabrata* lacks the ability to utilize exogenously provided glutathione, owing to the lack of a glutathione transporter, despite possessing a functional Dug complex for the degradation of glutathione. We also demonstrate that in *C. glabrata*, γ-glutamyl cysteine synthase (*GSH1*) is an essential enzyme, and that in *C. albicans*, despite the ability of *Cagcs1*Δ strains to grow in the presence of exogenous glutathione, *CaGCS1* is essential for virulence. The demonstration of the essentiality of γ-glutamyl cysteine synthase for the virulence of these yeast pathogens makes this enzyme an attractive target for antifungal therapy.

METHODS

Materials. All chemicals and reagents were of analytical reagent grade and were procured from different commercial sources. Oligonucleotide primers were synthesized by Sigma-Genosys, India. Medium components were purchased from BD (Difco). Restriction enzymes, DNA polymerases and other DNA-modifying enzymes were obtained from New England Biolabs. Gel extraction kits and plasmid miniprep columns were obtained from Qiagen.

Strains, media and growth conditions. The *Escherichia coli* strain DH5α was used as a cloning host. *S. cerevisiae*, *C. glabrata* and *C. albicans* strains used in the study are described in Table 1. *S. cerevisiae* and *C. glabrata* strains were regularly maintained on yeast extract peptone dextrose (YPD) medium. Synthetic defined minimal medium contained yeast nitrogen base, ammonium sulphate and glucose, supplemented with methionine, cysteine, glutathione, homocysteine, histidine, leucine, lysine (as per requirement) and uracil at 50 mg l^{-1}. Yeast transformations were carried out using the modified lithium acetate method, as described for *C. glabrata* (Gietz *et al.*, 1992), and for *C. albicans* using electroporation (Reuss *et al.*, 2004).

Cloning of *CgMET15*. The *CgMET15* (*CAGL0D06402g*) ORF along with its 5′ untranslated region (5′ UTR) (600 bp) was PCR-amplified from the genomic DNA of *C. glabrata* using primers CgMET15-600F and CgMET15R, yielding a 2 kb product with an *Sma*I site. This PCR product was then digested with *Sma*I and cloned into pGRB2.2 to yield plasmid pGRB2.2-CgMET15.

Subcloning of *ScGSH1* and *HGT1* from *S. cerevisiae* into *C. glabrata* expression vector pGRB2.2. Plasmid p416TEF-ScGSH1 (Sharma *et al.*, 2000) was digested with *Bsp*DI and *Hin*dIII to release the 2 kb insert from vector backbone of p416TEF (5.5 kb). Subsequently, this insert of 2 kb was blunted and then ligated at the *Sma*I site of pGRB2.2 to yield plasmid pGRB2.2-ScGSH1. Plasmid p416TEF-ScHGT1 (Kaur *et al.*, 2009) was digested with *Bam*HI and *Eco*R1 to release the 2.4 kb insert from the vector backbone of p416TEF (5.5 kb). Subsequently, this insert was cloned into pGRB2.2 at the same sites (*Bam*HI and *Eco*RI) to yield plasmid pGRB2.2-ScHGT1.

Construction of strains

Construction of the Cgmet15Δ strain. The *CgMET15* gene was disrupted in the wild-type background by homologous recombination. Briefly, 850 bp of the 5′ UTR of *MET15* was PCR-amplified from wild-type genomic DNA using primer pair OgRK17 and OgRK18, and digested and cloned upstream of the hygromycin-resistance gene (*hph*) (under the PGK1 promoter) in the *Kpn*I and *Hin*dIII sites of a plasmid containing the *URA3* gene as a selection marker (plasmid pRK9). Next, the 3′ UTR of *MET15* (about 750 bp) was amplified with primers OgRK19 and OgRK20 from wild-type genomic DNA, digested with *Spe*I and *Sac*I, and cloned downstream of the *hph* gene in the *Spe*I/*Sac*I sites of pRK9, so that the *hph* gene was flanked by the 5′ and 3′ UTRs of *MET15* (plasmid pRK13). For a one-step replacement strategy, plasmid pRK13 was digested with *Bcg*1 (sites engineered during primer design), the linear fragment carrying the HYG cassette (*hph* gene flanked by the 5′ and 3′ UTRs of *MET15*) was transformed into wild-type strains, and transformants were selected for hygromycin resistance. Hygromycin-resistant transformants were then screened for methionine auxotrophy. Replacement of the *MET15* ORF with the *hph* gene was verified by PCR using a primer external to the cloned fragment [at both the 5′ (OgRK21) and 3′ (OgRK22) ends] and a primer that annealed within the plasmid (OgRK45 and OgRK46). In addition, the lack of amplification with primers (OgRK23 and OgRK24) that annealed to the region within

Table 1. List of strains and plasmids used in this study

Strain or plasmid	Derived from strain:	Genotype or description	Source or reference
Strains			
ABC733 (BY4741)		*S. cerevisiae MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Euroscarf
ABG2367 (Cg462)		*C. glabrata* wild-type strain	Laboratory of R. Kaur
ABG2370 (YRK4)	ABG2367	*C. glabrata met15Δ::hph* Hyg^R *ura3Δ::*Tn*903* $G418^R$	Laboratory of R. Kaur
ABG2703	ABG2370	*C. glabrata met15Δ::hph* Hyg^R *ura3Δ::*Tn*903* $G418^R$ *dug3Δ::hisG-URA3-hisG*	This study
ABG2704	ABG2703	*C. glabrata met15Δ::hph* Hyg^R *ura3Δ::*Tn*903* $G418^R$ *dug3Δ::hisG*	This study
ABG2810	ABG2370	*C. glabrata met15Δ::hph* Hyg^R *ura3Δ::*Tn*903* $G418^R$ *gsh1Δ::CgMET15* + plasmid pGRB2.2-ScGSH1	This study
ABA2240 (Sc 5314)		*C. albicans* wild-type strain	Laboratory of K. Ganesan
ABA2772 (YB204)		*C. albicans ura3-iro1Δ::imm434/ura3-iro1Δ::imm434 Δgcs1::hisG/Δgcs1::hph*	Dr S. O. Kang
ABA2878	ABA2772	*C. albicans ura3-iro1Δ::URA3-IRO1/ura3-iro1Δ::imm434 Δgcs1::hisG/Δgcs1::hph*	This study
ABA2993	ABA2878	*C. albicans ura3-iro1Δ::URA3-IRO1/ura3-iro1Δ::imm434 Δgcs1::GCS1/Δgcs1::hph*	This study
Plasmids			
p416TEF		CEN-vector bearing a *URA3* marker and TEF-promoter-MCS-terminator for *S. cerevisiae* expression and Amp^r marker for selection in *E. coli*	Mumberg *et al.* (1995)
pGRB2.2		CEN-vector bearing *URA3* marker and PGK1-promoter-MCS-terminator for *C. glabrata* expression and Amp^r marker for selection in *E. coli*	Laboratory of R. Kaur
pBSK		*E. coli* expression vector (Stratagene)	
p416TEF-ScGSH1		*S. cerevisiae GSH1* gene cloned in *Bsp*DI and *Hin*dIII sites of p416TEF	Sharma *et al.* (2000)
p416TEF-ScHGT1		*S. cerevisiae HGT1* gene cloned in *Bam*HI and *Eco*RI sites of p416TEF	Kaur *et al.* (2009)
pGRB2.2-ScHGT1		*S. cerevisiae HGT1* gene cloned in *Bam*HI and *Eco*RI sites of pGRB2.2	This study
pGRB2.2-ScGSH1		*S. cerevisiae GSH1* gene cloned in *Sma*I site of pGRB2.2	This study
pGRB2.2-CgMET15		*C. glabrata MET15* gene along with its promoter (600 bp) cloned in *Sma*I site of pGRB2.2	This study
pAP599		Plasmid containing *hph* expression cassette for hygromycin resistance (Hyg^R) under the PGK1 promoter flanked by FRT sites followed by the MCS region and *URA3* as a selection marker	Laboratory of R. Kaur
pRK9		5′ UTR of *MET15* (850 bp) cloned in *Kpn*I and *Hin*dIII sites of plasmid pAP599	This study
pRK13		3′ UTR of *MET15* (750 bp) cloned in *Spe*I and *Sac*I sites of plasmid pRK9 so that the *hph* gene is flanked by the 5′ and 3′ UTRs of *MET15*	This study
pBSK-CgDug3		*CgDUG3* along with its 5′ UTR (593 bp) and 3′ UTR (1017 bp) cloned in *Eco*RV and *Kpn*I sites of pBSK	This study
pBSK-CgDUG3ΔURA3:HisG-URA3-HisG		Plasmid pBSK-CgDug3 digested with *Sna*BI and *Eco*NI followed by blunting and ligation with the hisG-URA3-hisG cassette	This study
PHUKH3		Plasmid containing hisG-URA3-hisG cassette.	Earley & Crouse (1996)
pBSK-CgGSH1		*CgGSH1* along with its 5′ UTR (629p) cloned in *Xba*I and *Xho*I sites of pBSK	This study
pBSK-Cggsh1Δ:CgMET15		Plasmid pBSK-CgGSH1 digested with *Sma*I, followed by ligation with *CgMET15* (released from plasmid pGRB2.2-*CgMET15* using *Sma*I)	This study

the ORF of *MET15* was taken as final evidence of the disruption of *MET15* with the *hph* gene.

Construction of Cgdug3Δ. *CAGL0J11484g*, the orthologue of *ScDUG3*, was PCR-amplified from the genomic DNA of *C. glabrata* using primers CgDug3F and CgDug3R, yielding a 2.7 kb fragment that was then digested with *Eco*RV and *Kpn*I, and ligated to an *Eco*RV- and *Kpn*I-digested pBSK vector to generate plasmid pBSK-CgDug3. pBSK-CgDug3 was digested with *Sna*BI and *Eco*NI, blunted, and ligated with the hisG-URA3-hisG cassette [obtained from plasmid PHUKH3 (Earley & Crouse, 1996) by *Pvu*II digestion], to yield plasmid pBSK-CgDug3Δ:HisG-URA3-HisG. The disruption cassette was then released with *Afl*II and *Nhe*I and transformed into a *C. glabrata met15Δura3Δ* background, and transformants were selected on minimal medium lacking uracil and containing methionine as the sulphur source. Screening was done using diagnostic PCR. To remove the URA marker from the disruptant ABG2703 (*Cgmet15Δura3Δdug3Δ::*hisG-URA3-hisG), 5-fluoroorotic acid (FOA) selection was performed by

using 0.1 % FOA and selecting transformants on minimal medium containing uracil (1.2 mg 100 ml^{-1}) and methionine to yield strain ABG2704 (*Cgura3*Δ*Cgdug3*Δ : *hisG*).

Construction of Cggsh1Δ : CgMET15. *CgGSH1* (*CAGL0L03630g*) was PCR-amplified using primers CgGSH1DF and CgGSH1DR to yield a 3 kb fragment that was digested with *Xba*I and *Xho*I and cloned into pBSK to yield plasmid pBSK-CgGSH1. pBSK-GSH1 was then digested with *Sma*I, and the *CgMET15* ORF with its own promoter was inserted into this plasmid to generate plasmid pBSKCggsh1Δ : CgMET15. Before disrupting *CgGSH1* in a *C. glabrata met15*Δ background, *S. cerevisiae GSH1* on plasmid pGRB2.2 (pGRB2.2-ScGSH1) was transformed in a *C. glabrata met15*Δ background, and transformants were selected on minimal medium lacking uracil and containing methionine. A *Cgmet15*Δ strain carrying the pGRB2.2-ScGSH1 plasmid was then used as the host strain to create a *CgGSH1* disruption using the Cggsh1Δ : CgMET15 disruption cassette that was excised from pBSK-Cggsh1Δ : CgMET15 using *Sna*BI and *Xho*I. Transformants were selected on minimal medium lacking uracil and any organic sulphur source except ammonium sulphate for the selection of the marker *CgMET15*. Screening for disruptants was done using diagnostic PCR.

Reintegration of the *URA3* and *IRO1* genes in the ABA2772 (*ura3*Δ *iro1*Δ *gcs1*Δ) strain of *C. albicans*. In order to study the virulence of the *C. albicans gcs1*Δ strain it was essential to reintegrate the *URA3* and *IRO1* genes in their native locus. Primers CaURA1134F and CaURA3792R were used to obtain a PCR product of 2.6 kb, which was then transformed in strain ABA2772 (*ura3*Δ *iro1*Δ *gcs1*Δ) by electroporation (Reuss *et al.*, 2004). Transformants were selected on minimal medium supplemented with 1 mM glutathione and lacking uracil. Diagnostic PCR was carried out with the transformants to check the locus of the reintegrated *URA3* and *IRO1* genes using a primer upstream of the reintegration cassette (CaURA1041F) and one specific to the reintegration cassette (CaURA3792R) to obtain strain ABA2878 ($CaURA3^{+}$ IRO^{+} *gcs1*Δ).

Reintegration of CaGCS1. Strain ABA2878 was used for the reintegration of *CaGCS1*. The *CaGCS1* ORF (orf19.5059) was PCR-amplified from the genomic DNA of *C. albicans* using primers CaGSH1F and CaGSH1R, and transformed into strain ABA2878, and subsequently the transformants were selected on medium lacking glutathione to obtain strain ABA2993 ($CaURA3^{+}$ IRO^{+} $GCS1^{+}$). Diagnostic PCR was also done to confirm the reintegration of *GCS1*.

Mouse virulence assay. All animal experiments were approved by the institutional animal ethics committee of the Institute of Microbial Technology (IMTECH). Male BALB/c mice 6–8 weeks of age were used for the survival experiment and were obtained from the IMTECH animal house. To investigate the virulence of the *Cagcs1*Δ strain in comparison with the *C. albicans* wild-type strain, both strains were grown in minimal medium supplemented with 1 mM glutathione for 16 h, followed by washing both strains with 1× PBS and finally resuspending them in the same buffer. To correlate the number of cells to 1 OD_{600} unit, haemocytometer cell counting was done. Mice were individually injected intravenously with 5×10^5 cells of *C. albicans* wild-type and *gcs1*Δ and then observed for survival. All mouse experiments were carried out twice with a set of seven to 10 mice per strain in each experiment.

Growth assays by dilution spotting. For dilution spotting assays, the different strains were grown overnight in minimal medium with nutrient supplements added as required, and were reinoculated into fresh medium to OD_{600} 0.1 and grown for 6 h. The exponential phase cells were harvested, washed with water and resuspended in water to OD_{600} 0.2. They were then serially diluted to 1 : 10, 1 : 100 and 1 : 1000. Of these cell suspensions, 10 µl of each dilution was spotted onto the appropriate plates. Plates were incubated for 2 days and photographed.

End-point dilution survival assay. THP1 (human monocyte) cells were treated with 16 nM phorbol 12-myristate 13-acetate (PMA) to differentiate them to macrophages and seeded at 2×10^5 cells per well in 96-well flat-bottomed tissue culture plates. Cells were incubated at 37 °C under 5 % CO_2, and RPMI medium was replaced with fresh medium after 12 h of PMA treatment. Overnight cultures of *C. albicans* wild-type and *gcs1*Δ mutant, grown in either YPD or YPD + 1 mM glutathione, were collected, washed with PBS and vortexed to disrupt any cell clumps. The OD_{600} of *C. albicans* cell suspensions was taken, followed by diluting the cell suspension to 2×10^6 cells ml^{-1} in PBS. A 200 µl volume of the suspension was added to two wells of a 96-well plate and serial fourfold dilutions were made in PBS. To infect THP1 cells at an m.o.i. of 1 : 4, 25 µl of the original cell suspension (2×10^6 cells ml^{-1}) was added to wells containing differentiated THP1 cells (2×10^5 cells) in 150 µl RPMI medium. As a control, an equal number of cells was incubated in RPMI medium containing either no glutathione or 1 mM glutathione. A total of five different m.o.i. values, 1 : 4, 1 : 16, 1 : 64, 1 : 256 and 1 : 1024, were used for the end-point dilution survival assay, which involved counting *C. albicans* colonies from the THP1 seeded wells where they could be visualized and comparing that number with the number of colonies that appeared in the wells of the same dilution in the absence of macrophages. After 90 min co-incubation with macrophage cells, extracellular yeasts were removed by three PBS washes followed by the addition of either RPMI medium or RPMI medium supplemented with 1 mM glutathione. The co-culturing of macrophages and *C. albicans* cells was continued for an additional 24 h, and the total number of *C. albicans* colonies at different m.o.i. values that appeared in wells seeded with or without THP1 cells was counted under the microscope. *C. albicans* survival was quantified by dividing the number of colonies formed in the presence of THP1 cells by the number of colonies formed in RPMI medium, and was expressed as percentage growth inhibition. The end-point survival assays were carried out twice, and each experiment was done in triplicate.

Macrophage viability assay. Macrophage viability was determined after 24 h by the trypan blue staining method. Briefly, at various time points post-infection of THP1 cells, supernatant was aspirated from the wells and 40 µl trypan blue (0.2 %) was added to wells. After 3–5 min incubation at room temperature, trypan blue was removed from the wells, and cells were washed twice with PBS and observed under the microscope. At 8 and 24 h post-infection, a minimum of 100 macrophages was counted to determine the presence of live (unstained) and dead (blue) cells.

RESULTS

The *C. glabrata met15*Δ strain is a strict organic sulphur auxotroph that can utilize cysteine and methionine but not glutathione

To gain insights into glutathione utilization by *C. glabrata*, we created a disruption of the *CgMET15* (*CAGL0D06402g*) gene. The *MET15* gene encodes *O*-acetyl homoserine thiolase (OAHSH), and disruption of this enzyme in *S. cerevisiae* leads to strict organic sulphur auxotrophy, which has greatly facilitated the study of organic sulphur utilization in this yeast. A similar disruption made in *C. albicans*, however, revealed that in *C. albicans*, the

disruptants have a severe (but not total) growth defect on inorganic sulphate (Viaene *et al.*, 2000), suggesting that a secondary pathway of sulphate assimilation through cysteine synthase (OASSH) might be present in this yeast.

On examination of the *C. glabrata met15*Δ strain, it was observed that it was unable to grow on inorganic sulphate, and like its *S. cerevisiae* counterpart, was a strict organic sulphur auxotroph (Supplementary Fig. S1). Furthermore, *C. glabrata met15*Δ could utilize either cysteine or methionine as a sulphur source (Fig. 2), suggesting the presence of both forward and reverse transulphuration pathways in this yeast (Fig. 1). However, when we tested the growth of the *C. glabrata met15*Δ strain on glutathione as a sulphur source, it was found that the strain could not utilize glutathione as a sulphur source (Fig. 2). This was quite distinct from the behaviour of *S. cerevisiae met15*Δ strains, which can efficiently utilize glutathione as a sulphur source.

C. glabrata lacks a glutathione transporter but retains a functional Dug pathway required for glutathione utilization

Glutathione utilization in *S. cerevisiae* has been shown to require transport through a high-affinity glutathione transporter (Hgt1p), followed by its degradation through the Dug complex pathway for glutathione degradation (involving Dug1p, Dug2p and Dug3p) (Bourbouloux *et al.*, 2000; Ganguli *et al.*, 2007). Putative orthologues of the Dug complex proteins (Dug1p, Dug2p and Dug3p) can be identified in *C. glabrata* by *in silico* analysis. Earlier comparative studies of transporters from different yeasts had already indicated that *C. glabrata* lacks any member of the oligopeptide transporter family to which Hgt1p belongs (Dujon *et al.*, 2004). Our analysis also confirmed that even remote homologues of the glutathione transporter Hgt1p are not found in this yeast. This is in contrast to other yeasts, which contain at least two to three members of this family. The possible absence of a glutathione transporter correlated well with the inability of a *Cgmet15*Δ strain to grow on glutathione; however, the presence of the glutathione degradation machinery was intriguing, considering the fact that this complex has been presumed to have evolved for the utilization of exogenous glutathione.

These observations needed further investigation. To examine therefore whether the inability of the *C. glabrata met15*Δ strain to utilize glutathione as a sulphur source was a consequence of glutathione not being transported inside the cell in the absence of an Hgt1p orthologue, we expressed the *S. cerevisiae* glutathione transporter Hgt1p in *C. glabrata met15*Δ strains and examined the transformants for their ability to grow on glutathione. Interestingly, the presence of Hgt1p enabled *C. glabrata* to utilize glutathione as a sulphur source (Fig. 3). This indicates that *C. glabrata* contains a functional glutathione utilization pathway, except for the glutathione transporter. To see whether this utilization of glutathione occurred through the Dug complex, which we have previously shown to have evolved for the utilization of exogenous glutathione, we disrupted the *CgDUG3* gene (*CAGL0J11484g*), which encodes one of the proteins of the Dug complex. On deleting *CgDUG3* it was found that *C. glabrata* failed to utilize exogenous glutathione, implying that the Dug complex is indeed functional in *C. glabrata* (Fig. 3). The presence of a functional Dug complex in *C. glabrata* suggests that apart

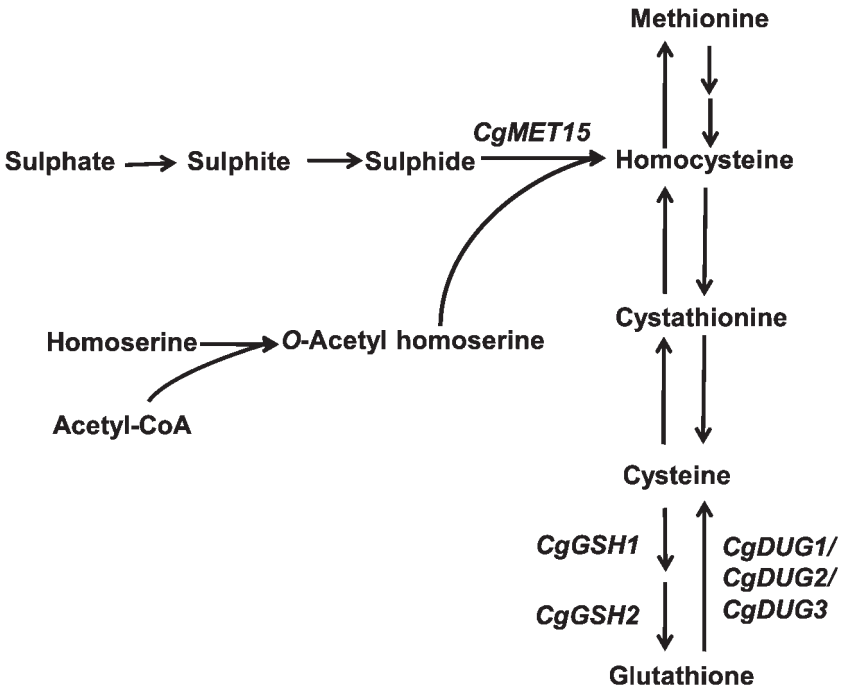


Fig. 1. Schematic showing the sulphur assimilatory pathways in *C. glabrata*.

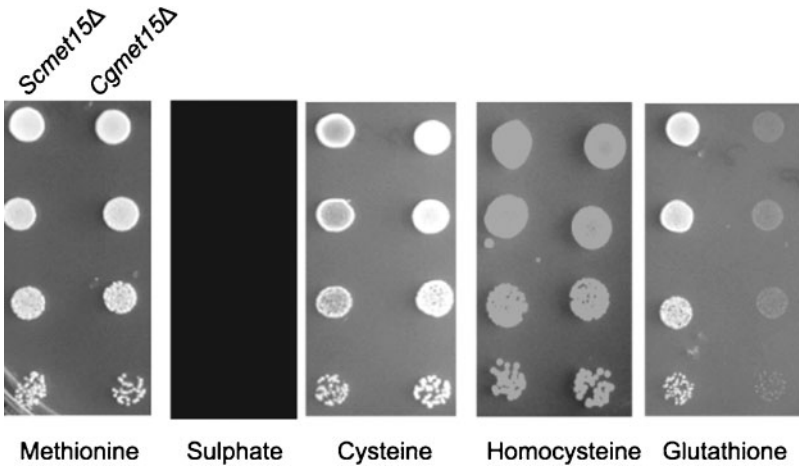


Fig. 2. Growth of *C. glabrata met15*Δ (ABG2370) and *S. cerevisiae met15*Δ (ABC733) strains on different sulphur sources. The *met15*Δ strains were grown in minimal medium (containing leucine, histidine, uracil and methionine), and cells were harvested, washed and resuspended to OD_{600} 0.2. Serial dilutions to OD_{600} 0.2, 0.02, 0.002 and 0.0002 were made, and 10 μl of each dilution was spotted onto minimal medium containing 200 μM methionine, ammonium sulphate (Sulphate), cysteine, homocysteine or glutathione as the sulphur source. Plates were photographed after 2 days.

from exogenous glutathione utilization, the Dug complex is also involved in intracellular glutathione homeostasis.

GSH1, γ-glutamyl cysteine synthase, is an essential enzyme in *C. glabrata*

As glutathione is an essential metabolite for the growth of eukaryotic cells, the absence of a glutathione transporter in *C. glabrata* suggested that the enzyme γ-glutamyl cysteine synthase (*GSHI*) might be essential in this yeast. However, the possibility also existed that glutathione was not an essential metabolite in this yeast, as is the case in *E. coli* (Murata & Kimura, 1982). To examine these possibilities, we created a knockout of the *C. glabrata GSH1* gene using *CgMET15* as a marker in cells harbouring a copy of the *S. cerevisiae GSH1* gene (*CAGL0L03630g*) on a *URA3* plasmid. The essentiality of the *CgGSH1* gene was then examined by removing the *ScGSH1*-containing plasmid using FOA (Fig. 4). Even in the presence of 100 μM glutathione, the cells lacking *ScGSH1* could not grow, indicating that *CgGSH1* is essential in *C. glabrata* (data not shown). At significantly higher concentrations of glutathione, however, we did observe some growth upon prolonged incubation of *C. glabrata* strains carrying a knockout of the *CgGSH1* gene, indicating that at high concentrations of glutathione alone, small amounts are taken up by *C. glabrata* cells, allowing their growth (data not shown).

The *C. albicans gcs1*Δ strain shows decreased growth rates and eventual growth stasis upon glutathione depletion

The essentiality of glutathione biosynthesis in *C. glabrata* prompted us to re-examine the importance of glutathione

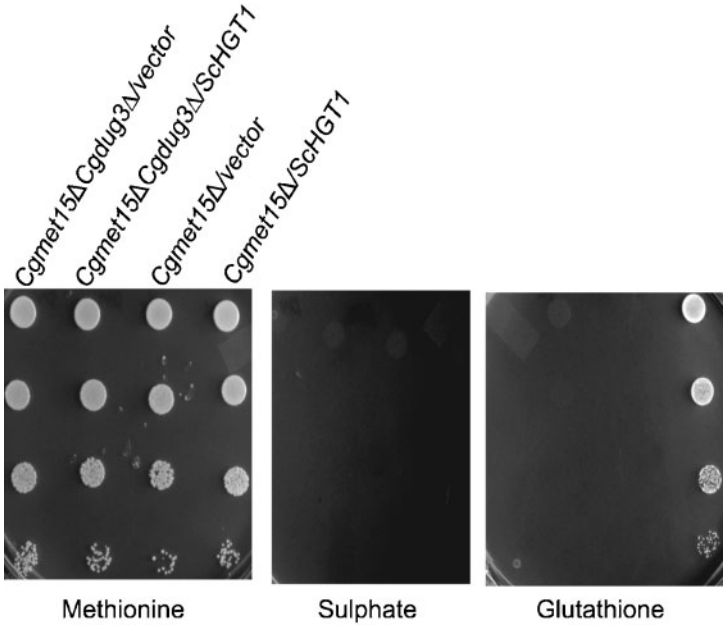


Fig. 3. Expression of a glutathione transporter from *S. cerevisiae* permits growth on glutathione in a *C. glabrata met15*Δ (ABG2370) strain but not in a *C. glabrata dug3*Δ *met15*Δ (ABG2704) strain. *C. glabrata met15*Δ and *met15*Δ *dug3*Δ strains were transformed with plasmid pGRB2.2 (vector, lanes 3 and 1, respectively) or plasmid pGRB2.2-PGK1-HGT1 (*ScHGT1*, lanes 4 and 2, respectively). The transformants were grown in minimal medium (containing methionine), and cells were harvested, washed and resuspended to OD_{600} 0.2. Serial dilutions of OD_{600} 0.2, 0.02, 0.002 and 0.0002 were then spotted onto minimal medium containing 200 μM glutathione, methionine or ammonium sulphate (Sulphate) (having no organic sulphur source).

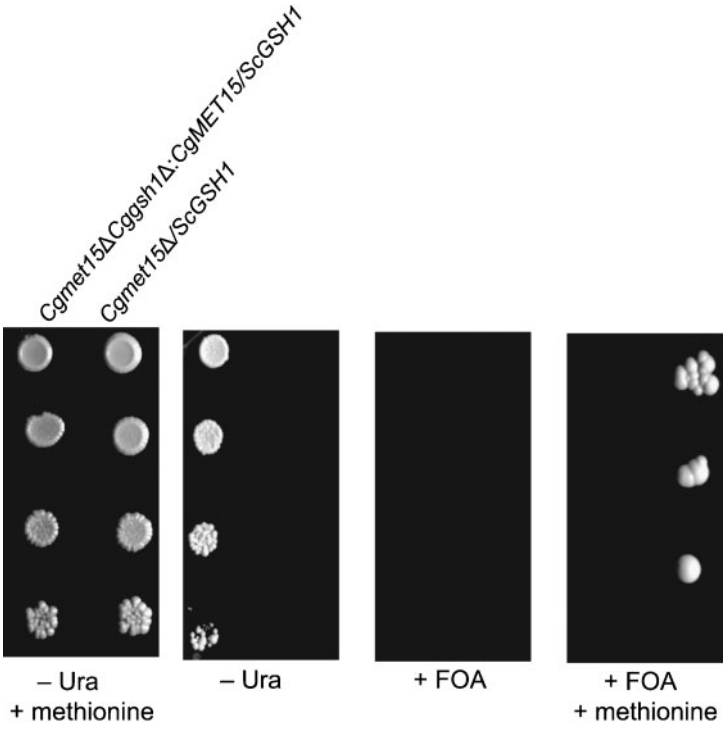


Fig. 4. *CgGSH1* is an essential gene in *C. glabrata*. *Cgmet15Δgsh1Δ : CgMET15* (ABG2810) and *Cgmet15Δ* (ABG2370) strains of *C. glabrata* were transformed with *S. cerevisiae GSH1* on plasmid pGRB2.2 under the PGK1 promoter (lanes 1 and 2, respectively). The disruptant and transformant were then grown in minimal medium without or with methionine, respectively, and cells were harvested, washed and resuspended to OD_{600} 0.2. Serial dilutions of OD_{600} 0.2, 0.02, 0.002 and 0.0002 were then spotted onto a different minimal medium (containing ammonium sulphate). Methionine, uracil and FOA (the plates containing FOA had uracil at a concentration of 1.2 mg 100 ml^{-1}) were added as indicated.

biosynthesis in *C. albicans* more rigorously. Earlier researchers have been able to create a *gcs1Δ* knockout in *C. albicans* (Baek *et al.*, 2004). The *Cagcs1Δ* strain was a glutathione auxotroph on plates, similar to its counterparts in the yeasts *S. cerevisiae* and *Schizosaccharomyces pombe*, which indicates that *C. albicans* can acquire its glutathione requirements from the extracellular medium in the absence of endogenous glutathione biosynthesis. Although *Schizosaccharomyces pombe gcs1Δ* strains show growth stasis immediately upon glutathione depletion, *S. cerevisiae gsh1Δ* cells show a delayed growth stasis upon glutathione depletion and manage to grow for up to seven or eight generations comparably with the wild-type without glutathione (Sharma *et al.*, 2000). We therefore decided to investigate the behaviour of the *C. albicans* strain in terms of its growth profiles in liquid broth. These growth studies with *Cagcs1Δ* were carried out in strains that were made *URA3*$^+$ and *IRO1*$^+$. (The previously created *Cagcs1Δ* strain was in the *C. albicans* CAI4 background, which lacks the *URA3* gene and also a part of the adjacent *IRO1* gene, involved in iron metabolism.) As both the *URA3* and the *IRO1* gene of *C. albicans* have been shown to have a role in pathogenesis (Chibana *et al.*, 2005; Lay *et al.*, 1998), we reintegrated these genes in their native locus to make the strains *URA3*$^+$ and *IRO1*$^+$, as described in Methods, before carrying out all subsequent experiments.

Upon examination of the growth characteristics of the *Cagcs1Δ* strain, we observed that in medium lacking glutathione, the strain grew more slowly than the wild-type, but despite that it did succeed in growing for five to six generations before entering into growth stasis (Fig. 5a). Upon reinoculation of the *Cagcs1Δ* strain (grown for 24 h in glutathione-free medium) into fresh glutathione-free medium, complete growth stasis was observed (Fig. 5b). The substantial viability of the cells even after 24 h growth in glutathione-free medium (up to 78 % viability) indicated that the cells were not undergoing cell death and were primarily experiencing growth stasis (data not shown). When the growth was carried out in medium that was supplemented with 1 mM glutathione, the *Cagcs1Δ* cells significantly recovered their growth (Fig. 5b). However, even in the presence of 1 mM glutathione, growth was still slower than that of the corresponding wild-type. Increasing the glutathione concentration in the medium to 5 mM did not further increase the growth of the strain (data not shown).

The *C. albicans gcs1Δ* strain shows reduced survival in human macrophages and also displays attenuated killing of differentiated THP1 cells

The decreased growth seen in *Cagcs1Δ* strains even when grown in medium supplemented with high levels of glutathione highlighted the importance of glutathione biosynthesis even in *C. albicans*, despite its ability to obtain glutathione from the external medium. Professional phagocytes (macrophages and neutrophils) constitute the first line of defence against yeast pathogens, so to examine the importance of endogenous glutathione biosynthesis as a response to the initial immune response of the host, we assessed the survival of *C. albicans* wild-type and *Cagcs1Δ* cells in differentiated human monocytic THP1 cells by an end-point dilution survival assay (Rocha *et al.*, 2001).

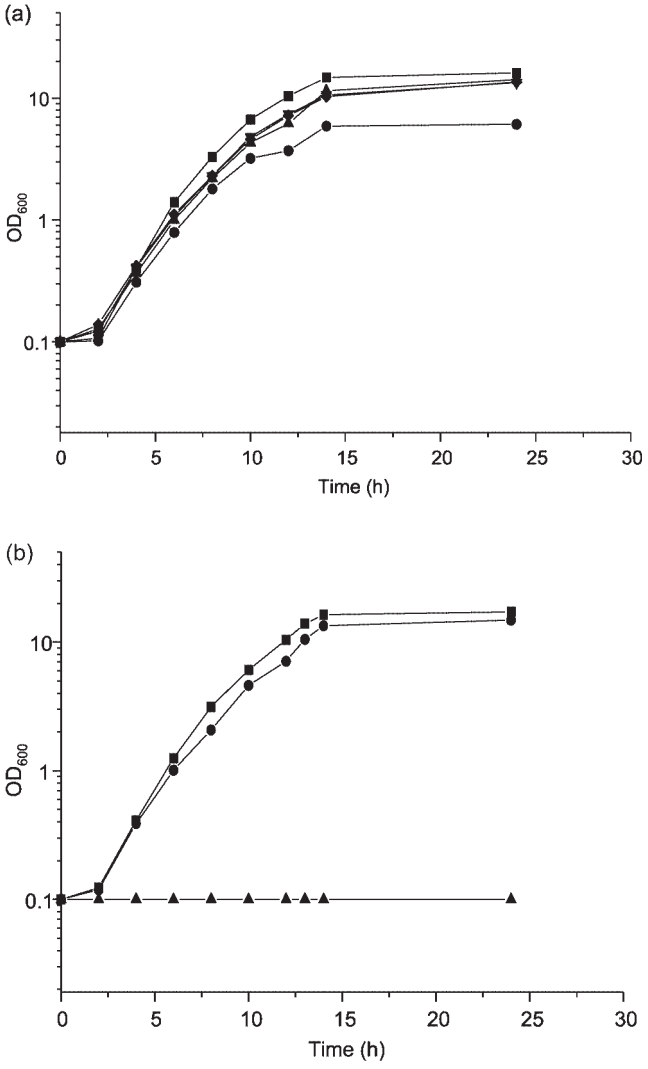


Fig. 5. A *C. albicans Cagcs1*Δ strain (ABA2878) shows delayed growth stasis upon glutathione depletion. (a) Growth of *C. albicans* wild-type (ABA2240), *Cagcs1*Δ (ABA2878) and *CaGCS1* reintegrated strains (ABA2993) on minimal medium with 1 mM glutathione or without glutathione. Cells were grown overnight in minimal medium containing glutathione, washed twice with minimal medium and reinoculated to OD_{600} 0.1. OD_{600} was measured on a Shimadzu UV-1800 spectrophotometer. *C. albicans* wild-type (■), *Cagcs1*Δ (●), *Cagcs1*Δ + 1 mM glutathione (▲), *CaGCS1* (▼), *CaGCS1* + 1 mM glutathione (◆). (b) *Cagcs1*Δ (ABA2878) cells were grown in glutathione-free medium for 24 h and freshly reinoculated into minimal medium with (●) or without glutathione (▲); *C. albicans* wild-type (■) was the control.

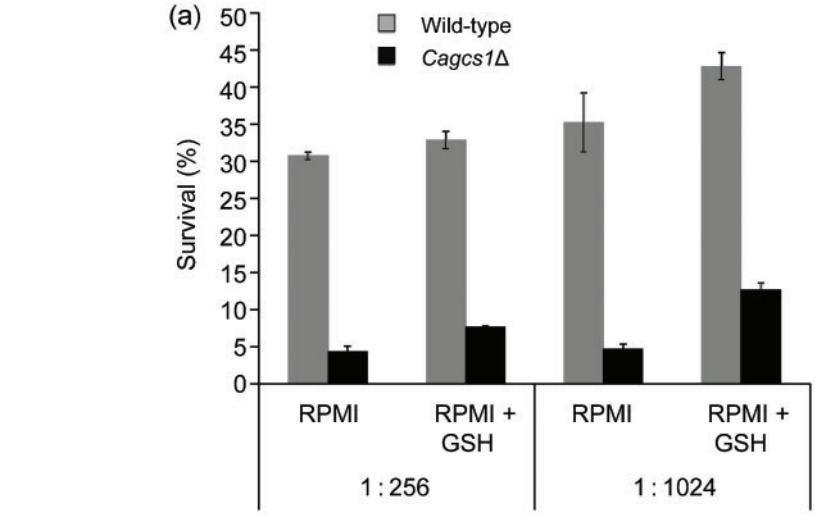
A wide range of m.o.i. values (from 1 : 4 to 1 : 1024) was used to study the interaction of *C. albicans* with macrophages. No significant differences were seen in the internalization rates (about 75 %) for *C. albicans* wild-type and *Cagcs1*Δ cells (data not shown). Co-incubation with macrophages impaired the growth of both *C. albicans* wild-type and *Cagcs1*Δ cells. Growth inhibition of 55–70 % was seen for the wild-type *C. albicans* cells as compared with growth in RPMI medium at m.o.i. values of 1 : 256 and 1 : 1024 (Fig. 6a). Growth of the *Cagcs1*Δ strain, in contrast, was more severely inhibited upon co-incubation with macrophages, with *Cagcs1*Δ cells displaying 3–5 % survival under similar conditions (Fig. 6a). This reduced colony formation phenotype of the *Cagcs1*Δ strain was partially rescued by supplementing the RPMI medium with 1 mM glutathione (7–15 % survival) (Fig. 6a). Although colonies formed by the *Cagcs1*Δ strain in the presence of macrophages were smaller than those of the wild-type (which could be due to the slow growth rate of the strain), importantly, no increase in the number of colonies was observed with prolonged incubation (data not shown). Interestingly, there was no difference in the rate of germ-tube formation in wild-type and *Cagcs1*Δ cells, as both strains exhibited morphology switching within the first 2 h of co-incubation with THP1 cells (Fig. 6b and data not shown). Taken together, these results suggest that *Cagcs1*Δ cells are unable to survive and escape from the macrophage antimicrobial response.

To investigate whether *Cagcs1*Δ cells would also be defective in lysing and killing macrophages, macrophage viability was assessed by trypan blue staining 24 h post-infection; wells with lower m.o.i. values were used due to the invisibility of THP1 cells in higher m.o.i. wells. We observed that THP1 cells infected with the *Cagcs1*Δ strain were able to retain their viability, and only 1–13 % of the macrophages stained blue even after 24 h of co-culture at different m.o.i. values (Fig. 6b and data not shown). In contrast, when infected with the *C. albicans* wild-type strain under similar conditions, 64–81 % of THP1 cells were unable to efflux trypan blue, indicating significant cell death (Fig. 6b and data not shown). The reduced killing of THP1 cells by the *Cagcs1*Δ strain even at a higher m.o.i. (or when co-cultured with THP1 in medium lacking glutathione) suggests that *C. albicans Cagcs1*Δ cells are attenuated for virulence.

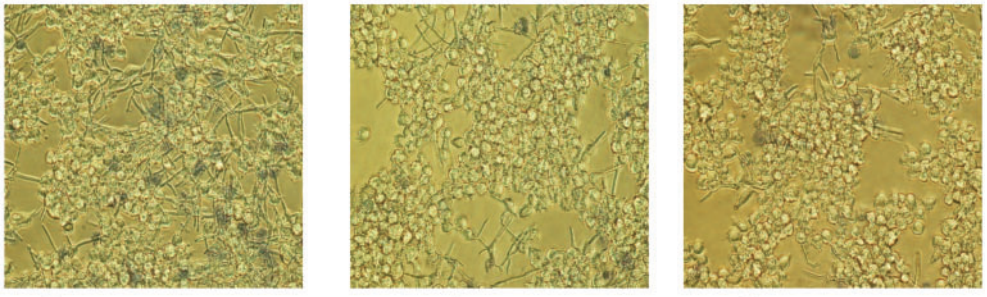
GCS1 is essential for virulence of *C. albicans* in mice

The reduced survival of the *C. albicans gcs1*Δ strain in a macrophage cell line prompted us to examine whether glutathione biosynthesis might also play a role in the virulence of *C. albicans* in a systemic murine model of candidiasis. BALB/c mice were infected intravenously with an inoculum of 5×10^5 yeast cells and mice survival was monitored as a function of time.

As shown in Fig. 7(a), mice infected with wild-type *C. albicans* could not survive beyond 9 days, with death onset seen as early as day 4. In contrast, all mice infected with the *C. albicans gcs1*Δ strain remained healthy and were still alive 15 days post-infection. To examine whether the decreased mouse mortality seen with the *C. albicans gcs1*Δ strain was linked to reduced fungal dissemination within the host and colonization of different organs, we



(b)



C. albicans wild-type with glutathione | *Cagcs1*Δ | *Cagcs1*Δ + 1 mM glutathione

Viability of THP1 cells (%)

m.o.i.	*C. albicans* strain	Glutathione (–)	Glutathione (+)
1 : 64	Wild-type	21 ± 4.51	19 ± 5.00
	*Cagcs1*Δ	98 ± 1.00	87 ± 3.05
1 : 256	Wild-type	36 ± 4.36	35 ± 5.51
	*Cagcs1*Δ	99 ± 1.00	93 ± 0.58

Fig. 6. Survival of *C. albicans* wild-type (ABA2240) and *gcs1*Δ (ABA2878) cells in differentiated THP1 cells, as determined by an end-point dilution survival assay. (a) THP1 cells were infected with *C. albicans* wild-type and *gcs1*Δ cells as described in Methods, and yeast colonies observed in wells with macrophages were counted and expressed as the ratio with respect to colonies seen in wells containing RPMI medium alone 24 h post-infection. One representative experiment (out of two) is shown. Experiments were performed in triplicate (means ± SD). (b) THP1 cells were infected with yeast cells at an m.o.i. of 1 : 16, and images were taken 8 h post-infection with an upright microscope (×40 magnification). The viability of THP1 cells was measured by trypan blue staining 24 h post-infection. One representative experiment (out of two) is shown. Experiments were performed in triplicate (means ± SD).

quantified the fungal load in different tissues (kidney, liver and spleen) at various time points (day 2, day 3, day 4 and day 6) post-tail vein injection. Although no significant c.f.u. were recovered for *C. albicans* wild-type and *Cagcs1*Δ strains from the liver and spleen at any time point, renal loads were vastly different for the two strains throughout the course of infection. While about 10^6 yeasts could be recovered from the kidneys of mice infected with wild-type *C. albicans* cells at day 6, the kidneys harvested from mice injected with *Cagcs1*Δ cells were almost sterile (Fig. 8). Taken together, these results suggest that *CaGCS1* is required for virulence in *C. albicans*.

To confirm that the avirulent phenotype seen in *Cagcs1*Δ strains was a consequence of the deletion of the *GCS1* gene, we integrated a copy of the *CaGCS1* gene in the double disruptant strain. These strains regained the ability to grow in glutathione-free medium, although the total growth seen was still a little lower than that of the wild-type (Fig. 5a). Next, to examine whether the *C. albicans* strain carrying a reintegrated copy of *CaGCS1* would be virulent in the murine model of systemic candidiasis, we injected the mice intravenously either with the wild-type strain or with the single-copy *CaGCS1* reconstituted strain. We observed that the strain carrying a reintegrated copy of *CaGCS1* behaved like the wild-type, and a parallel mice survival pattern was seen in the two strains (Fig. 7b). This suggests that the capability to synthesize glutathione, even with a single copy of the Gcs1p enzyme, is sufficient for *Cagcs1*Δ cells to regain their virulence attributes in mice. The results clearly demonstrate that even a single copy of the gene allows the *Cagcs1*Δ strain to regain not only its ability to synthesize glutathione but also simultaneously its virulence.

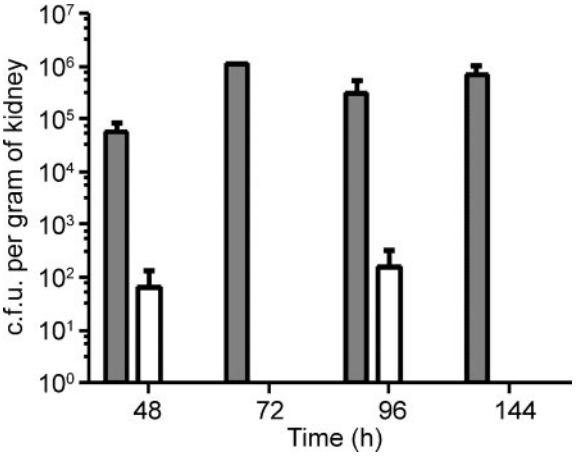


Fig. 8. Organ load experiments. BALB/c mice were infected by the intravenous route with 5×10^5 cells of the *Cagcs1*Δ (ABA2878, white bars) and wild-type (ABA2240, grey bars) strains. Mice were sacrificed at specified time intervals, and organs were isolated and crushed in a tissue homogenizer and plated on YPD+1 mM glutathione medium. Results for kidney tissue are shown.

DISCUSSION

In this study we have examined the essentiality of glutathione biosynthesis in two fungal pathogens, *C. albicans* and *C. glabrata*, and have uncovered a link between glutathione biosynthesis and their virulence, raising the possibility of exploiting metabolic strategies to attenuate *Candida* infection. In *C. glabrata*, the absence of a glutathione transporter showed that glutathione biosynthesis is an essential process in this yeast. In *C. albicans*, although a *Cagcs1*Δ strain could grow on plates when supplied with exogenous glutathione, and in liquid broth showed only a delayed growth stasis when depleted for glutathione, the strain was completely defective in its ability to cause infection in mice.

The complete avirulence of *Cagcs1*Δ strains in mice experiments was surprising, considering the ability of these strains otherwise to grow on exogenous glutathione. With abundant glutathione in the human host, one would have expected *C. albicans* to have exploited the host glutathione for its survival. In a recent study on inositol, also an essential metabolite that can be both endogenously synthesized and transported by specific transporters, it was observed that merely disrupting the biosynthesis gene (*INO1*) does not have any effect on the virulence capabilities unless the transporters (*ITR1/ITR2*) are disrupted as well (Chen *et al.*,

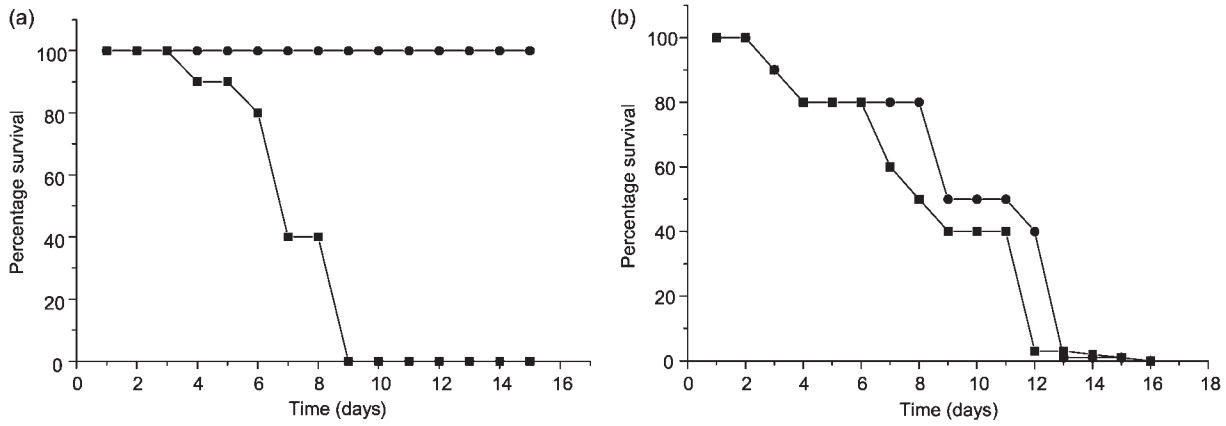


Fig. 7. Comparison of virulence of a *C. albicans gcs1*Δ strain (ABA2878) and a *Cagcs1*Δ strain containing a single copy of *GCS1* (ABA2993) with that of a *C. albicans* wild-type strain (ABA2240) in mice. (a) Percentage survival of BALB/c mice following intravenous challenge with 5×10^5 cells of *C. albicans* wild-type (ABA2240, ■) and *Cagcs1*Δ mutant (ABA2878, ●). (b) Percentage survival of BALB/c mice following intravenous challenge with 5×10^5 cells of *C. albicans* wild-type (ABA2240, ■) and *Cagcs1*Δ::*GCS1* strains (ABA2993, ●).

2008). One explanation for the striking difference seen in the case of glutathione may be the inefficient uptake of glutathione by this yeast. *C. albicans* contains eight members of the oligopeptide transporter family, *CaOPT1–CaOPT8*, many of which have been shown to transport oligopeptides (Reuss & Morschhäuser, 2006). Among these, *CaOPT1* has been considered to be a possible orthologue of the *S. cerevisiae* glutathione transporter *HGT1*, owing to the high sequence similarity. However, we have recently shown that the *CaOPT1* protein is in fact a very weak glutathione transporter, although glutathione is unlikely to be its primary substrate (Thakur & Bachhawat, 2010). This is especially important when one considers the low concentrations of glutathione in blood plasma [reported to be about 1–3 μM (Guttormsen *et al.*, 2004)]. Within the host, pathogens experience a variety of stress conditions that include exposure to ROS and hypoxia, in which glutathione plays very vital roles (Ernst & Tielker, 2009). The increased requirement for and inadequate supply of glutathione in the absence of endogenous biosynthesis is likely to affect the ability of the pathogen to survive the harsh *in vivo* conditions. Glutathione limitation would thus be expected to significantly affect the virulence of the organism, and the results described here, in which *Cagcs1*Δ strains were completely avirulent, are in complete agreement with these expected increased requirements for glutathione *in vivo*. It is also possible that glutathione, which plays a central role under stress conditions, is also important for other processes specifically required for virulence, although one such process, the ability to form hyphae, did not appear to be affected. Specific depletion of the fungal glutathione would thus clearly impinge on fungal survival, and in this context, it is interesting to note that an antifungal compound from garlic, diallyl sulphide, and the protein kinase inhibitor staurosporine, have both been shown to cause their antifungal activity by depletion of glutathione in *C. albicans* (Castro *et al.*, 2010; Lemar *et al.*, 2007).

C. glabrata appears to be the only yeast that lacks any member of the glutathione transporter family (the oligopeptide transporter family; OPT) and the reason for this absence is not clear. Comparative evolutionary genomics analysis has revealed that whole-genome duplication in *C. glabrata* was followed by extensive gene loss (Dujon *et al.*, 2004). Favouring the reductive gene evolution theory, this yeast seems to have lost several genes related to cellular metabolism (Kaur *et al.*, 2005). This gene loss may reflect the diverse selective pressures encountered inside the human host and the successful adaptation of *C. glabrata* to its new ecological niche. The lack of the nicotinic acid biosynthetic pathway in *C. glabrata* has recently been shown to be linked with the expression of the genes required for efficient colonization of the host (Domergue *et al.*, 2005). In light of these findings, the loss of *HGT1* in *C. glabrata* might stem from the fact that despite the presence of the transporter, biosynthesis would still be essential, since particularly in blood plasma, glutathione levels are exceedingly low to meet the much higher glutathione requirement *in vivo*. What is even more interesting, however, is that in spite of 'losing' the glutathione transporter, *C. glabrata* has retained a functional glutathione utilization machinery (Dug complex). Prior to this observation, the Dug pathway was presumed to have evolved along with the high-affinity glutathione transporter for the utilization of exogenous glutathione. Its functional presence in *C. glabrata* thus suggests that the Dug pathway has not evolved solely for the utilization of exogenous glutathione, and also plays a role in intracellular glutathione homeostasis.

The essentiality of Gsh1p in *C. glabrata* and its requirement for virulence in *C. albicans* make γ-glutamyl cysteine synthase (Gcs1p) a possible candidate for antifungal drug development in addition to being previously considered as an anti-protozoal target. The γ-glutamyl cysteine synthases have evolved into three different lineages. Yeasts share the same lineage as their protozoan and mammalian counterparts, but even so, significant differences exist between the human and the yeast enzymes. The mammalian enzyme is heterodimeric, with catalytic and regulatory subunits, while *C. glabrata* and *C. albicans*, like *S. cerevisiae*, seem to lack the regulatory subunit (Biterova & Barycki, 2009). *C. albicans* Gcs1p has a length of 772 amino acids and appears to contain a 90 amino acid insertion when compared with the sequences of *C. glabrata* and *S. cerevisiae*. To determine whether this is a genuine insertion, we examined the Gcs1p sequences of other *Candida* species, such as *Candida dubliniensis* and *Candida tropicalis*. A similar insertion was found in these species, thus suggesting that the insertion in *C. albicans* is in fact a genuine insertion rather than a sequence artefact. Although approximately 40 % identity exists between the *Ca*Gcs1p/*Cg*Gsh1p enzyme and the catalytic subunit of human *GCS*, the differences in subunit structure (absence of a regulatory subunit) and the extra portion of about 90 amino acids in *C. albicans* raise possibilities for further study and exploitation.

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