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Mutants in the *Candida glabrata* Glycerol Channels Are Sensitized to Cell Wall Stress

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Many fungal species use glycerol as a compatible solute with which to maintain osmotic homeostasis in response to changes in external osmolarity. In *Saccharomyces cerevisiae*, intracellular glycerol concentrations are regulated largely by the high osmolarity glycerol (HOG) response pathway, both through induction of glycerol biosynthesis and control of its flux through the plasma membrane Fps1 glycerol channel. The channel activity of Fps1 is also controlled by a pair of positive regulators, Rgc1 and Rgc2. In this study, we demonstrate that *Candida glabrata*, a fungal pathogen that possesses two Fps1 orthologs and two Rgc1/-2 orthologs, accumulates glycerol in response to hyperosmotic stress. We present an initial characterization of mutants with deletions in the *C. glabrata FPS1* (CAGL0C03267 [www.candidagenome.org]) and *FPS2* (CAGL0E03894) genes and find that a double mutant accumulates glycerol, experiences constitutive cell wall stress, and is hypersensitive to treatment by caspofungin, an antifungal agent that targets the cell wall. This mutant is cleared more efficiently in mouse infections than is wild-type *C. glabrata* by caspofungin treatment. Finally, we demonstrate that one of the *C. glabrata RGC* orthologs complements an *S. cerevisiae rgc1 rgc2* null mutant, supporting the conclusion that this regulatory assembly is conserved between these species.

Under conditions of high osmolarity stress, many fungal species maintain osmotic equilibrium by producing and retaining high concentrations of glycerol as a compatible solute (18, 20, 30, 34). The intracellular glycerol concentration is regulated in *Saccharomyces cerevisiae* in part by the plasma membrane aquaglyceroporin, Fps1 (13, 26, 37, 39). Increased external osmolarity induces Fps1 closure, whereas decreased osmolarity causes channel opening, both within seconds of the change in external osmolarity (39). This channel, which functions as a homotetramer (2), is required for surviving a hypoosmotic shock, when yeast cells must export glycerol rapidly to prevent bursting (26, 39). Fps1 is also required for controlling turgor pressure during fusion of mating yeast cells (33).

Relative to other characterized aquaglyceroporins, Fps1 possesses N-terminal and C-terminal cytoplasmic extensions that are important for its regulation (11, 38). The pathway responsible for regulation of Fps1 in response to changes in osmolarity has not been fully delineated but involves the mitogen-activated protein (MAP) kinase Hog1 (high osmolarity glycerol response) (13, 39), which binds to the N-terminal domain of Fps1 (29). Hog1 is activated in response to hyperosmotic stress to mediate both the biosynthesis of glycerol and its retention within the cell (13). Fps1 activity is also controlled by a pair of positive regulators, named Rgc1 and Rgc2 (for regulator of the glycerol channel 1 and 2; *YPR115W* and *YGR097W*, respectively) (1). Rgc1 and Rgc2 are phosphorylation targets of Hog1, and their positive regulation of Fps1 is inhibited by Hog1 phosphorylation (1). Loss of either *FPS1* or *RGC1* and *RGC2* function results in excess turgor pressure and consequent cell wall stress, to which the cell responds by fortifying the cell wall (1). Additional cell wall stress imposed upon these mutants results in cell lysis. Although the fungal kingdom is replete with Rgc orthologs, they are not represented in metazoans, suggesting that the Rgc-Fps pathway may be an attractive target for antifungal-drug development.

Candida species are the most common cause of systemic fungal infections in humans (4, 5). Although *Candida albicans* infections are the most prevalent among these, *Candida glabrata* is the second most common cause of such infections, accounting for approximately 15% of *Candida* bloodstream infections worldwide (17). Despite its status as an opportunistic human pathogen, *C. glabrata* is phylogenetically more closely related to the baker's yeast, *S. cerevisiae*, than it is to other *Candida* species associated with human disease (17).

The most recently introduced class of antifungal agents is the echinocandins, which include caspofungin, micafungin, and anidulafungin. These are lipopeptides that interfere with cell wall biosynthesis by inhibition of β-1,3-glucan synthase, the enzyme responsible for synthesizing the major structural polymer of the fungal cell wall (23). This class of antifungals is effective against *Candida* (particularly *C. albicans* and *C. glabrata*) and *Aspergillus* species (7). Significantly, *S. cerevisiae rgc1 rgc2* null mutants are hypersensitive to caspofungin treatment because of their increased requirement for β-1,3-glucan synthase activity (1).

The *C. glabrata* genome possesses two orthologs of *S. cerevisiae FPS1*, hereinafter designated *CgFPS1* and *CgFPS2*. These genes encode the only clear Fps1 orthologs that include the regulatory N-terminal and C-terminal extensions that have been identified among pathogenic fungal species. Additionally, *C. glabrata* pos-

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TABLE 1 *S. cerevisiae* and *C. glabrata* strains

Strain	Relevant genotype	Source
DL3187	*MAT***a** S288c (BY4742) *his3*Δ *leu2*Δ *ura3*Δ *lys2*Δ	Research Genetics
DL3188	*MAT*α S288c *rgc1*Δ::*KanMX* *rgc2*Δ::*KanMX*	1
DL3207	*MAT***a** S288c *rgc1*Δ::*KanMX* *rgc2*Δ::*KanMX*	1
DL3226	*MAT***a** S288c *fps1*Δ::*KanMX*	Research Genetics
DL3229	*MAT*α S288c *fps1*Δ::*KanMX*	Research Genetics
DL3799	*MAT*α S288c *fps1*Δ::*ScFPS1-Flag-HPH*	This study
DL3800	*MAT*α S288c *fps1*Δ::*CgFPS1-Flag-HPH*	This study
DL3801	*MAT*α S288c *fps1*Δ::*CgFPS2-Flag-HPH*	This study
DL3802	*MAT*α S288c *rgc1*Δ::*KanMX* *rgc2*Δ::*ScRGC2-Flag-HPH*	This study
DL3803	*MAT*α S288c *rgc1*Δ::*KanMX* *rgc2*Δ::*CgRGC1-Flag-HPH*	This study
DL3804	*MAT*α S288c *rgc1*Δ::*KanMX* *rgc2*Δ::*CgRGC2-Flag-HPH*	This study
BG2	*C. glabrata* wild type	6
DL3614	*C. glabrata* BG14 *ura3*Δ::Tn*903* G418r	27
DL3616	*C. glabrata* BG14 *fps1*Δ::*frt*, no. 1	This study
DL3618	*C. glabrata* BG14 *fps1*Δ::*frt*, no. 2	This study
DL3675	*C. glabrata* BG14 *fps2*Δ::*frt*, no. 1	This study
DL3677	*C. glabrata* BG14 *fps2*Δ::*frt*, no. 2	This study
DL3679	*C. glabrata* BG14 *fps1*Δ::*frt fps2*Δ::*frt*, no. 1	This study
DL3681	*C. glabrata* BG14 *fps1*Δ::*frt fps2*Δ::*frt*, no. 2	This study
BG3075	*C. glabrata* BG14 *fps1*Δ::*frt fps2*Δ::*frt URA3*	This study
BG3129	*C. glabrata* BG14/pGRB2.0	This study
BG3130	*C. glabrata* BG14 *fps1*Δ::*frt* *fps2*Δ::*frt*/pGRB2.0	This study
BG3123	*C. glabrata* BG14 *fps1*Δ::*frt* *fps2*Δ::*frt*/pGRB2.0-*CgFPS1*	This study

TABLE 2 Plasmids

Plasmid	Description	Source
p2769	pGRB2.0 *C. glabrata* expression vector, *URA3*	8, 28
pNAT	pPCR2.1 with NAT^R cassette, flanked by FRT sites	This study
p2823	pAG32; integration vector, *hphMX4*	9
p3055	pRD16-*FLP1* recombinase vector	28
p3057	pGRB2.0-*ScFPS1 C. glabrata* vector, *ScFPS1*	This study
p3058	pGRB2.0-*CgFPS1 C. glabrata* vector, *CgFPS1*	This study
p3059	pGRB2.0-*CgFPS2 C. glabrata* vector, *CgFPS2*	This study
p3060	pGRB2.0-*ScPRM5pro-lacZ*	This study
p3061	pAG32-*ScFPS1* integration vector, *ScFPS1*	This study
p3062	pAG32-*CgFPS1* integration vector, *CgFPS1*	This study
p3063	pAG32-*CgFPS2* integration vector, *CgFPS2*	This study
p3064	pAG32-*ScRGC2* integration vector, *ScRGC2*	This study
p3065	pAG32-*CgRGC1* integration vector, *CgRGC1*	This study
p3066	pAG32-*CgRGC2* integration vector, *CgRGC2*	This study

sesses two *RGC* orthologs, hereinafter designated *CgRGC1* and *CgRGC2*. As a first step toward understanding the functions of these genes in a fungal pathogen, we report here the initial characterization of *C. glabrata* mutants with deletions of *FPS1* and *FPS2*.

MATERIALS AND METHODS

Yeast strains and growth conditions. The *S. cerevisiae* strains used in this study were all derived from the Research Genetics background S288c (Research Genetics, Inc., Huntsville, AL) and are listed in Table 1. Yeast cultures were grown in YPD (1% Bacto yeast extract, 2% Bacto peptone, 2% glucose) or SD (synthetic dextrose; 0.67% yeast nitrogen base, 2% glucose) medium supplemented with the appropriate nutrients to select for plasmids. Chromosomal gene integrations were created by replacing endogenous open reading frames with cassettes created in integration vector pAG32 (hphMX4) (9). Cassettes were constructed to contain a C-terminally Flag-tagged version of the replacement gene ligated at its 3′ end to the hphMX4 gene, which confers hygromycin B resistance. Linear PCR products amplified from the engineered cassettes were transformed into the appropriate haploid yeast strain. Integrants were selected on plates containing hygromycin B, and gene replacement confirmed by PCR analysis across the integration junctions.

All *C. glabrata* deletion strains were derived from a *ura3* derivative (BG14) of the clinical isolate BG2 (6) and are listed in Table 1. Gene deletions were created in strain BG14 by homologous recombination. A cassette containing the *C. albicans NAT1* (Ca*NAT1*) gene (35), which confers nourseothricin resistance, and flanked by FLP recombination target (FRT) recombination sites was generated by PCR and cloned into pPCR2.1 (Life Technologies, Carlsbad, CA) to generate pNAT. To delete genes, the NAT-FRT cassette was amplified by PCR using primers that included 50 nucleotides of 5′ and 3′ noncoding sequence from the *FPS1* or *FPS2* gene. These fragments were used to target the cassette to the integration site. Deletions were selected on plates containing nourseothricin and confirmed by PCR analysis across the integration junctions. To create the *fps1*Δ *fps2*Δ double mutant, the *NAT* cassette was recombined out by transforming the *fps1*Δ::*NAT* mutant with a vector expressing FLP1 recombinase (pRD16) (28). Flp1-induced recombination at the FRT sites recombined out the *NAT* cassette, resulting in restoration of nourseothricin sensitivity and allowing the generation of a second deletion by the same method. Deletion strains lacking the *NAT* gene were created in this way and used for phenotypic analyses. For mouse infection studies, *URA3* was restored to the *fps1*Δ::*FRT fps2*Δ::*FRT ura3*Δ::*Tn*903 strain either by transformation with the *CgURA3* gene or by transformation with pGRB2.0-based plasmids (28) followed by selection for uracil prototrophy.

Plasmids. Plasmids for the expression of *S. cerevisiae FPS1* (*ScFPS1*), *CgFPS1*, and *CgFPS2* were created by PCR amplification of genomic DNA of the coding regions of these genes with their respective promoters using forward primers containing a NotI site and reverse primers containing a SalI site. The amount of 5′ sequence included varied among these genes, being 800 bp for *ScFPS1* and 650 bp for *CgFPS1* and *CgFPS2*. DNA fragments were ligated into the *C. glabrata* expression vector pGRB2.0, a *URA3* CEN/ARS plasmid (28). This yielded pGRB2.0-*ScFPS1* (p3057), pGRB2.0-*CgFPS1* (p3058), and pGRB2.0-*CgFPS2* (p3059).

The *C. glabrata* cell wall stress reporter plasmid was constructed by amplifying the Sc*PRM5-lacZ* cassette from p1366 (14) using a forward primer containing an XbaI site and a reverse primer containing an XhoI site. The cassette was ligated into pGRB2.0 to yield pGRB2.0-*PRM5-lacZ* (p3060). The plasmids used for gene integration were created by amplifying the coding regions of the genes indicated below, which were ligated into pAG32 (p2823). *ScFPS1* was amplified using primers containing SalI and BglII sites (pAG32-*ScFPS1*; p3061), *CgFPS1* with primers containing PvuII and SalI sites (pAG32-*CgFPS1*; p3062), *CgFPS2* with primers containing SalI and BglII sites (pAG32-*CgFPS2*; p3063), *ScRGC2* with primers containing HindIII and PacI sites (pAG32-*ScRGC2*; p3064), *CgRGC1* with primers containing PacI and BglII sites (pAG32-*CgRGC1*; p3065), and *CgRGC2* with primers containing SalI and BglII sites (pAG32-*CgRGC2*; p3066) of pAG32. Plasmids are listed in Table 2. Primer sequences are available upon request.

Quantitative reverse transcriptase (qRT)-PCR. *C. glabrata* strain BG2 was grown to mid-log phase in YPD. A zero-time aliquot was centrifuged, washed once with diethyl pyrocarbonate (DEPC)-treated water, and flash frozen for subsequent RNA extraction. For hyperosmotic shock,

cells were centrifuged, and pellets were resuspended in either YPD or YPD supplemented with 1.8 M sorbitol. These cultures were grown at 30°C, and samples were taken at 1-h, 2-h, 4-h, and 20-h intervals. Each sample was centrifuged and washed once with DEPC-treated water before being flash frozen for RNA extraction.

RNA was obtained from each sample by acid phenol extraction, followed by ethanol precipitation. Samples were treated twice with DNase (New England BioLabs) to remove any genomic DNA contamination. Samples of 8 μg of RNA were used for cDNA synthesis in 20-μl reaction mixtures, using 1× cDNA buffer, reverse transcriptase, and oligo(dT) primers. Reaction mixtures without reverse transcriptase were used to determine genomic DNA contamination in subsequent qRT-PCRs. Any remaining RNA was removed by RNase H (New England BioLabs) treatment. The resulting cDNA was diluted 1:10 in sterile water.

PCR primers (Eurofins Mwg Operon, Inc., Huntsville, AL) specific for CgFps1 and CgFps2 were designed to produce amplicon sizes of 158 and 131 bp, respectively, with sequences as follows: CgFps1_QRTFwd, TTGCCATCCACATACCATCC; CgFps1_QRTRev, GAGTCCTGTTGTGCTTTACC; CgFps2_QRTFwd, TTGTCGGCTGATATCTTCCC; and CgFps2_QRTRev, AAGGCCCATAGAGCAAGACG. Standard curves for each primer set were created using 10-fold serial dilutions of BG2 genomic DNA, and values were normalized to the total histone H3 transcript level in each sample. PCR products were amplified using 5 μl of cDNA diluted 10× in 25-μl reaction mixtures containing 1× polymerase buffer, 1 mM $MgCl_2$, 200 μM deoxynucleoside triphosphates (dNTPs), 0.5 μM each primer, 1× EvaGreen nucleic acid dye (Biotium), and 0.25 units of *Taq* polymerase. The thermal cycling conditions were as follows (Bio-Rad CFX96 real-time system): initial denaturation at 95°C followed by 40 cycles of 95°C (10 s), 60°C (30 s), 72°C (30 s). A melting curve was performed starting at 65°C to test for the presence of nonspecific products.

Phenotypic analysis of the *C. glabrata fps*Δ mutants. Glycerol accumulation assays and β-galactosidase assays were carried out as described previously (1). Cell wall stress was imposed by exposing log-phase cells to 40 μg/ml calcofluor white for 5 h. Caspofungin was purchased from Merck.

Mouse infections. Infection of mice with *C. glabrata* was carried out as described previously (3, 27). Briefly, mice were infected by tail vein injection with either wild-type (BG2) or *fps1*Δ *fps2*Δ (BG3075) cells suspended in 0.2 ml of phosphate-buffered saline (PBS). In a second experiment, mice were infected with either wild-type (BG14) or *fps1*Δ *fps2*Δ (DL3679) cells bearing a *URA3*-containing vector or a plasmid expressing *CgFPS1*. In both experiments, 4 days after infection, mice were treated by intraperitoneal injection with caspofungin (0.2 ml PBS containing 2 mg/kg of body weight or 25 mg/kg) or 0.2 ml PBS alone (mock treatment). Three days after caspofungin administration, the mice were sacrificed and their kidneys, livers, and spleens were recovered. The organs were homogenized, and dilutions of the samples from the kidney, spleen, and liver were plated on YPD plates for recovery of viable fungal cells. CFU per organ were counted after 48 h of growth at 30°C.

RESULTS

***Candida glabrata FPS1* and *FPS2*.** The *C. glabrata* genome possesses two orthologs of the *S. cerevisiae FPS1* gene. The most closely related of these (CAGL0C03267) encodes a predicted protein of 652 amino acids that shares 66% sequence identity with *Sc*Fps1 through the C-terminal two-thirds of the protein with reduced identity through the predicted N-terminal cytoplasmic domain. We designate this gene *CgFPS1* herein. The other ortholog (CAGL0E03894) encodes a predicted protein of 602 amino acids that possesses a shorter N-terminal cytoplasmic domain than either *Sc*Fps1 or *Cg*Fps1 and shares 54% sequence identity with *Sc*Fps1 through the remainder of the protein. We designate this gene *CgFPS2* herein.

We constructed single *fps1*Δ and *fps2*Δ mutants and a double *fps1*Δ *fps2*Δ mutant in the *C. glabrata* strain BG14 (27). We tested these mutants for several distinctive phenotypes displayed by *S. cerevisiae fps1*Δ mutants. First, mutants with mutations in *ScFPS1* or its positive regulators, *ScRGC1* and *ScRGC2*, retain excess intracellular glycerol, which causes elevated turgor pressure and constitutive cell wall stress (1, 26, 39). These mutants are consequently hypersensitive to growth at elevated temperature (35 to 40°C) and to growth inhibition by the cell wall biosynthesis inhibitor caspofungin (1).

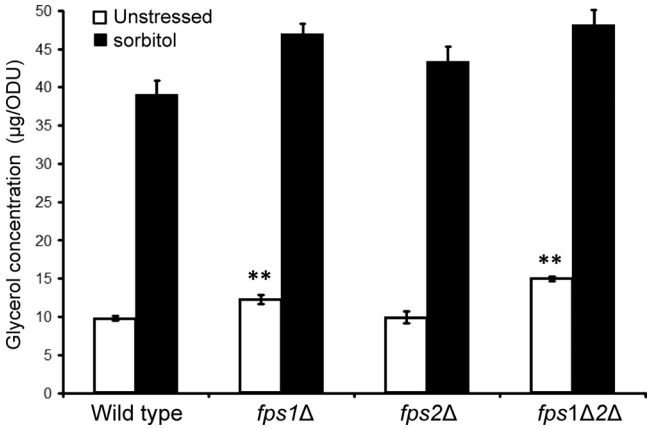

FIG 1 Retention of glycerol by *C. glabrata* in response to hyperosmotic stress and in *fps*Δ mutants. Glycerol concentrations of log-phase cells were determined in triplicate. *C. glabrata* strains were grown to mid-log phase in YPD and diluted into YPD with or without sorbitol (to 1.8 M) to induce hyperosmotic shock (15 min). Each value represents the mean and standard deviation from three independent experiments. Strains were the wild type (BG14) and *fps1*Δ (DL3616), *fps2*Δ (DL3675), and *fps1*Δ *fps2*Δ (*fps1*Δ*2*Δ) (DL3679) mutants. Statistical significance was determined using two-sample *t* tests with a two-tailed distribution. ODU, optical density units; **, $P < 0.005$.

Although accumulation of glycerol in response to hyperosmotic shock has been detected in many fungal species, it has not been demonstrated in *C. glabrata*. Therefore, we first measured the intracellular glycerol levels in cells exposed to hyperosmotic shock with 1.8 M sorbitol. The wild-type (BG14) strain accumulated approximately 4-fold more intracellular glycerol after 15 min (Fig. 1). This is comparable to the increase observed for wild-type *S. cerevisiae* exposed to the same treatment (approximately 6-fold) (1). This result demonstrates that *C. glabrata*, like many other fungal species, accumulates glycerol as a compatible solute in response to hyperosmotic shock. We also compared basal glycerol retention in the mutants with single and double mutations of the *CgFPS* genes. The *fps1*Δ mutant retained a slightly greater glycerol concentration than the wild-type strain, whereas the *fps2*Δ mutant did not (Fig. 1). However, the effect of deleting both genes appeared to be additive, because the *fps1*Δ *fps2*Δ mutant retained approximately 50% more glycerol than the wild-type strain. This is a considerably smaller difference than that observed for an *S. cerevisiae fps1*Δ mutant (approximately 12-fold) (1). The *S. cerevisiae fps1*Δ mutant accumulates so much glycerol that it fails to increase its glycerol levels further in response to hyperosmotic shock (1). This was not the case for the *C. glabrata fps1*Δ *fps2*Δ mutant (Fig. 1). These results suggest that the *Cg*Fps1 and *Cg*Fps2 proteins function as glycerol channels that contribute to the maintenance of osmotic balance and that *Cg*Fps1 is more important than *Cg*Fps2 in this regard.

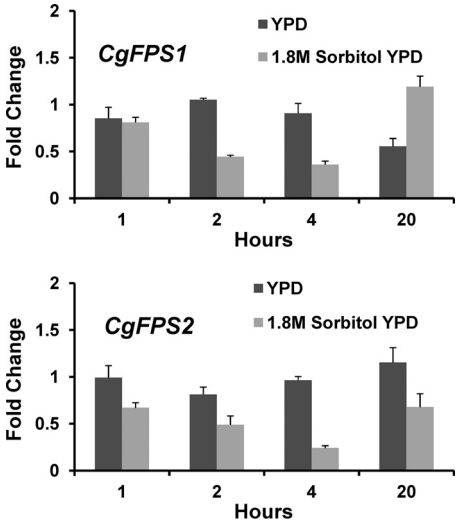

FIG 2 Expression of *C. glabrata FPS1* and *FPS2*. Wild-type (BG2) cells were grown to mid-log phase in YPD and diluted into YPD with or without sorbitol (to 1.8 M) to induce hyperosmotic shock for the indicated times. RNA was extracted and subjected to qRT-PCR analysis. Values are presented as relative to the value for the zero-time sample and represent the means and standard deviations of technical triplicate samples.

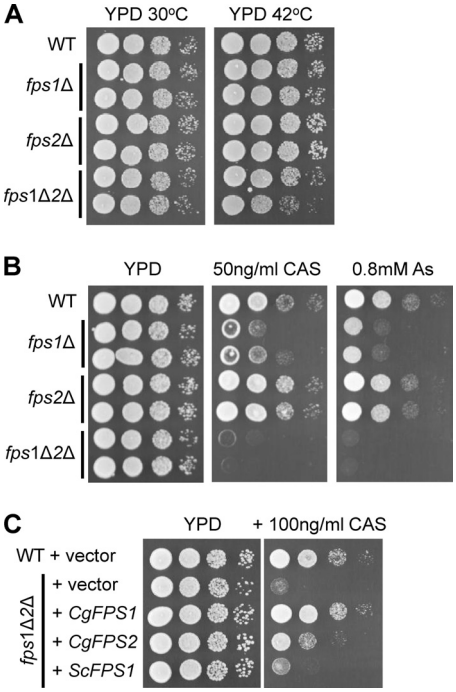

FIG 3 Phenotypes of *C. glabrata fps*Δ mutants. (A) Growth of mutants at elevated temperature. A wild-type (WT) *C. glabrata* strain (BG14) and pairs of independently derived *fps1*Δ (DL3616 and DL3618), *fps2*Δ (DL3675 and DL3677), and *fps1*Δ *fps2*Δ (*fps1*Δ*2*Δ) (DL3679 and DL3681) cells were grown to mid-log phase in YPD, and 10-fold serial dilutions were spotted onto YPD plates and incubated at 30°C or 42°C for 3 days. (B) Sensitivity of mutants to caspofungin and arsenite. The same strains as in the experiments whose results are shown in panel A were spotted onto YPD plates with or without caspofungin (CAS) or arsenite (As) prior to incubation at 30°C for 3 days. (C) Complementation of the caspofungin sensitivity of a *C. glabrata fps1*Δ *fps2*Δ mutant. Strain DL3679 was transformed with vectors expressing various *FPS* genes under the control of their endogenous promoters, and transformants were treated as described above.

We examined the levels of *CgFPS1* and *CgFPS2* mRNA in response to treatment with 1.8 M sorbitol using quantitative RT-PCR. For both genes, we detected a modest decrease in mRNA levels at 2 h and 4 h after osmotic shock (Fig. 2). The mRNA levels of both genes returned to normal after 20 h in 1.8 M sorbitol-containing medium. These results suggest that *C. glabrata* may temporarily diminish the expression of the glycerol channels, as well as control their activity.

Despite retention of elevated intracellular glycerol, none of the *C. glabrata* mutants displayed strong temperature sensitivity for growth up to 42°C (Fig. 3A). Growth of the *fps1*Δ *fps2*Δ double mutant was only very slightly impaired at elevated temperature. However, the *fps1*Δ mutant was hypersensitive to caspofungin treatment (Fig. 3B). Moreover, the *fps1*Δ *fps2*Δ double mutant displayed an even greater degree of hypersensitivity to caspofungin than the *fps1*Δ mutant, supporting the conclusion that *CgFPS2* is partially redundant with *CgFPS1* but of lesser importance to the maintenance of proper turgor pressure. To confirm that the caspofungin sensitivity of the *fps1*Δ *fps2*Δ mutant was the consequence of loss of *FPS1/-2* function, we reintroduced either *CgFPS1* or *CgFPS2* into the *fps1*Δ *fps2*Δ mutant on a centromeric plasmid. The *CgFPS1* gene complemented the caspofungin sensitivity of the *fps1*Δ *fps2*Δ mutant fully, whereas the *CgFPS2* gene partially complemented the mutant, supporting the conclusion that *FPS1* is more important than *FPS2* (Fig. 3C). The *ScFPS1* gene failed to complement the *C. glabrata fps1*Δ *fps2*Δ mutant when provided in the context of its own promoter. However, we do not know if its failure to complement reflects a problem with the expression or function of the encoded protein. Collectively, our results support the conclusion that these putative channel proteins serve the same function with regard to glycerol transport in *C. glabrata* as in *S. cerevisiae* and that *CgFPS1* is more important than *CgFPS2* for the maintenance of glycerol homeostasis.

The Fps1 glycerol channel of *S. cerevisiae* is also the principal point of entry for the toxic metalloid arsenite, and loss of function of this channel confers resistance to this agent (1, 40, 41). Surprisingly, not only were the *C. glabrata fps1*Δ and *fps2*Δ mutants not resistant to arsenite, but these mutations sensitized *C. glabrata* to the metalloid (Fig. 3B). This suggested both that the Fps1 and Fps2 channels are not significant ports for arsenite entry in *C. glabrata* and that their loss may induce the opening of channels that do allow the entry of arsenite.

We next examined cell wall stress in the *fps1*Δ *fps2*Δ mutant. This was done using a cell wall stress-responsive reporter from *S. cerevisiae* (*PRM5-lacZ*) (14) cloned into a *C. glabrata* centromeric vector. Although not optimized for expression in *C. glabrata*, this reporter responded to cell wall stress induced by calcofluor white, a chitin antagonist (Fig. 4A). Basal cell wall stress was elevated in the *fps1*Δ *fps2*Δ mutant (Fig. 4B), as predicted by its hypersensitivity to caspofungin. These results, taken together, support the conclusion that *Cg*Fps1 and *Cg*Fps2, like *Sc*Fps1, function to maintain glycerol homeostasis.

As a final test of the conservation of function of *Cg*Fps1 and *Cg*Fps2 with *Sc*Fps1, we integrated *CgFPS1* or *CgFPS2* at the *S. cerevisiae FPS1* locus such that the coding region replaced that of *ScFPS1* and was under the control of the endogenous *ScFPS1* promoter. The *CgFPS1* gene but not the *CgFPS2* gene was able to complement the loss of *ScFPS1* function for growth at 40°C (Fig.

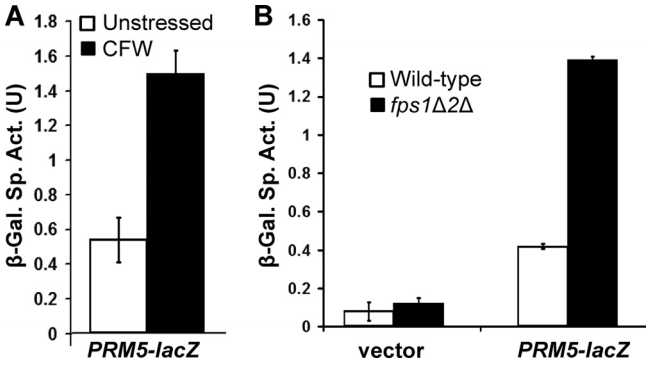

FIG 4 Cell wall stress in a *C. glabrata fps1*Δ *fps2*Δ mutant. (A) Activation of a cell wall stress reporter. An *S. cerevisiae* cell wall stress reporter, *PRM5-lacZ*, is induced in *C. glabrata* by the cell wall antagonist calcofluor white (CFW). Wild-type (BG14) cells, transformed with the pGRB2.0-*PRM5*-lacZ transcriptional reporter plasmid (p3060), were grown to mid-log phase in selective medium and treated with calcofluor white (40 μg/ml) for 5 h at 30°C, followed by cell lysis and measurement of β-galactosidase (β-Gal.) activity. Each value represents the mean and standard deviation from three independent transformants. (B) The *C. glabrata fps1*Δ *fps2*Δ (*fps1*Δ*2*Δ) mutant is under constitutive cell wall stress. Wild-type (BG14) cells and an *fps1*Δ *fps2*Δ mutant (DL3679) were transformed with pGRB2.0-*PRM5*-lacZ or pGRB2.0 (vector), and β-galactosidase activity was measured from log-phase cells growing at 30°C. Each value represents the mean and standard deviation of three separate experiments.

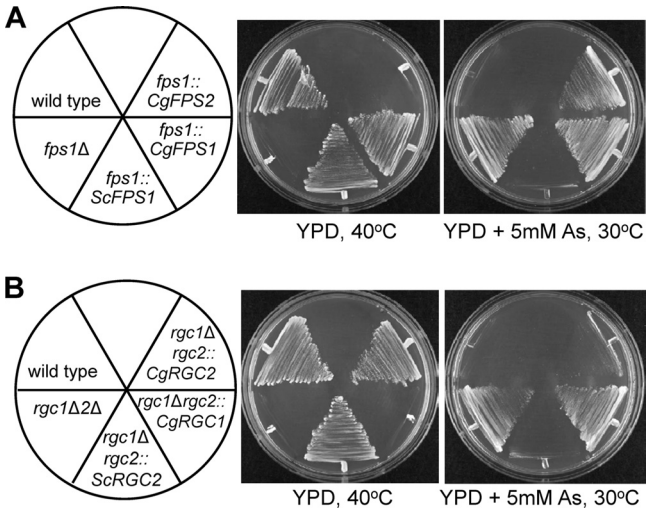

FIG 5 Heterologous complementation of *S. cerevisiae fps1*Δ and *rgc1*Δ *rgc2*Δ (*rgc1*Δ*2*Δ) mutant phenotypes by *C. glabrata* genes. (A) Complementation of an *fps1*Δ mutant. *S. cerevisiae FPS1* was replaced by integration with the indicated *FPS* genes such that the integrated genes were expressed under the control of the endogenous *ScFPS1* promoter. The indicated strains were streaked onto YPD plates with or without arsenite (As) and incubated at 40°C or 30°C, respectively, for 3 days. Strains were the wild type (DL3187) and *fps1*Δ (DL3226), *fps1*::*ScFPS1* (DL3799), *fps1*::*CgFPS1* (DL3800), and *fps1*::*CgFPS2* (DL3801) mutants. (B) Complementation of an *rgc1*Δ *rgc2*Δ mutant. The *RGC2* gene of *S. cerevisiae* was replaced by integration with the indicated *RGC* genes in an *rgc1*Δ mutant background such that the integrated genes were expressed under the control of the endogenous *ScRGC2* promoter. Temperature and arsenite phenotypes were determined as described for panel A. Strains were the wild type (DL3187) and *rgc1*Δ *rgc2*Δ (DL3207), *rgc1*Δ *rgc2*::*ScRGC2* (DL3802), *rgc1*Δ *rgc2*::*CgRGC1* (DL3803), and *rgc1*Δ *rgc2*::*CgRGC2* (DL3804) mutants.

5A), providing strong support for the conclusion that *CgFPS1* functions as a glycerol facilitator. Interestingly, the expression of *CgFPS1* did not restore arsenite sensitivity to the *S. cerevisiae fps1*Δ mutant (Fig. 5A), supporting the conclusion that arsenite does not enter through these *C. glabrata* channels.

Because the *fps1*Δ *fps2*Δ mutation sensitized *C. glabrata* to caspofungin treatment, we asked if the *fps1*Δ *fps2*Δ mutant was cleared by caspofungin treatment more effectively than was the wild type in the context of a mouse infection. This experiment was carried out with two sets of strains. In one experiment, mice were infected by tail vein injection with wild-type *C. glabrata* (BG2) or the *fps1*Δ *fps2*Δ mutant (BG3075). Four days postinfection, mice were given a single dose of caspofungin (either 2 mg/kg or 25 mg/kg in PBS) by intraperitoneal injection or were mock treated with PBS. Three days posttreatment with caspofungin, mice were sacrificed and their spleens, kidneys, and livers were harvested and homogenized for detection of viable *C. glabrata* by measurement of CFU. Comparable numbers of wild-type and *fps1*Δ *fps2*Δ mutant cells were detected in the kidneys and spleens of untreated mice, with somewhat lower numbers of the *fps1*Δ *fps2*Δ mutant recovered from the liver (Fig. 6A). Importantly, treatment with either dose of caspofungin was more effective at clearing the *fps1*Δ *fps2*Δ mutant than the wild type from all three organs. We repeated the experiment using a second set of strains, to ensure that restoration of *FPS1* would reverse the clearance phenotype (Fig. 6B). Mice were infected by tail vein injection with BG14 (*ura3*) cells transformed to Ura^+ with empty pGRB2.0 vector, the *fps1*Δ *fps2*Δ mutant transformed with pGRB2.0 (BG3130), or the *fps1*Δ *fps2*Δ mutant transformed with pGRB2.0-*CgFPS1* (BG3123). Four days postinfection, the mice were treated with caspofungin as described above, and they were sacrificed 3 days after the caspofungin treatment. Comparable numbers of wild-type, mutant, and restored cells were recovered from all three organs. As in the first experiment, treatment with caspofungin was noticeably more effective at clearing the *fps1*Δ *fps2*Δ mutant than the wild-type cells. The *fps1*Δ *fps2*Δ mutant transformed with pGRB2.0-*CgFPS1* behaved like the wild type in this assay, demonstrating that loss of *FPS1* and *FPS2* sensitizes *C. glabrata* to the antifungal effect of caspofungin treatment (Fig. 6B).

***Candida glabrata RGC1* and *RGC2*.** In *S. cerevisiae*, an *rgc1*Δ *rgc2*Δ mutant displays a phenotype similar to that of an *fps1*Δ mutant, because loss of function of these functionally redundant Fps1 regulators results in greatly diminished Fps1 channel activity (1). The *C. glabrata* genome also possesses two *RGC* orthologs, which we hereinafter designate *CgRGC1* (CAGL0B04213) and *CgRGC2* (CAGL0G03179), based both on respective sequence similarities and syntenic organization. The predicted *Cg*Rgc2 protein, at 1,062 amino acids, is similar in length to the *S. cerevisiae* Rgc proteins, whereas the predicted *Cg*Rgc1 protein is substantially shorter, at 901 amino acids. The *Sc*Rgc2 protein shares 45% sequence identity with *Cg*Rgc2 across the entire protein and 38% with *Cg*Rgc1 across the central 600 amino acids. The *Sc*Rgc1 protein shares 40% sequence identity with *Cg*Rgc2 across the entire protein and 41% with *Cg*Rgc1 over the central 600 amino acids.

We tested the *C. glabrata RGC1* and *RGC2* genes for their ability to complement the phenotypes of an *S. cerevisiae rgc1*Δ *rgc12*Δ mutant. As with the *CgFPS* genes, we integrated *CgRGC1* or *CgRGC2* at the *ScRGC2* locus such that the coding region replaced that of *ScRGC2* and was under the control of the endogenous *ScRGC2* promoter. The *CgRGC2* gene but not the *CgRGC1* gene was able to complement the loss of *ScRGC1* and *ScRGC2* function for growth at 40°C and to restore arsenite sensitivity to this mutant

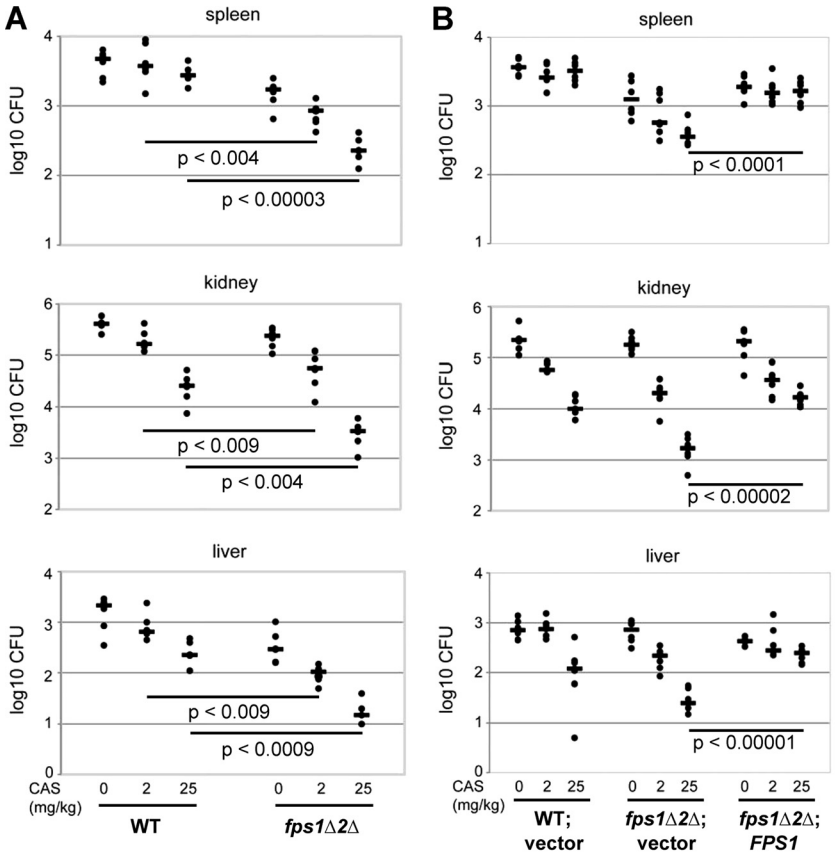

FIG 6 Caspofungin clearance of *C. glabrata* from mice infected with a wild-type strain or an *fps1*Δ *fps2*Δ mutant. (A) Groups of mice were infected by tail vein injection with either 2.2×10^7 wild-type (WT) cells (BG2) or 0.9×10^7 *fps1*Δ *fps2*Δ (*fps1*Δ2Δ) mutant cells (BG3075). Mice were treated with the indicated dose of caspofungin (CAS) by intraperitoneal injection (6 mice per group) 4 days postinfection. Three days posttreatment, viable fungal cells were measured from spleens, kidneys, and livers. (B) Groups of mice were infected with either 2.3×10^7 BG14 (wild type, *ura3*) cells transformed to Ura^+ with empty vector pGRB2.0 (vector), 2.1×10^7 DL3679 (*fps1*Δ *fps2*Δ *ura3*) cells transformed to Ura^+ with pGRB2.0, or 1.6×10^7 DL3679 cells transformed with the *CgFPS1* expression plasmid (p3058, *FPS1*). Mice were treated with the indicated dose of caspofungin by intraperitoneal injection (7 mice per group) 4 days postinfection. Three days posttreatment, viable fungal cells were measured from spleens, kidneys, and livers. Statistical significance was determined using two-sample *t* tests with a two-tailed distribution. The ends of each bar indicate the sample pairs for which the significance value is presented. Bars within groups show the means.

(Fig. 5B). These results indicate that at least *Cg*Rgc2 is capable of activating the *Sc*Fps1 channel and suggest that it serves the same function in the regulation of the *Cg*Fps channels. It is possible that the shorter *Cg*Rgc1 protein has lost this function.

DISCUSSION

Many fungal species use glycerol as a compatible solute with which to maintain osmotic homeostasis in response to changes in external osmolarity. In *S. cerevisiae*, intracellular glycerol concentrations are regulated largely by the high osmolarity glycerol (HOG) response pathway, both through induction of glycerol biosynthesis and control of its flux through the plasma membrane Fps1 glycerol channel. Fps1 closes in response to hyperosmotic shock and opens in response to hypoosmotic shock (26, 39). Although the HOG signaling pathway is highly conserved among pathogenic fungi (10, 19, 20, 21, 34, 36), only the *C. glabrata* genome possesses clear orthologs of *FPS1*. However, it has not been demonstrated that *C. glabrata* retains glycerol in response to hyperosmotic stress. Here, we demonstrate that *C. glabrata*, like many other fungal species, elevates its intracellular glycerol concentration in response to hyperosmotic shock.

***Candida glabrata FPS1* and *FPS2*.** In this work, we studied mutants with deletions of the two genes encoding the presumptive *C. glabrata* glycerol channels, Fps1 and Fps2. We found that the *C. glabrata FPS1* and *FPS2* genes encode functionally additive glycerol channels, with *FPS1* playing a greater role than *FPS2* for the maintenance of osmotic homeostasis. *C. glabrata* cells lacking both *FPS1* and *FPS2* retained excess intracellular glycerol in the absence of hyperosmotic stress and displayed constitutive cell wall stress as judged by a transcriptional reporter for cell wall stress and by hypersensitivity to caspofungin, an antifungal agent that targets cell wall biogenesis. The increase in glycerol concentration in the *C. glabrata fps1 fps2* null mutant compared to its level in the wild-type was considerably smaller than that observed in an *S. cerevisiae fps1* null mutant (1, 26, 39). It is not clear why loss of these *C. glabrata* proteins resulted in only a modest increase in basal glycerol retention, but that result suggests that either this species may have additional ports for glycerol efflux not possessed by *S. cerevisiae* or it has more efficient metabolic mechanisms for glycerol removal. Whatever the explanation, this comparatively weak effect may explain why the *C. glabrata* mutant was not sensitive to growth inhibition at elevated temperature, a cell wall stress condition in *S. cerevisiae* (15, 25).

We also tested the ability of the *C. glabrata FPS* genes to function in *S. cerevisiae* by expressing them under the control of the *ScFPS1* promoter. *CgFPS1* but not *CgFPS2* was able to complement the turgor pressure-induced temperature sensitivity of an *S. cerevisiae fps1* null mutant, perhaps reflecting the greater degree of conservation between *S. cerevisiae FPS1* and *CgFPS1* as compared to *CgFPS2*. The predicted N-terminal cytoplasmic domain of the *Cg*Fps2 protein is shorter than that of Fps1 from either species. Because the MAP kinase Hog1 binds to this domain of *Sc*Fps1 (29), it is possible that *Cg*Fps2 lacks an important regulatory site for control of osmotic homeostasis by Hog1.

In *S. cerevisiae*, Fps1 is also the port through which trivalent metalloids, such as arsenite and antimonite, enter the cell (1, 40, 41). Mutants lacking *Sc*Fps1 are consequently resistant to the toxic effects of these metalloids. Therefore, we were surprised to find that not only were the *C. glabrata fps* null mutants not more resistant than wild-type to arsenite, they were sensitized to this agent. Consistent with this, *CgFPS1* failed to restore arsenite sensitivity to the *S. cerevisiae fps1* null mutant, despite its ability to complement the growth defect. We conclude from these results that the *C. glabrata* Fps1 and Fps2 channels are not entry ports for arsenite. The enhanced sensitivity of the *C. glabrata fps1 fps2* null mutant to arsenite may reflect compensatory activation of another glycerol efflux mechanism that may also allow entry of arsenite.

***C. glabrata RGC1* and *RGC2*.** *S. cerevisiae* possesses two paralogous positive regulators of Fps1 channel activity, Rgc1 and Rgc2 (1). Their loss of function strongly impairs Fps1 activity, although the mechanism of their action is not yet known. Rgc proteins are found widely among fungal species, including those that do not possess Fps1 proteins, indicating that these proteins can serve other functions. Therefore, we tested the ability of the *CgRGC1* and *CgRGC2* genes to complement an *S. cerevisiae rgc1 rgc2* null mutant. Our finding that *CgRGC2* complemented the Fps1-related phenotypes of this mutant reveals that at least this ortholog is capable of regulating Fps1 and suggests that the roles of both the Rgc and Fps proteins in the maintenance of glycerol homeostasis are conserved between *S. cerevisiae* and *C. glabrata*.

Caspofungin sensitivity and clearance of *C. glabrata* infection. Because the *C. glabrata fps1 fps2* null mutant was hypersensitive to caspofungin treatment *in vitro*, we compared the efficacy of caspofungin treatment of this mutant to its efficacy against the wild type in the clearance of *C. glabrata* infections in mice. This mutant was reproducibly more effectively cleared from the spleen, kidney, and liver of infected animals by caspofungin.

Three echinocandins (caspofungin, micafungin, and anidulafungin) are currently available for clinical use. Although the first of these received FDA approval in 2002 (caspofungin) (32), clinical isolates of *C. albicans* and *C. glabrata* carrying mutations that confer reduced echinocandin sensitivity began to be reported shortly thereafter (12, 16, 22, 24, 31). In those cases examined, the mutations mapped to the β-1,3-glucan synthase catalytic subunit Fks1 or its paralog Fks2 (16, 32). Our results suggest the possibility of developing drugs that target Fps1 and Fps2 channel activity to enhance *C. glabrata* echinocandin sensitivity or to resensitize clinical isolates with reduced echinocandin sensitivity. Such drugs could target either the channel proteins directly or the Rgc1 and Rgc2 positive regulators. We have found recently that *Sc*Rgc2 maintains *Sc*Fps1 in an open conformation by binding to its C-terminal cytoplasmic domain (J. Lee and D. E. Levin, unpublished data). Pharmacological intervention with this interaction would be expected to induce closure of the glycerol channel.

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