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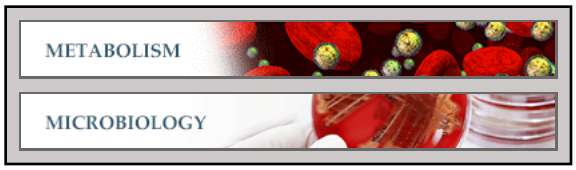

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Glutathione Utilization by *Candida albicans* Requires a Functional Glutathione Degradation (DUG) Pathway and OPT7, an Unusual Member of the Oligopeptide Transporter Family[S]

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Background: Glutathione biosynthesis is essential for pathogenesis in *Candida albicans*. Exogenous glutathione utilization is not known.
Results: The DUG pathway of glutathione degradation and an unusual transporter, OPT7, function in glutathione utilization.
Conclusion: Glutathione utilization is efficient but not essential for survival *in vivo*.
Significance: It enables one to understand how this pathway contributes toward homeostasis of redox and sulfur in a yeast pathogen.

Candida albicans **lacks the ability to survive within its mammalian host in the absence of endogenous glutathione biosynthesis. To examine the ability of this yeast to utilize exogenous glutathione, we exploited the organic sulfur auxotrophy of *C. albicans met15*Δ strains. We observed that glutathione is utilized efficiently by the alternative pathway of glutathione degradation (DUG pathway). The major oligopeptide transporters OPT1–OPT5 of *C. albicans* that were most similar to the known yeast glutathione transporters were not found to contribute to glutathione transport to any significant extent. A genomic library approach to identify the glutathione transporter of *C. albicans* yielded OPT7 as the primary glutathione transporter. Biochemical studies on OPT7 using radiolabeled GSH uptake revealed a K_m of 205 μM, indicating that it was a high affinity glutathione transporter. OPT7 is unusual in several aspects. It is the most remote member to known yeast glutathione transporters, lacks the two highly conserved cysteines in the family that are known to be crucial in trafficking, and also has the ability to take up tripeptides. The transporter was regulated by sulfur sources in the medium. OPT7 orthologues were prevalent among many pathogenic yeasts and fungi and formed a distinct cluster quite remote from the *Saccharomyces cerevisiae* HGT1 glutathione transporter cluster. *In vivo* experiments using a systemic model of candidiasis failed to detect expression of OPT7 *in vivo*, and strains disrupted either in the degradation (*dug3*Δ) or transport (*opt7*Δ) of glutathione failed to show a defect in virulence.**

Candida albicans is the most important yeast pathogen to cause systemic fungal infections in humans (1–3). The ability of this yeast to effectively survive the harsh host environments suggests the presence of stress response capabilities that are likely to be unique to this yeast. Oxidative stress is one of the major stress conditions faced by yeast pathogens *in vivo* (4), and an effective response is required by the yeast pathogen to defend itself from the host immune system. *C. albicans* has both a thioredoxin-dependent and a glutathione-dependent pathway of redox homeostasis (5). Although the redox functions of these pathways have been shown to partially overlap in *S. cerevisiae* (6, 7), disrupting either the thioredoxin pathway (Trx1 knockouts) (8) or the glutathione biosynthetic pathway alone in *C. albicans* (Gsh1 knockouts) leads to attenuated virulence (9).

The abundance of glutathione in the human host and the presence of apparent homologues of glutathione utilization pathways of *S. cerevisiae* in *C. albicans* have made the essentiality of glutathione biosynthesis for the survival of *C. albicans in vivo* surprising. Two possible explanations could be put forward to explain these observations. The first is that the high levels of intracellular glutathione that are required by these yeast pathogens *in vivo* might demand continuous glutathione biosynthesis. Glutathione levels in all eukaryotic cells reach up to 10 mM (10), and these levels might be difficult to reach only

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables S1–S3 and Figs. S1 and S2.
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from extracellular sources. A second explanation for the essentiality of glutathione biosynthesis *in vivo* might be the absence of an efficient glutathione utilization pathway in *C. albicans*, as a consequence of which glutathione, though abundant in the environment, would be inefficiently utilized by the yeast. Exogenous glutathione utilization requires both a transporter and a degradation pathway. Glutathione transporters in several yeasts have now been identified and found to belong to the peptide transporter clade of the oligopeptide transporter superfamily (11–13). Glutathione degradation has been shown to require the alternative pathway (DUG pathway) of glutathione degradation (14) whose homologues are present in all yeasts and fungi barring *Schizosaccharomyces pombe*. The DUG pathway involves the proteins DUG1, DUG2, and DUG3. The DUG1 has been shown to be a Cys-Gly peptidase (15), whereas DUG2 and DUG3 form a complex to cleave the γ-glutamyl linkage of glutathione.[8] The *C. albicans* genome has revealed the presence of several members of the oligopeptide transporter (OPT)[9] family, OPT1–OPT8 (16). Of these, OPT1–OPT7 belonged to the peptide transporter (PT) clade, and among these OPT1 is the closest in similarity to the *Saccharomyces cerevisiae* glutathione transporter, HGT1. Despite the strong similarity to HGT1, OPT1 was shown not to play a role in glutathione transport as seen from heterologous complementation experiments (13). Thus it was possible that *C. albicans* lacks the ability to efficiently transport and utilize glutathione. This would be akin to the situation in *Candida glabrata* that completely lacks the homologues of the oligopeptide transporter superfamily belonging to the PT clade and in fact fails to transport and utilize glutathione when provided from external sources (9).

Considering the importance of glutathione and redox pathways for yeast pathogens *in vivo*, we sought to resolve these issues, and in this study we have investigated glutathione transport and utilization in *C. albicans*. The construction of a *met15*Δ strain in *C. albicans* that was an organic sulfur auxotroph revealed that *C. albicans* could utilize glutathione as a sulfur source efficiently, and we have used this phenotype for facilitating the further analysis of glutathione utilization in this yeast. We investigated the role of the OPTs as well as the DUG pathway, and our studies revealed that *C. albicans* can utilize glutathione efficiently, and the transport is dependent on an unusual member of the OPT family, OPT7, whereas degradation was dependent on the DUG pathway. The possible importance of these pathways in the virulence and survival of *C. albicans in vivo* was also investigated in a mouse model of systemic candidiasis.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents—All of the chemicals used in this study were analytical grade and obtained from commercial sources. Media components were purchased from Difco (Detroit, MI) Sigma-Aldrich, HiMedia (Mumbai, India), Merck India Ltd. (Mumbai, India), and USB Corporation (Cleveland, OH). Oligonucleotides were purchased from Sigma-Genosys (Bangalore, India). Restriction enzymes, vent DNA polymerase, and other DNA modifying enzymes were obtained from New England Biolabs, (Beverly, MA). DNA sequencing kit (ABI PRISM 310 XL with dye termination cycle sequencing ready reaction kit) was obtained from PerkinElmer Life Sciences. Gel extraction kits and plasmid miniprep columns were obtained from Qiagen or Sigma. [^{35}S]GSH (specific activity, 1000 Ci mmol^{-1}) was purchased from Bhabha Atomic Research Centre (Mumbai, India). HA tag (6E2) mouse monoclonal antibody and horse anti-mouse HRP-linked antibody were bought from Cell Signaling (Danvers, MA). Alexa Flour® 488-conjugated goat anti-mouse antibody was obtained from Molecular Probes (Eugene, OR).

Strains, Media, and Growth Conditions—The *Escherichia coli* strain DH5α was used as a cloning host. *S. cerevisiae* and *C. albicans* strains used in the study are described in (Table 1). *S. cerevisiae* and *C. albicans* strains were regularly maintained on yeast extract, peptone, and dextrose medium (YPD). Synthetic defined minimal medium (SD) contained yeast nitrogen base, ammonium sulfate, and dextrose supplemented with histidine, leucine, and lysine (as per requirement) at 50 mg/liter. Glutathione was added as required (17). For *C. albicans*, synthetic defined medium contained ammonium chloride instead of ammonium sulfate and was supplemented with either methionine, cysteine, cystine, or glutathione (as per requirement). Mycophenolic acid plates were prepared by adding 1 mg/ml of mycophenolic acid in YPD medium. The molecular techniques used in the study were according to standard protocols (18).

Construction of Disruption Cassettes of MET15 in C. albicans—The *MET15* gene of *C. albicans* was cloned by amplifying the ORF including 100 bp upstream and downstream of the ORF by PCR with SmaI sites introduced into the primers and cloning into SmaI site of pBSK. For preparing the two disruption cassettes for each of the alleles, we excised the SAT1 flipper from pSFS2a by ApaI-SacI, blunted and cloned into the MscI and BsmI sites. A second disruption cassette was made by PCR amplifying an internal piece of the MET15 clone corresponding to, but internal to, the site and inserting the SAT1 flipper into the HpaI-BstEII site of this smaller MET15 fragment. To use the first cassette for disruption, we evicted the same with SmaI and transformed into the appropriate *C. albicans* strain. To create a disruption in the second allele, we first evicted the SAT1 flipper from the first strain by growth in maltose medium as described earlier (19). The second disruption cassette was excised with SmaI sites and then transformed into the strain containing a disruption in the first allele. The *MET15* gene was disrupted in this manner in both alleles of *C. albicans* SC5314 to yield strain ABA 2490, in OPT7M4AB to yield strain ABA 3031, in OPT1M4AB to yield strain ABA 2546, in OPT12345M4AB to yield strain ABA 2548, and in ABA 2558 to yield strain ABA 2592. The disruptions were confirmed by diagnostic PCR, and the absence of a third allele was also confirmed by diagnostic PCR. The SAT1 flipper was evicted using maltose-containing medium as described previously.

[8] H. Kaur and A. K. Bachhawat, unpublished observations.

[9] The abbreviations used are: OPT, oligopeptide transporter; PT, peptide transport; SD, synthetic defined minimal medium; MES, 4-morpholineethanesulfonic acid; IVET, *in vivo* expression technology; MPA, mycophenolic acid.

TABLE 1
Strains used in this study

Strain	Genotype	Sources or references
ABC 817	*MATa his3Δ1 leu2Δ0 met15*Δ-0 *ura3Δ0 hgt1*Δ:: *LEU2*	Lab strain
SC5314	*C. albicans* wild-type strain	Ref. 34
OPT1M4A and B	*opt1-1*Δ::*FRT/opt1-2*Δ::*FRT*	Ref. 19
ABA 2490	*met15-1*Δ::*FRT/met15-2*Δ::*FRT*	This study
OPT7M4A and B	*opt7-1*Δ::*FRT/opt7-2*Δ::*FRT*	Ref. 16
OPT12345M4A and B	*opt1-1*Δ::*FRT/opt1-2*Δ::*FRT* *opt23-1*Δ::*FRT/opt23-2*Δ::*FRT* *opt4-1*Δ::*FRT/opt4-2*Δ::*FRT* *opt5-1*Δ::*FRT/opt5-2*Δ::*FRT*	Ref. 16
ABA 2558	*dug3-1*Δ:FRT::*dug3-2*Δ:FRT	This study
ABA 2592	*dug3-1*Δ:FRT::*dug3-2*Δ:FRT, *met15-1*Δ:FRT::*met15-2*Δ:FRT	This study
ABA 2546	*opt1-1*Δ::*FRT/opt1-2*Δ::*FRT* *met15-1*Δ::*FRT/met15-2*Δ::*FRT*	This study
ABA 3031	*opt7-1*Δ::*FRT/opt7-2*Δ::*FRT* *met15-1*Δ::*FRT/met15-2*Δ::*FRT*	This study
ABA 2548	*opt1-1*Δ::*FRT/opt1-2*Δ::*FRT* *opt23-1*Δ::*FRT/opt23-2*Δ::*FRT* *opt4-1*Δ::*FRT/opt4-2*Δ::*FRT* *opt5-1*Δ::*FRT/opt5-2*Δ::*FRT* *met15-1*Δ::*FRT/met15-2*Δ::*FRT*	This study
S5FI2A	*Ura3*Δ::*imm434/ura3*Δ::*imm434* *ACT1/act1*::*FRT-MPAR-FRT* *SAP5-2/sap5-1*::*SAP5P-ecaFLP-URA3*	Ref. 29
S2FI1A[S]	*ura3*Δ::*imm434/ura3*Δ::*imm434* *SAP2/sap2*::*SAP2P-FLP-URA3* , ACT1/act1::FRT	Ref. 30
S2UI1A	*ura3*Δ::*imm434/ura3*Δ::*imm434* *ACT1/act1*::*FRT-MPAR-FRT* *SAP2/sap2*::*SAP2P-FLP-URA3*	Ref. 30
OPT7FLIA	*ura3*Δ::*imm434/ura3*Δ::*imm434* *ACT1/act1*::*FRT-MPAR-FRT* *OPT7-2/opt7-1*::*OPT7P-ecaFLP-URA3*	This study
CBF1M4A	*ura3*Δ::*imm434/*Δ*ura3*::*imm434* Δ*cbf1*::*FRT/*Δ*cbf1*::*FRT*	Ref. 28

Construction of Disruption Cassettes of DUG3 in C. albicans—The *DUG3* gene of *C. albicans* was cloned by PCR amplifying the ORF including 100 bp upstream and downstream of the ORF with SmaI sites introduced into the primers and cloning into the SmaI site of pBSK. For preparing the two disruption cassettes for each of the alleles, we excised the SAT1 flipper from pSFS2a by ApaI-SacI, blunted and cloned into the NdeI-HpaI sites. A second disruption cassette was made by PCR amplifying an internal piece of the DUG3 clone corresponding to, but internal to, the NdeI-HpaI site and inserting the SAT1 flipper into the BclI-MscI site of this smaller DUG3 fragment. To use the first cassette for disruption, we evicted the same with SmaI and transformed into the appropriate *C. albicans* strain. To create a disruption in the second allele, we first evicted the SAT1 flipper from the first strain by growth in maltose medium as described earlier. The second disruption cassette was excised with SmaI sites and then transformed into the strain containing a disruption in the first allele. In this way both alleles of DUG3 of *C. albicans* SC5314 were disrupted to yield strain ABA 2558. The disruptions were confirmed by diagnostic PCR, and the absence of a third allele was also confirmed by diagnostic PCR. The SAT1 flipper was evicted using maltose-containing medium.

Cloning of OPT7 in S. cerevisiae Expression Vector—The ORF *OPT7* was PCR-amplified from *C. albicans* genomic DNA using the primer pairs OPT7F and OPT7R (supplemental Table S3). The 2.3-kb PCR product obtained was digested with Xba1 and BamHI and cloned downstream of the TEF promoter in the single copy, URA3-based expression vector to construct *p416TEF-OPT7* (20). *OPT7* was tagged with HA epitope by PCR mutagenesis. The reverse primer had the HA tag before the stop codon.

Growth Assay by Dilution Spotting—For growth assays, *S. cerevisiae* strains were grown overnight in minimal medium without uracil, whereas *C. albicans* strains were grown in SD minimal medium. The cells were reinoculated in fresh medium to an A_{600} of 0.1 and grown up to exponential phase. The exponential phase cells were harvested washed with water and resuspended in water to an A_{600} of 0.2. These were serially diluted to 1:10, 1:100, and 1:1000. 10 μl of these cell resuspensions were spotted on minimal medium containing different concentration of either glutathione, γ-Glu-Cys, α-Glu-Cys, Cys-Gly, Leu-Tyr-Met-Arg, GSSG, or methionine. The plates were incubated at 30 °C for 2–3 days, and photographs were taken.

Glutathione Transport Assay—The *S. cerevisiae* ABC 817 strain (*met15*Δ*hgt1*Δ) transformed with appropriate plasmids was grown in minimal medium containing methionine and other supplements, without uracil. *C. albicans* strains *met15*Δ, *met15*Δ-*opt7*Δ, and *cbf1*Δ were grown in SD minimal medium containing 20 μM to 40 μM methionine (nonrepressive sulfur conditions) or 1 mM methionine (repressive sulfur conditions) overnight. These cultures were reinoculated in the same medium and allowed to grow till they reached exponential phase. The cells were harvested and washed and put on ice in a pH 5.5 MES-buffered medium, until the transport was initiated. Transport experiments were carried out with [^{35}S]GSH as

described earlier. The results were expressed as nanomoles of glutathione·mg·protein^{-1} min^{-1} as described previously (21).

For saturation kinetics, the initial rate of glutathione uptake was measured at different concentrations of glutathione with specific activity kept constant at each concentration. The initial rate of glutathione uptake was determined by measuring the radioactive glutathione in the cell. After subtracting background uptake observed with vector alone from uptake with *P416TEF-OPT7*, the data were plotted. The Lineweaver-Burke best fit line was plotted and was used to calculate the kinetic parameters K_m and V_{max}. The experiment was repeated a minimum of two times in duplicate at each glutathione concentration.

Inhibition studies were done with different peptides, Glu-Cys, Cys-Gly, Leu-Gly, Met-Gly, Gly-Gly-Gly, GSH, and GSSG. The inhibitors were added at 10-fold excess along with the assay medium, and the initial rates of glutathione were measured. For the studies done with the metabolic inhibitors, the cells were preincubated with the metabolic inhibitor for 15 min at 30 °C before measuring the initial rate of glutathione uptake.

For determining the pH optima, the initial rates of glutathione uptake in the OPT7 were determined at different pH levels ranging from 3.5 to 7.5. The different pH levels were maintained using different buffers: 20 mM acetate-sodium acetate (pH 3.5, 4.0, and 4.5), 20 mM MES/KOH (pH 5.0, 5.5, and 6.0) and 20 mM HEPES/KOH (pH 6.5, 7.0, and 7.5). In each case, the buffer also contained 0.5 mM $CaCl_2$, 0.25 mM $MgCl_2$, and 2% glucose.

Indirect Immunofluorescence of the HA-tagged OPT7 by Confocal Microscopy—For localization studies, the *P416TEF-OPT7HA* construct was transformed in the *S. cerevisiae met15*Δ*hgt1*Δ strain, and indirect immunofluorescence was performed using a previously published protocol (21).

Multiple Sequence Analysis and Phylogenetic Analysis—The OPT sequences were retrieved from Entrez. The multiple sequence alignment of the protein sequences was generated using the ClustalX program using default parameters (22). The MEGA 3.1 software (23) was used to visualize the phylogenetic tree of the family.

Experimental Infection in Mice—Experimentation with mice was done under the health and safety executive guidelines for level 2 biohazard containment. Male BALB/c mice aged 6–8 weeks were used for the survival and IVET experiment. To investigate the virulence of *C. albicans opt7*Δ and *dug3*Δ strains in comparison with *C. albcians* WT (SC5314), both of the strains were grown on minimal medium for 16 h; further, the cells were washed twice with 1× PBS and resuspended in the same buffer. To correlate the number of cells to 1 A_{600}, hemocytometer cell counting was done. The mice were intravenously injected with 5×10^5 cells of *C. albicans* WT SC5314, *opt7*Δ, and *dug3*Δ and individually and observed for survival. For the *in vivo* expression technology experiments, two mice were sacrificed after every 2 days, the kidneys were isolated and homogenized in buffer, and an aliquot (after dilutions) was plated on selective plates.

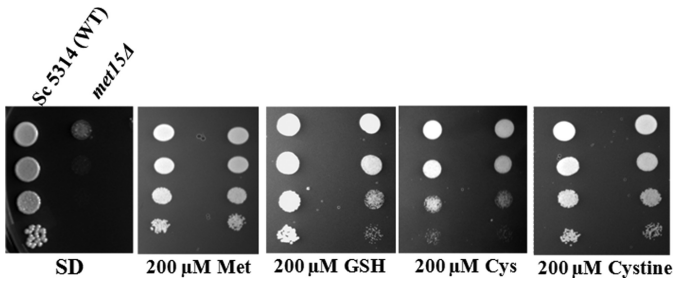

FIGURE 1. ***C. albicans* met15Δ are organic sulfur organic auxotrophs and can use glutathione, cysteine, and cystine as sulfur sources.** The *C. albicans* WT and *met15*Δ strains were grown to exponential phase in minimal medium containing methionine, harvested, washed, resuspended in water, and serially diluted to give 0.2, 0.02, 0.002, and 0.0002 A_{600} of cells. 10 μl of these dilutions were spotted on minimal ammonium chloride medium containing methionine, glutathione, cysteine, and cystine. The photographs were taken after 2 days of incubation at 30 °C.

RESULTS

*C. albicans met15*Δ *Are Organic Sulfur Organic Auxotrophs and Can Use Glutathione as a Source of Sulfur*—The *MET15* gene of *S. cerevisiae* codes for *O*-acetyl homoserine thiolase, an enzyme in the sulfur assimilation pathway. Deletion of this gene has been found to lead to organic sulfur auxotrophy in both *S. cerevisiae* and *C. glabrata*. *met15*Δ strains have been previously created in *C. albicans*, and these disruptants were shown to be methionine auxotrophs, although prolonged growth on minimal medium permitted growth even on inorganic sulfate (24). However, the growth on other sulfur compounds was never examined. We decided to recreate the *met15*Δ in a WT background to re-evaluate the earlier findings, and further to examine whether the strain would be useful to evaluate growth on glutathione. The *met15*Δ strain in SC5314 background was created as described under "Experimental Procedures." These strains were not only methionine auxotrophs as described previously, but organic sulfur auxotrophs because the strain did not grow on inorganic sulfate but were able to grow on cysteine, cystine, and glutathione as a source of sulfur (Fig. 1). They could also grow on homocysteine (data not shown). However, prolonged growth of *met15*Δ led to growth even in the absence of any organic sulfur sources in accordance with the previous observations that it was not a strict auxotrophy (24). Despite this, and considering that the growth on organic sulfur sources was much faster, they have proved useful in investigating growth on sulfur sources. The ability to grow on other sulfur sources was shared with *S. cerevisiae* and *C. glabrata*. The only difference was that the *C. glabrata met15*Δ strain could not utilize glutathione as a sulfur source, but *C. albicans met15*Δ strain could efficiently utilize glutathione as a sulfur source. We used this glutathione-dependent growth of *met15*Δ strains of *C. albicans* to identify and investigate the glutathione transport and degradation pathways.

The Oligopeptide Transporters (OPT1–OPT5) Are Not Involved in Glutathione Transport in C. albicans—The first step in the glutathione utilization pathway of yeasts is transport. In *S. cerevisiae* glutathione transport is mediated by the glutathione transporter Hgt1p, which belongs to the family of oligopeptide transporters (11). The absence of glutathione utilization in *C. glabrata* was shown to be due to the absence of homologues of HGT1 (9). Analysis of the *C. albicans* genome

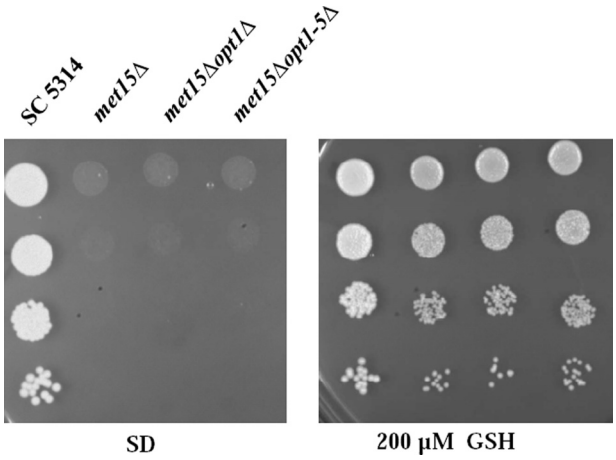

FIGURE 2. **OPT1-OPT5 are not involved in glutathione transport in *C. albicans*.** The *C. albicans* WT *met15*Δ, *opt1*Δ, and *opt1*Δ-*opt5*Δ strains were grown to exponential phase in minimal medium containing methionine, harvested, washed, and resuspended to an A_{600} = 0.2. Serial dilutions were spotted on minimal medium containing glutathione as described under "Experimental Procedures" and in the legend to Fig. 1.

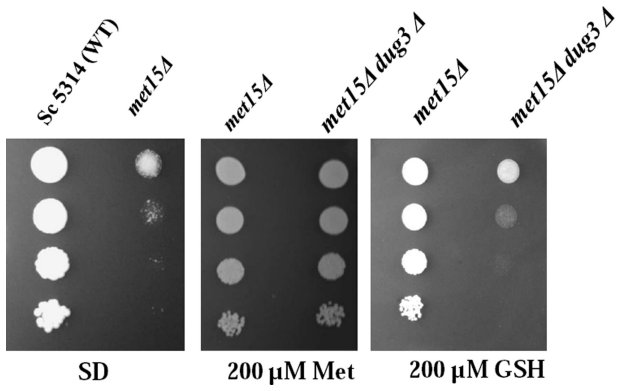

FIGURE 3. **DUG pathway is essential for glutathione degradation in *C. albicans*.** The C. *albicans* WT, *met15*Δ, and *met15*Δ-*dug3*Δ strains were grown to exponential phase in minimal medium containing methionine, harvested, washed, and resuspended to an A_{600} = 0.2. Serial dilutions were spotted on minimal ammonium chloride medium containing methionine and glutathione as described under "Experimental Procedures" and in the legend to Fig. 1.

has revealed the presence of eight members of the oligopeptide transporter family. Seven of these belong to the peptide transporter clade (OPT1–OPT7), whereas OPT8 belonged to the more remote YSL (yellow stripe-like) clade. The PT clade members include the previously identified ORFs: *OPT1*, *OPT2*, *OPT3*, *OPT4*, and *OPT5*, which were found to be induced during nitrogen limiting conditions and shown to transport different oligopeptides of different sequence and sizes, as well as two other hypothetical ORFs, OPT6 and OPT7 (16). The OPT1 of *C. albicans*, which shows maximum similarity and identity to HGT1 (25), did not display significant capability to transport glutathione during heterologous complementation in *S. cerevisiae* (13). To evaluate the contribution of OPT1 and the combined contribution of OPT1 to OPT5 in glutathione uptake in its native host, we created *met15*Δ knockouts in previously deleted *opt1*Δ and *opt1–5*Δ mutant strains, the latter being deleted for five OPTs (16). We observed that both *opt1*Δ*met15*Δ and *opt1–5*Δ *met15*Δ strains showed a very marginal (almost negligible) growth defect on glutathione as compared with *met15*Δ strains grown on glutathione (Fig. 2). This indicated that OPT1, as observed previously by heterologous complementation in *S. cerevisiae met15*Δ*hgt1*Δ, was not contributing to glutathione transport in *C. albicans* in any significant manner. Furthermore, none of the other four OPTs, OPT2–OPT5, were contributing to glutathione transport either. We corroborated these findings biochemically by carrying out glutathione uptake assays in different strains carrying the different OPT genes under control of the ADH1 promoter that had been integrated into an *opt123*Δ mutant strain (16). We compared the accumulation of radiolabeled glutathione ([^{35}S]GSH) in the *opt12345*Δ delete strain with the OPT1–OPT5 genes expressed under constitutive ADH1 promoter. Although OPT1, OPT2, and OPT5 led to a slight accumulation of radiolabeled glutathione as compared with the deletion strain opt*12345*Δ, the contributions did not appear to be significant (data not shown). These observations further confirm that OPT1 to OPT5 family members of *C. albicans* do not function as glutathione transporters of the cell.

The DUG Pathway Is Essential for Glutathione Degradation in C. albicans—The ability of *C. albicans met15*Δ to grow on glutathione as a sulfur source in the absence of involvement of the OPTs OPT1–OPT5 was surprising. The immediate question was whether glutathione needed to be transported inside to be utilized or whether it was being degraded extracellularly following the transport of the constituents inside. We addressed this issue by arguing that if glutathione was being transported inside, then it would most likely involve the alternative degradation pathway, the DUG pathway that required the DUG1/DUG2/DUG3 proteins. In *S. cerevisiae* exogenous utilization of glutathione as a sulfur source has been shown to be mediated by the alternative pathway of glutathione degradation (DUG pathway) (14).

Putative orthologues of the Dug proteins (Dug1p, Dug2p, and Dug3p) were identified in *C. albicans*, and to investigate the role of the Dug pathway in glutathione degradation in *C. albicans*, the DUG3 gene (19.5159) was deleted in a *met15*Δ background. This strain was severely compromised in its ability to utilize exogenous glutathione in *C. albicans*, clearly demonstrating that the Dug pathway is critical for utilization of extracellular glutathione in *C. albicans* (Fig. 3).

Isolation of OPT7 as a Glutathione Transporter in C. albicans—The absence of involvement of OPT1–OPT5 with the requirement for the DUG pathway suggested the presence of an unidentified transporter enabling efficient glutathione transport in *C. albicans*. To identify a possible transporter enabling growth on glutathione, we transformed a *S. cerevisiae met15*Δ*hgt1*Δ strain that lacked the ability to grow on glutathione as a sulfur source with a *C. albicans* genomic library made in an *S. cerevisiae* expression vector Yep13. The transformants were selected on glutathione-containing plates (100 and 150 μM), and seven clones appeared on these plates after 4 days. Four of these were confirmed as genuinely complementing the glutathione transport defect in *S. cerevisiae met15*Δ*hgt1*Δ strains. Plasmids were isolated and retransformed into *S. cerevisiae met15*Δ*hgt1*Δ strains, and after reconfirmation of complementation, the inserts were sequenced at the ends. All four clones contained one common ORF, *OPT7* (ORF 19.5673). Sequencing of the clone revealed that it had four polymorphic

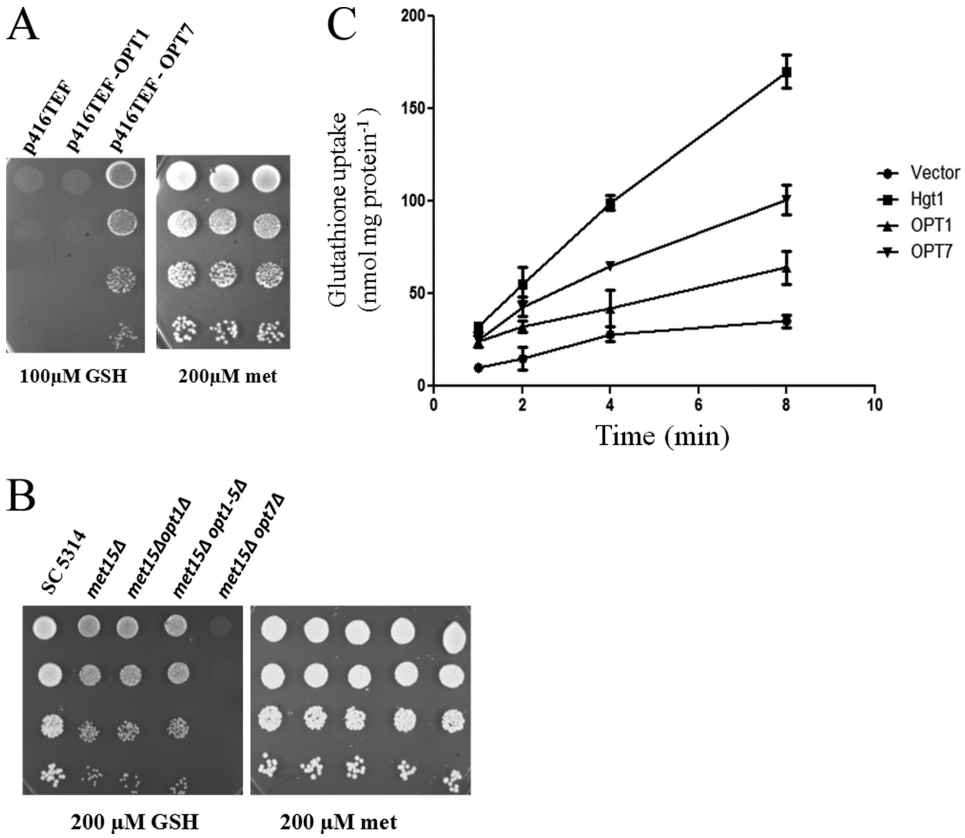

FIGURE 4. *A*, *OPT7* ORF expressed from TEF promoter complements the growth defect of *S. cerevisiae met15Δ hgt1Δ even* at low glutathione concentrations. Plasmid bearing *OPT7*, *OPT1*, and *HGT1* under the TEF promoter and the corresponding vector p416TEF were transformed into *met15Δhgt1Δ* (ABC 817), were grown to exponential phase in minimal medium containing methionine, harvested, washed, resuspended in water, and serially diluted to give A_{600}. Dilutions were spotted on minimal medium containing methionine and glutathione as described under "Experimental Procedures." *B*, disruption of *OPT7* in *C. albicans met15Δ* background results in growth defect at glutathione. The *met15Δ* and *met15Δ-opt7* strains were grown to exponential phase in minimal medium containing methionine, harvested, washed, and resuspended to an A_{600} = 0.2. Serial dilutions were spotted on minimal ammonium chloride medium containing glutathione as described under "Experimental Procedures." *C*, *OPT7* expressed in *S. cerevisiae met15Δ hgt1Δ* strain led to uptake of [^{35}S]glutathione. *met15Δhgt1Δ* strain of *S. cerevisiae* was transformed with *TEF-OPT7*, *TEF-OPT1*, and *TEF-HGT1* and with the corresponding vector control p416TEF. Transformants were grown in SD medium containing 200 μM methionine, and log phase grown cells were then harvested and used for the transport experiment as described under "Experimental Procedures." The data shown are the means of one experiment done in duplicate (each of the experiment repeated twice).

changes that differed from the sequence deposited in the Candida Genome Database, but corresponded to polymorphic changes in an allele of this ORF reported earlier (GenBank™ accession number DQ372680). To confirm whether the *OPT7* ORF was indeed responsible for complementing the defect of glutathione transport in *S. cerevisiae met15Δhgt1Δ*, we amplified this ORF from *C. albicans* genomic DNA by PCR, cloned it downstream of the TEF promoter, and checked for complementation in *S. cerevisiae*. As shown in Fig. 4*A*, *TEF-OPT7* complemented the glutathione transport defect of the *S. cerevisiae met15Δhgt1Δ* strain at low glutathione concentration.

To gain more direct information on the role of the OPT7 encoded protein in glutathione uptake, we measured the accumulation of [^{35}S]GSH in *S. cerevisiae met15Δhgt1Δ* cells. *OPT7* expressed from the TEF promoter led to a 2–3-fold higher accumulation of [^{35}S]GSH in cells as compared with the vector control (Fig. 4*C*). These observations further demonstrated the ability of OPT7 to transport glutathione.

To evaluate the involvement of OPT7 in GSH utilization in its native host *C. albicans*, we deleted *OPT7* in a *met15Δ* background and examined the ability of the *C. albicans met15Δopt7Δ* to utilize glutathione as a sulfur source. The *C. albicans met15Δ* and *met15Δopt7Δ* grew comparably on methionine as a sulfur source. However, a clear growth defect was observed for the *met15Δopt7Δ* mutant at low concentrations of glutathione (20, 50, 100, and 200 μM), as compared with the *met15Δ* (Fig. 4*B*). At higher glutathione concentrations, both strains could grow and could be a consequence of multiple OPTs contributing marginally to the transport that masked the phenotype at the higher glutathione concentrations. These results confirmed that *OPT7* is the major glutathione transporter in *C. albicans.*

The ability of OPT7 to take up glutathione from the extracellular environment also suggested that it was a plasma membrane transporter. To confirm this, we tagged OPT7 with a nine-amino acid HA tag at the C terminus and examined the localization in *S. cerevisiae*. The tagged construct was functional as seen from the complementation in the *S. cerevisiae met15Δhgt1Δ* (data not shown). Indirect immunofluorescence in using an anti-HA monoclonal antibody followed by the Alexa Fluor 488-conjugated secondary antibody images captured by confocal microscopy revealed a prominent signal on the plasma membrane with few intracellular patches (supplemental Fig. S1). These studies suggested that OPT7 localizes primarily to the plasma membrane.

OPT7 has a single CUG codon at position 575 predicted to code for serine in *C. albicans* and leucine in *S. cerevisiae*. We also mutated this codon so that it codes for serine in *S. cerevisiae* by site-directed mutagenesis. The Leu to Ser clone was functional and complemented the *S. cerevisiae met15*Δ*hgt1*Δ similar to the clone bearing the CUG codon with leucine at the position (data not shown).

Biochemical Characterization of OPT7 Reveals That It Is a Glutathione Transporter, with the Ability to Transport Tripeptides—To biochemically characterize *OPT7*, we exploited the *S. cerevisiae met15*Δ*hgt1*Δ strain as the host strain (11), transforming it with *TEF-OPT7* (and the corresponding vector) and thus eliminating any interference of other *C. albicans* OPTs contributing toward glutathione transport.

To determine the kinetic parameters of *OPT7*, the initial rate of glutathione transport of the transformants was measured over a range of glutathione concentrations. After deduction of the background glutathione uptake activity (associated with the *p416TEF* strain), a Lineweaver-Burke plot was obtained. The plot was linear (Fig. 5*A*), and determination of the kinetic parameters revealed a K_m of 205.5 ± 30.5 μM and V_{max} of 18.2 ± 4.9 nmol of glutathione·mg·protein^{-1} min^{-1}. We also determined the K_m without changing CUG codon (Leu to Ser) and obtained a value close but slightly higher at 314.5 ± 12.4 μM.

To examine the substrate specificity of the transporter, competitive transport studies were undertaken in which the initial rate of glutathione uptake was measured in the presence of a 10-fold excess of unlabeled competing ligand. The effect of a dipeptide, tripeptide, GSH, and GSSG was evaluated on the glutathione uptake by OPT7. Among the various compounds used, unlabeled glutathione was found to be the most effective competitor, blocking nearly 60% uptake with 10-fold and 70% with 20-fold excess, followed by GSSG and tripeptide (Gly-Gly-Gly) (Fig. 5*B*). The ability of GSSG to inhibit uptake mediated by OPT7 was similar to what has been observed with other high affinity glutathione transporters (11, 12) and is a reflection of the ability of the transporters to also transport glutathione conjugates. However, the significant inhibition by the tripeptide suggested that although OPT7 was principally a glutathione transporter, it might also be able to transport tripeptides as well. This possibility was examined by a growth assay in which we evaluated the ability of the *S. cerevisiae met15*Δ *hgt1*Δ strain (transformed with *p416TEF-OPT7*) to grow on the cysteine-containing tripeptide Glu-Cys-Gly and the methionine-containing tetrapeptide Leu-Tyr-Met-Arg in addition to GSSG as a source of sulfur for growth. We observed that Glu-Cys-Gly and GSSG substrates were efficiently used as a sulfur source by OPT7, but not by OPT1. The latter transformants were not able to grow on these peptides. In contrast, although we did not observe any growth of the OPT7-transformed cells on the tetrapeptide Leu-Trp-Met-Arg, OPT1 grew efficiently on this tetrapeptide as expected from previous observations on OPT1 (16). The observations with OPT7 are also in contrast to the *S. cerevisiae* glutathione transporter, Hgt1p, which did not show any growth on Glu-Cys-Gly, unlike OPT7 (Fig. 5*C*). Put together, these observations, along with the inhibition studies, suggest that OPT7 mediates uptake of tripeptides in addition to glutathione and its conjugates and thus, despite being a glutathione transporter, has a broader specificity.

The pH dependence studies indicated that GSH uptake mediated by OPT7 was optimal at pH 5.0, and strongly decreases at higher pH and lower pH (data not shown). Preincubation with the metabolic inhibitors, such as sodium azide and carbonyl cyanide *p*-chlorophenylhydrazone, resulted in a considerable loss in glutathione uptake (Fig. 5*B*), suggesting that OPT7-mediated glutathione uptake is likely to be an energy-dependent process.

Glutathione Transport by OPT7 Is Significantly Repressed When Cells Are Pregrown in Methionine—Glutathione transporters *of S. cerevisiae* and *S. pombe* have been shown to be regulated by the sulfur regulatory network, a further demonstration that their true function is in glutathione transport. Considering the ability of OPT7 to take up tripeptides as well, it was important to obtain information on its regulation so as to throw light on its true physiological function.

Genome wide expression profiling studies have revealed that *OPT7* is induced 5.6-fold by cadmium (26). Cadmium causes depletion of glutathione levels and has been shown to cause sulfur pathway derepression in *S. cerevisiae* (27). To investigate whether OPT7 regulation is under sulfur limitation, glutathione uptake was measured in both *met15*Δ and *met15*Δ*opt7*Δ strains after growing it in sulfur-starved and sulfur-rich medium. In a *met15*Δ strain, uptake of GSH was maximum in nonrepressing sulfur conditions and considerably repressed in the presence of sulfur-rich medium. Moreover, the fold induction in the OPT7 expression between the poor methionine and the rich methionine medium was 3-fold, whereas it was just 1.5-fold in the *met15*Δ*opt7*Δ background (Fig. 6*A*). These studies suggest that OPT7 is under sulfur regulation. Interestingly, when we examined the promoter of *OPT7*, we observed a CBF1 binding motif, TCACGTG, at position −145. Importantly, this motif was not present in any of the other *C. albicans* OPTs but was interestingly also observed in the *DUG3* promoter at position −166. CBF1 disruptions in *C. albicans* have been shown to lead to cysteine and methionine auxotrophy (28), and a regulatory role in sulfur metabolism is likely to be played by this protein in *C. albicans*, similar to the role of the orthologous protein in *S. cerevisiae*.

To investigate a possible role for CBF1 in the glutathione utilization pathway, we initially examined the growth of *C. albicans cbf1*Δ in different sulfur sources. We observed that the strain could grow on cysteine and methionine (as previously reported (28)), but it does not grow on glutathione (Fig. 6*B*), clearly implicating a role in glutathione utilization. We further measured the transport capabilities of glutathione uptake in the *cbf1*Δ, *met15*Δ, and *met15*Δ*opt7*Δ strains after growing it in sulfur-starved medium. Significantly reduced glutathione transport activity was observed in the *cbf1*Δ strain of *C. albicans* and was comparable with the transport in the *met15*Δ*opt7*Δ (Fig. 6*C*). These results strongly suggest that the sulfur regulatory factor CBF1 is essential for expression of OPT7 in glutathione utilization.

Glutathione Transport and Glutathione Utilization Are Not Essential for Virulence of C. albicans in a Mouse Model of Systemic Candidiasis—To determine whether the ability to transport and utilize exogenous glutathione was essential for the

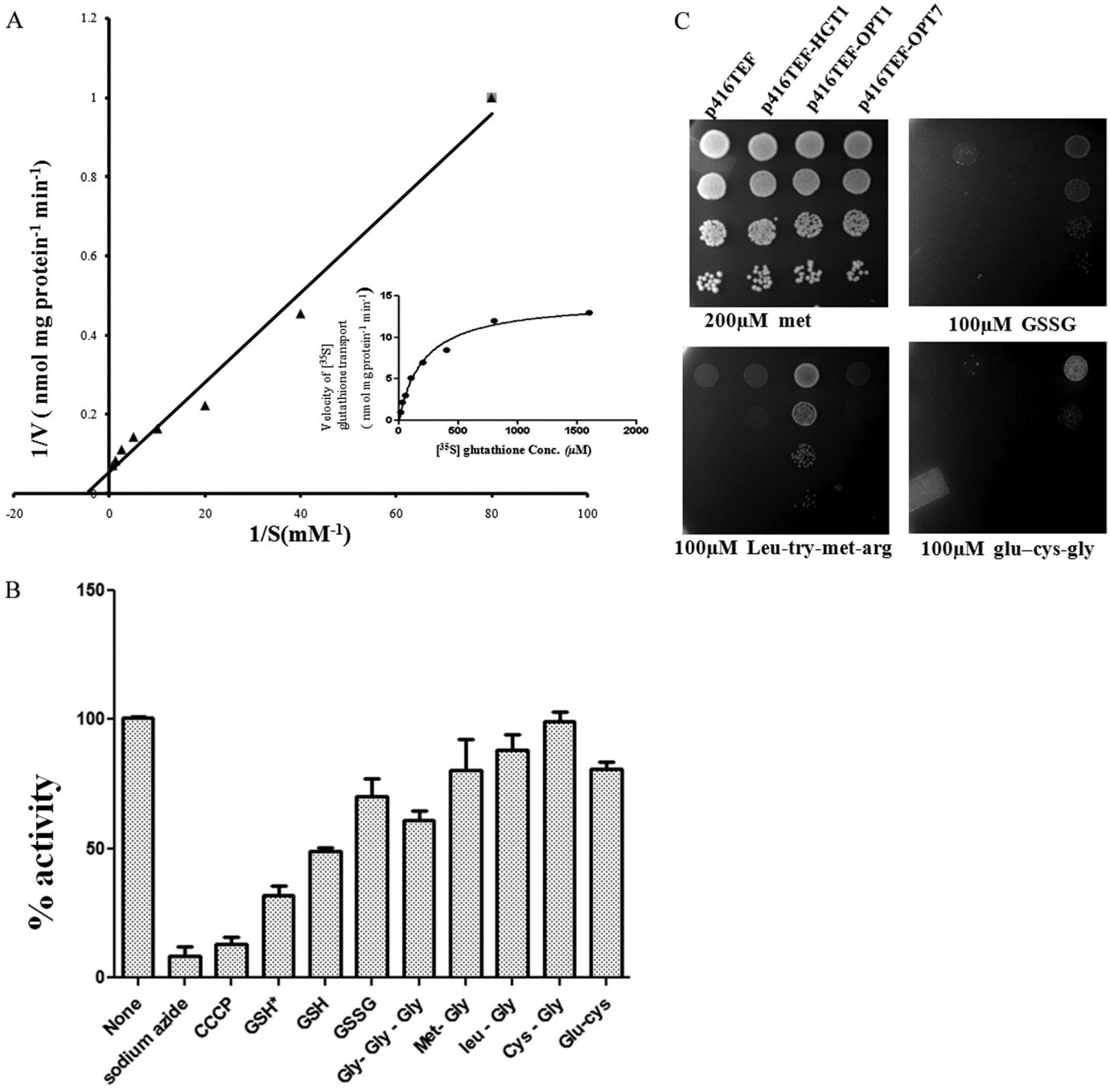

FIGURE 5. *A*, Lineweaver-Burke plot for glutathione uptake in *met15*Δ strain to determine kinetic parameters of OPT7. The initial rates of glutathione uptake were measured at glutathione concentrations ranging from 12.5 to 1600 μM, by harvesting cells incubated with radiolabeled glutathione at 30- and 180-s time intervals described under "Experimental Procedures." The data are representative from three experiments (done in duplicate). *Inset*, Michaelis-Menten curve for the glutathione uptake mediated by OPT7. Michaelis-Menten plot drawn using Prism version 5.02 (GraphPad Software, San Diego, CA). *B*, OPT7 is an active transporter, and it is specific for glutathione, tripeptide, and glutathione derivatives. Inhibition of glutathione uptake by different compound was measured in *S. cerevisiae met15*Δ*hgt1*Δ strain transformed with the glutathione transporter *OPT7* in the presence of different inhibitors added at 10-fold (*, 20-fold) excess over the labeled substrate (200 μM). The cells were harvested at 30- and 180-s intervals. For the metabolic inhibitors, the cells were preincubated with 100 μM carbonyl cyanide *p*-chlorophenylhydrazone (*CCCP*) and sodium azide for 15 min before measuring the initial rate of glutathione uptake at 30 and 180 s. The results were normalized to rate of uptake measured in the absence of any other compound. The data are representative of two different experiments and shown as the means ± S.D. *C*, comparison of utilization of tripeptides and tetrapeptides as a sulfur source by *OPT7*, *OPT1*, and *HGT1* expressed in *S. cerevisiae met15*Δ*hgt1*Δ. The *S. cerevisiae met15*Δ*hgt1*Δ strain was transformed with *TEF-OPT7*, *TEF-OPT1*, and *TEF-HGT1* and with the corresponding vector control p416TEF. Transformants were grown in SD medium containing 200 μM methionine, and dilution spotting was done on γ-Glu-Cys, GSSG, α-Glu-Cys-Gly, and Leu-Tyr-Met-Arg as described under "Experimental Procedures" and in the legend to Fig. 1.

survival of *C. albicans in vivo*, we examined the virulence of *C. albicans* strains deleted either in OPT7 or DUG3 for their survival *in vivo* in a systemic murine model of candidiasis in BALB/c mice. The mice were infected intravenously with an inoculum of yeast (5×10^5 cells), and the survival of mice was monitored on a daily basis. The majority of mice infected with wild-type *C. albicans* could not survive beyond 11 days, with death onset seen as early as day 4. All of the mice infected with

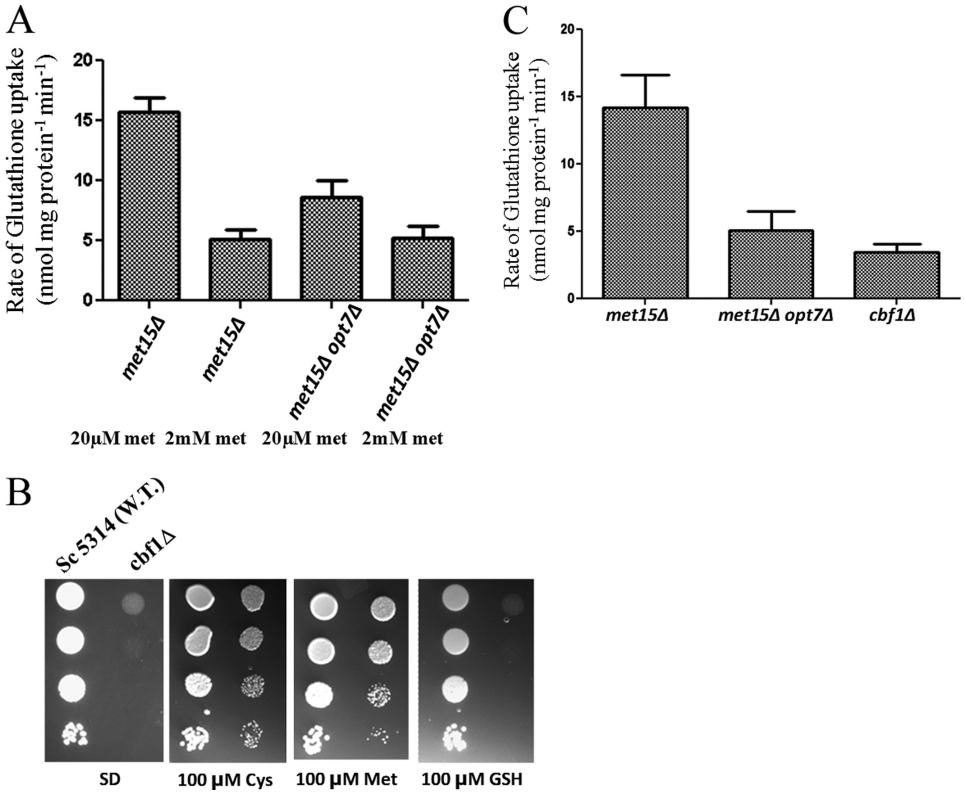

FIGURE 6. *A*, expression of OPT7 is under sulfur regulation. Strains *met15*Δ and *met15*Δ*opt7*Δ were grown in minimal ammonia medium containing 20 μM methionine (nonrepressive sulfur) and 1 mM methionine (repressive sulfur), and the initial rate of [^{35}S]glutathione uptake was calculated by measuring the radiolabeled glutathione accumulated in the cells at 30- and 180-s time intervals. *B*, *C. albicans cbf1*Δ can use cysteine and methionine as sulfur sources but fail to grow on glutathione. The *C. albicans* WT and *cbf1*Δ strains were grown to exponential phase in minimal medium containing methionine, harvested, washed, resuspended in water, and serially diluted to give 0.2, 0.02, 0.002, and 0.0002 A_{600} of cells. 10 μl of these dilutions were spotted on minimal ammonium chloride medium containing methionine, cysteine, and glutathione. The photographs were taken after 4 days of incubation at 30 °C. *C*, *C. albicans cbf1*Δ strain defective in glutathione uptake. *C. albicans met15*Δ, *met15*Δ*opt7*Δ, and *cbf1*Δ were grown in minimal ammonia medium containing 40 μM methionine (nonrepressive sulfur). The initial rate of [^{35}S]glutathione uptake was calculated by measuring the radiolabeled glutathione accumulated in the cells at 30- and 180-s time intervals.

the *C. albicans opt7*Δ and *dug3*Δ strains behave similar to the wild-type strain (Fig. 7). These results revealed that glutathione transport and utilization are not required for virulence in *C. albicans.*

To examine whether the *OPT7* gene was expressed *in vivo*, we employed the IVET developed for *C. albicans* (29, 30). Based on this technique, the *OPT7* promoter was fused to FLP recombinase, and expression of the FLP recombinase would lead to eviction of a mycophelonic resistance cassette flanked by FRT sites that was integrated into the same strain (29). The expression of *OPT7 in vivo* would lead to expression of FLP recombinase and eviction of the MPA resistance cassette that would be seen as appearance of small colonies (sensitive colonies) on MPA plates. In contrast, a lack of induction would lead to a lack of FLP recombinase expression and consequently the appearance of only large colonies reflecting the MPA resistance cassette being intact.

As controls, we used strains carrying the SAP5 fusions to FLP recombinase described earlier (29). These are known to be expressed *in vivo*. The mice were injected with each of these fusion constructs along with control strains S2UF1A (which contains the mycophenolic acid cassette and gives rise to large sized colonies on MPA-containing plates) and S2F1^{s} (which lacks the mycophenolic acid cassette and gives rise to small sized colonies on MPA-containing plates). Two mice were sacrificed at 2, 4, and 6 days, and the right and left kidneys were separately homogenized; aliquots of the extract were plated on YPD medium containing mycophenolic acid (1 mg/ml). We observed that in the case of SAP5, we could observe numerous small colonies appearing at different time points, indicating *in vivo* expression even after 2 days post-infection. In contrast, in the case of OPT7 only large-sized colonies, and no small colonies were observed even after 6 days post-infection (Table 2). The results suggested that OPT7 was not being induced *in vivo* in the infection model used in this study.

DISCUSSION

The work described in this study demonstrates the presence of an efficient transport and degradation pathway for glutathione in *C. albicans*. The pathway involves OPT7, a member of the oligopeptide transporter family, and DUG3, a component of the DUG pathway of glutathione degradation (14). The K_m of OPT7 for glutathione was 205 μM and reflects a reasonably high affinity glutathione transporter. The study seems to suggest that the essentiality of glutathione biosynthesis for the survival of this yeast *in vivo* may not be a consequence of an inefficient pathway of glutathione utilization. A possible explanation is that it is a consequence of the demand of high glutathione levels for the yeast pathogen *in vivo* that cannot be met from external sources.

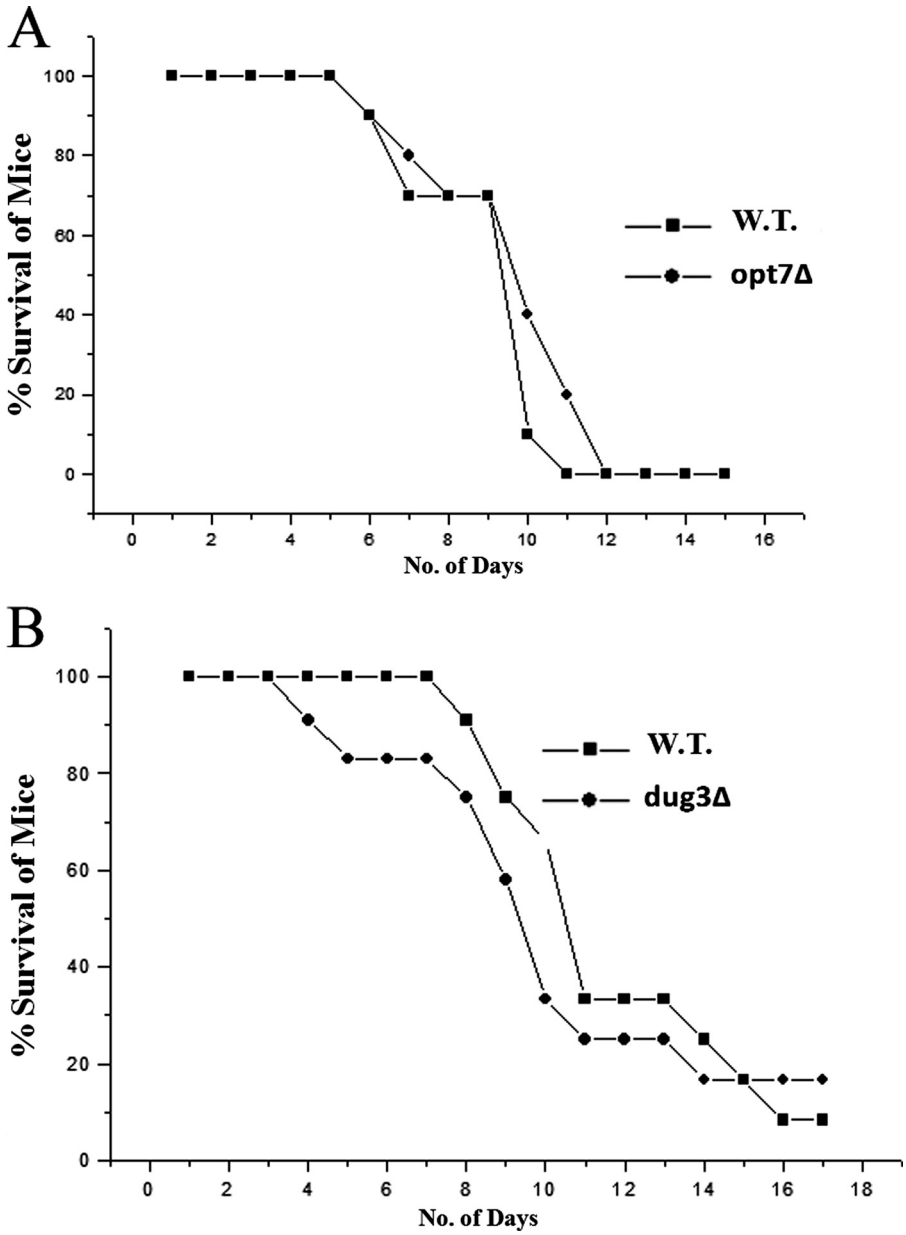

FIGURE 7. **Comparison of virulence of *C. albicans opt7*Δ (*A*), *dug3*Δ (*B*), and WT in mice.** Survival (%) for mice injected with *C. albicans* is shown. Ten BALB¢ male mice were injected with 5×10^5 cells of *opt7*Δ, *dug3*Δ, and WT.

TABLE 2

OPT7 expression *in vivo* by IVET

The mice were injected with 5×10^5 cells/mice of *C. albicans* with the strains S2U11A,S2F11A^S, S5F12A, and OPT7FL1AB. The mice were sacrificed at 2, 4, and 6 days post-infection, the right and left kidneys were isolated and crushed in tissue homogenizer, and different dilutions were plated on indicator plates containing 1 mg·ml^{-1} MPA. A percentage of small colonies (identified in expression) are shown (two mice/time point); however, for the S5FI2A strain, at 4 days, only one mouse was sacrificed.

Strains	Percentage of small colonies after infection at different days		
	2 days	4 days	6 days
	%	%	%
S2U11A	0	0	0
S2F11A^S	100	100	100
S5FI2A	44	85	100
OPT7FLIA	0	0	0

The observation that among the seven OPTs (of the peptide transporter clade of the OPT superfamily), it was OPT7, the most remote OPT to the known glutathione transporters (HGT1 of *S. cerevisiae* and PGT1 of *S. pombe*), that was the glutathione transporter in *C. albicans* was completely unexpected and intriguing. OPT7 is unusual not only in its remoteness to HGT1/PGT1 but in that it belongs to a different cluster that lacks the conserved disulfide bonding cysteine pairs (21) seen in the larger OPT family, including other *C. albicans* OPTs, OPT1–OPT6. Because disulfide-bonding cysteine residues are more conserved even than tryptophan and are rarely lost in evolution, it would be interesting to know the basis of this peculiar evolution. Previous studies on the oligopeptide transporter superfamily have demonstrated that in the PT clade almost all members contain two conserved cysteines at positions corresponding to Cys-622 and Cys-632 of Hgt1p and were indispensable for trafficking to the cell surface and its functionality (21). The only members of the PT clade that did not contain these two cysteines were a small cluster of members that

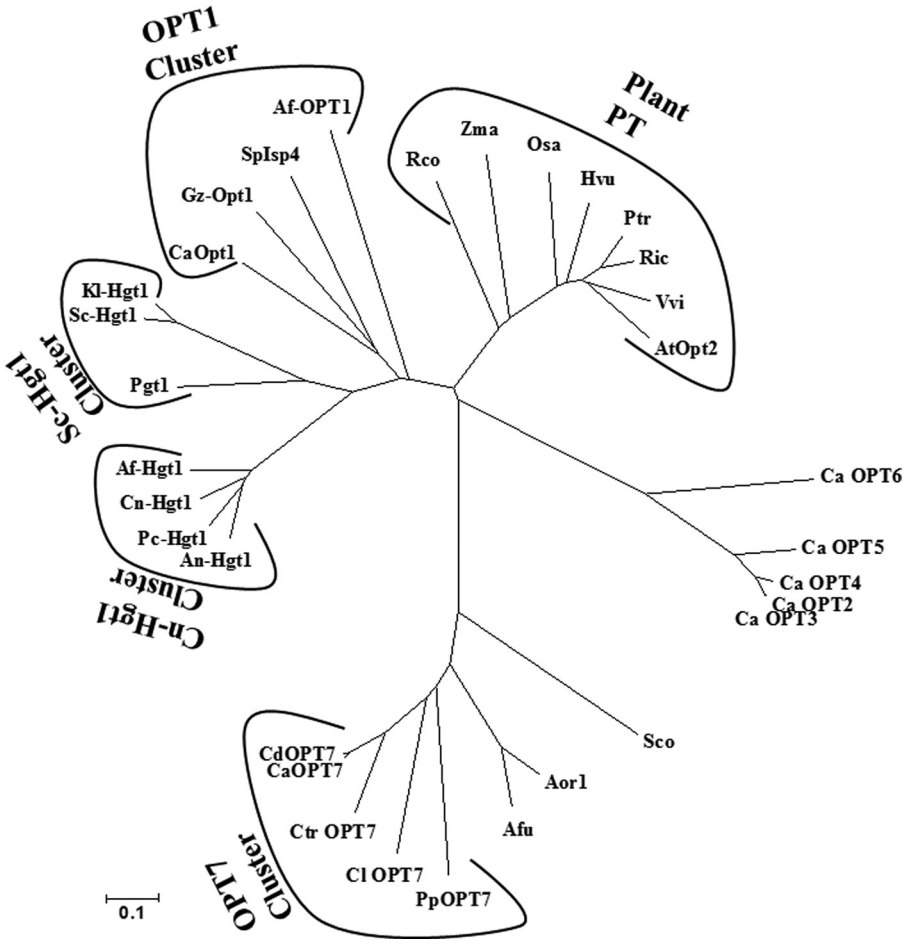

FIGURE 8. **The homologous proteins closely related to OPT7 of *C. albicans* form distinct clusters.** OPT7 homologous proteins were retrieved from Entrez for multiple sequence alignment of the representative members to construct phylogenetic tree (listed in supplemental Table S1).

included OPT7 (21). Multiple sequence alignment of all the OPTs, from OPT1 to OPT7 of *C. albicans*, revealed that in these seven members also, it was only OPT7 that did not contain these two conserved cysteines (data not shown). A phylogenetic tree of all the *Candida* OPTs along with the known glutathione transporters (Fig. 8 and supplemental Fig. S2 and Table S2) reveals that OPT7 transporters form a distinct cluster that was clearly quite remote from the others. It is interesting that although *C. albicans*, *Candida tropicalis*, and *Candida lusitaniae* have OPT7 transporters, *C. lusitaniae* also has a member in the HGT1 cluster. Because the *C. lusitaniae* member in the HGT1 cluster also contains the conserved Phe and Gln residues in transmembrane domain 9, it is likely to be a glutathione transporter (13, 31), and it would be interesting to see whether the *C. lusitaniae* OPT7 is also a glutathione transporter. The phylogenetic analysis also allows us to cluster the different OPTs, and it is interesting to observe a large number of members of OPT4 and OPT5 among *C. tropicalis*. Another unusual feature of OPT7 as a glutathione transporter is that unlike the high affinity glutathione transporters of *S. cerevisiae* and *S. pombe*, which only transport glutathione (and glutathione conjugates), OPT7 also displays the ability to transport tripeptides as shown above.

Interestingly, whereas orthologues of the glutathione transporters (HGT1 of *S. cerevisiae*/PGT1 of *S. pombe*) are present in almost all organisms in the fungal/plant kingdom, OPT7 orthologues were clearly absent in *S. cerevisiae* or *S. pombe* and seemed to be more prevalent in pathogenic organisms, being found in all the *Candida* spp. (barring *C. glabrata*, which lacks any members of the family), and also fungi belonging to the *Aspergilli* family.

The observation that neither OPT7 nor DUG3 was required for virulence was interesting. External glutathione can meet two requirements of the yeast pathogen. The first is the requirement of glutathione as a redox compound and in iron metabolism. The second use of glutathione is as a sulfur source. The lack of requirement of DUG3 clearly reflects that glutathione is not an important sulfur source *in vivo* (despite its abundance). The lack of requirement of OPT7 reinforces this aspect but additionally suggests that it cannot meet the high glutathione requirements *per se*.

Although OPT7 was regulated by sulfur sources, we could not observe any expression *in vivo* using the IVET approach. This observation is consistent with the observation that *OPT7* disruptants had no effect on virulence in the mouse model of systemic candidiasis and may also explain the need for glutathione biosynthesis *in vivo*. However, the observation also suggests that the cells were not experiencing a sulfur starvation *in vivo*. The other sulfur-regulated promoter that has been extensively used in *C. albicans* research is the MET3 promoter. This

promoter is derepressed many-fold in the absence of both cysteine and methionine. *In vivo* studies carried out with gene fused to this promoter have also revealed that the promoter is not induced to any significant extent *in vivo* (32, 33). This suggests that the yeasts do not face a sulfur starvation *in vivo*, at least in the mouse model of systemic candidiasis. The lack of OPT7 expression *in vivo* (or at least below detection levels of the assay) is in keeping with these results, but considering the higher prevalence of OPT7 among pathogenic yeasts, it will be interesting to see whether in other models of infection, similar or different observations would be observed.

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