Role of the Slt2 mitogen-activated protein kinase pathway in cell wall integrity and virulence in *Candida glabrata*

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ABSTRACT

The Slt2 mitogen-activated protein kinase pathway plays a major role in maintaining fungal cell wall integrity. In this study, we investigated the effects of *SLT2* deletion and overexpression on drug susceptibility and virulence in the opportunistic fungal pathogen *Candida glabrata*. While the Δ*slt2* strain showed decreased tolerance to elevated temperature and cell wall-damaging agents, the *SLT2*-overexpressing strain exhibited increased tolerance to these stresses. A mutant lacking Rlm1, a transcription factor downstream of Slt2, displayed a cell wall-associated phenotype intermediate to that of the Δ*slt2* strain. When *RLM1* was overexpressed, micafungin tolerance was increased in the wild-type strain and partial restoration of the drug tolerance was observed in the Δ*slt2* background. It was also demonstrated that echinocandin-class antifungals were more effective against *C. glabrata* under acidic conditions or when used concurrently with the chitin synthesis inhibitor nikkomycin Z. Finally, in a mouse model of disseminated candidiasis, the deletion and overexpression of *C. glabrata SLT2* resulted in mild decreases and increases, respectively, in the CFUs from murine organs compared with the wild-type strain. These fundamental data will help in further understanding the mechanisms of cell wall stress response in *C. glabrata* and developing more effective treatments using echinocandin antifungals in clinical settings.

INTRODUCTION

Candida glabrata is the second most common cause of invasive candidiasis after *Candida albicans* and is relatively resistant to azole antifungals (Pfaller & Diekema, 2007). Echinocandin-class antifungals inhibit the synthesis of β1,3-glucan, which is integral to the structure and function of the fungal cell wall (Chandrasekar & Sobel, 2006). In most instances, echinocandins, such as caspofungin and micafungin, are clinically effective against *C. glabrata* infections; however, a few in vitro studies have demonstrated that caspofungin is unable to kill this fungus completely in standard time-kill assays (Barchiesi, *et al.*, 2005, Barchiesi, *et al.*, 2005).

The Slt2 mitogen-activated protein kinase (MAPK) pathway has been well studied in *Saccharomyces cerevisiae* and is required for cell wall integrity (Levin, 2005, Lesage & Bussey, 2006). Mutant phenotypes of *SLT2* orthologs have been characterized in several pathogenic fungi, including *C. albicans* and *Cryptococcus neoformans* (Monge, *et al.*, 2006, Roman, *et al.*, 2007, Zhao, *et al.*, 2007). To date, very little is known about *C. glabrata* Slt2, although it has been reported that a Δ*slt2* strain is unable to increase the chitin content in the cell wall in response to caspofungin treatment and shows increased susceptibility to this drug (Cota, *et al.*, 2008). Although the involvement of Slt2 in virulence has been reported in several pathogenic fungi (Roman, *et al.*, 2007, Zhao, *et al.*, 2007), it has never been described in *C. glabrata*.

The ability to adapt to environmental conditions, such as elevated temperatures (body temperature), acidic conditions (gastrointestinal tract or vagina), and neutral to slightly alkaline conditions (bloodstream or organ tissues), is necessary for pathogenic organisms to survive or cause infections in mammalian hosts (Cooney & Klein, 2008). Although the

requirement of the Slt2 MAPK pathway for cellular adaptation to environmental stresses encountered in hosts has been demonstrated in several pathogenic fungi, species-dependent Δ*slt2* phenotypes also exist (Monge*, et al.*, 2006, Roman*, et al.*, 2007, Zhao*, et al.*, 2007). In this study, we characterized the phenotypes of a Δ*slt2* deletion mutant and an *SLT2*-overexpressing strain to investigate the roles of the Slt2 MAPK pathway in the environmental stress responses and virulence of *C. glabrata*.

MATERIALS AND METHODS

Strains and culture conditions.

The *C. glabrata* and *S. cerevisiae* strains used in this study are listed in Table 1. Cells were routinely propagated at 30°C in YPD medium (1% yeast extract, 2% peptone, and 2% dextrose) (Difco Laboratories, Detroit, MI) or in minimal medium (MIN), which consisted of a 0.7% yeast nitrogen base without amino acids (Difco Laboratories) and 2% dextrose, unless otherwise noted. *S. cerevisiae* BY4742 and Δ*slt2* strains were purchased from Open Biosystems (Huntsville, AL).

Plasmid and strain construction.

Sequence information of *C. glabrata* genes was obtained from the *C. glabrata* genome database Genolevures (http://www.genolevures.org/). The primers and plasmids used in this study are listed in Tables 2 and 3, respectively. The plasmids constructed using PCR products were verified by sequencing before use. Transformation of *C. glabrata* was performed using the LiAc protocol as described previously (Cormack & Falkow, 1999).

C. glabrata deletion strains were constructed using the one-step PCR–based technique described previously (Gola*, et al.*, 2003). Briefly, a 1-kb fragment containing *C. glabrata HIS3* was excised from pCgACH (Kitada*, et al.*, 1996) by digestion with *Xho*I and inserted into the *Xho*I site of pBluescript II SK+ (Stratagene, La Jolla, CA) to yield pBSK-HIS. A deletion construct was amplified from pBSK-HIS using primers tagged with the 100 bp sequences homologous to the flanking regions of the target ORF (Table 2). *C. glabrata* strain 2001HT (Δ*his3*, Δ*trp1*) (Kitada*, et al.*, 1995) was transformed with the deletion construct, and the resulting transformants were selected by histidine prototrophy. Both PCR and Southern

blotting were performed to verify whether the desired homologous recombination occurred at the target locus without ectopic integration in the *SLT2* deletion mutant TG151 (Δ*slt2*::*HIS3*, Δ*trp1*) and the *RLM1* deletion mutant TG181 (Δ*rlm1*::*HIS3*, Δ*trp1*).

To construct an overexpression plasmid for *SLT2* that could be used in *C. glabrata*, a 1025-bp *Sac*I-*Kpn*I fragment containing the *S. cerevisiae PGK1* promoter, a polylinker, and the *C. glabrata HIS3* 3'-UTR was excised from pGRB2.2 (Frieman, *et al.*, 2002) and inserted into the corresponding site of pCgACT (Kitada, *et al.*, 1996) to yield pCgACT-P. A 1625-bp *C. glabrata SLT2* region (+1 to +1625) containing the entire ORF (+1 to +1467) was amplified from the genomic DNA of *C. glabrata* wild-type strain CBS138 (Dujon, *et al.*, 2004) with the primer pair CgSLT2-F1 and CgSLT2-R1. The PCR product obtained was digested with *Sal*I and inserted into the *Sal*I site of pCgACT-P to generate pCgACT-PS2. To construct a *C. glabrata RLM1*-overexpressing plasmid, a 1908-bp *C. glabrata RLM1* region (+1 to +1908) containing the entire ORF (+1 to +1881) was amplified from the genomic DNA of CBS138 with the primer pair CgRLM1-F2 and CgRLM1-R2. The PCR product obtained was digested with *Bam*HI and inserted into the *Bam*HI site of pCgACT-P to generate pCgACT-PR1. *C. glabrata* strains (2001T, TG151 and TG181) were transformed with pCgACT-P, pCgACT-PS2, and pCgACT-PR1, and the resulting transformants were selected by tryptophan prototrophy.

Phenotypic analysis

The growth rates of *C. glabrata* strains were examined as described previously (Miyazaki, *et al.*, 2006). Briefly, cells were grown in MIN broth at 30 and 37°C with agitation and the optical density at 600 nm was monitored every two hours.

A spot dilution test was carried out as described previously (Miyazaki, *et al.*, 2006, Ram & Klis, 2006). Briefly, logarithmic-phase cells grown in MIN broth were harvested and adjusted to 2×10^7 cells/ml. Serial 10-fold dilutions were then prepared, and 5 µl of each dilution was spotted onto an agar plate containing either micafungin, Congo red or Calcofluor white at the desired concentrations. Plates were incubated at 30°C for 48 h, unless otherwise noted. Micafungin was kindly provided by Astellas (Tokyo, Japan) and was dissolved in distilled water. Caspofungin susceptibility was examined by Etest (AB BIODISK, Solna, Sweden) using RPMI 1640 medium (Gibco, Invitrogen Corporation, Grand Island, NY) supplemented with 2% glucose and 0.165 M morpholinepropanesulfonic acid (Sigma, St Louis, MO).

Time-kill assays were performed as described previously (Klepser, *et al.*, 1998, Pfaller, *et al.*, 2004). Nikkomycin Z (Sigma) was dissolved in distilled water at a stock concentration of 5 mg/ml. All sensitivity tests were repeated at least three times.

Virulence studies

Animal experiments were performed essentially as described previously (Chen, *et al.*, 2007, Kaur, *et al.*, 2007). Briefly, to prepare cells for injection, logarithmic-phase *C. glabrata* cells were harvested, washed, resuspended in sterile saline, and adjusted to 4×10^8 cells/ml after counting the number of cells using a hemocytometer. The actual CFU in the inocula were confirmed by plating serial dilutions of cell suspension on YPD plates. Groups of 10 female, 8-week-old BALB/c mice (Charles River Laboratories Japan, Inc., Japan) were injected with 0.2 ml of the *C. glabrata* cell suspension via the lateral tail vein. The mice were euthanized 7 days after injection and the spleen and liver were then excised. No mice died before euthanasia in our experiments. Appropriate dilutions of organ homogenates were plated on

YPD plates. Colonies were counted after 3 days of incubation at 30°C and the CFUs per organ were calculated. A *P*-value of <0.05 with the Mann-Whitney test was considered statistically significant.

RESULTS AND DISCUSSION

The predicted amino acid sequence of the *C. glabrata SLT2* ortholog (GenBank accession no. XP447735, Genolevures ID CAGL0J00539g) showed high homology to *SLT2* orthologs from several fungi, including *S. cerevisiae* (76.0% identity), *C. albicans* (53.8%), *C. neoformans* (49.1%), and *Aspergillus fumigatus* (54.9%), suggesting that Slt2 is highly conserved among model yeast and other clinically relevant pathogenic fungi.

To investigate the roles of Slt2 in *C. glabrata*, we constructed a Δ*slt2* strain in which the entire *SLT2* ORF was deleted. A *SLT2* reconstituted strain was then prepared by introducing an intact *SLT2* gene under control of the *PGK1* promoter allowing for overexpression in pCgACT-PS2 into the Δ*slt2* strain. As a preliminary experiment, it was confirmed by real-time PCR that the expression of *SLT2* was undetectable in the Δ*slt2* strain and was increased approximately ninefold in the *SLT2* reconstituted strain compared with the wild-type control (data not shown).

Slt2 is required for cell growth under cell wall-damaging conditions in *C. glabrata.*

The effects of *SLT2* deletion on cell growth at elevated temperatures are species dependent in fungi. For example, the loss of Slt2 function leads to growth defects at 37°C in both *S. cerevisiae* (Lee, *et al.*, 1993, Martin, *et al.*, 1993) and *C. neoformans* (Kraus, *et al.*, 2003), but not in *C. albicans* (Navarro-Garcia, *et al.*, 1995). In the present study, the growth of the *C. glabrata* Δ*slt2* strain was comparable to that of the wild-type control at both 30 and 37°C (Fig. 1). However, the Δ*slt2* strain showed modestly impaired growth at 41°C compared with the wild-type control, while overexpression of *SLT2* resulted in increased tolerance to this high temperature (Fig. 1b). Although cells are subjected to various stresses at elevated

temperatures (Attfield, 1997), it is known that the loss of Slt2 results in cell lysis due to a lack of cell wall integrity (Levin, 2005). To further analyze cell wall-associated phenotypes of the Δ*slt2* strain, cell growth was examined in the presence of chemical agents that cause cell wall damage by different mechanisms. The Δ*slt2* strain showed decreased tolerance to Congo red (inhibitor of chitin and β-glucan fiber formation), Calcofluor white (inhibitor of chitin polymer assembly), and micafungin (inhibitor of β1,3-glucan synthesis) compared with the wild-type control (Fig. 1b). The growth of the Δ*slt2* strain under the cell wall-damaging conditions could be rescued by the reintroduction of *SLT2* or addition of an osmotic stabilizer, 1 M sorbitol, to the growth medium. Taken together, these results suggest that Slt2p plays an important role in cell wall integrity in *C. glabrata*, and are consistent with previous findings in other fungi (Kraus*, et al.*, 2003, Levin, 2005).

Effects of environmental pH on echinocandin susceptibility in *C. glabrata*.

Although the loss of Slt2 in *S. cerevisiae* results in growth defects at both alkaline and acidic pHs (Serrano*, et al.*, 2004, Dudley*, et al.*, 2005, Mira*, et al.*, 2009, and Fig. 2a in this study), the growth of the *C. glabrata* Δ*slt2* strain was similar to that of the wild type at all the pH levels tested in this study (pH 2-8) (Fig. 2b). The effect of environmental pH on micafungin tolerance in *C. glabrata* was also examined. While all three strains grew well at pH 7 even in the presence of 0.01 μg/ml micafungin (Fig. 2b), their growth was impaired at pH 8, with only the Δ*slt2* strain exhibiting slightly decreased tolerance to micafungin under alkaline conditions. In contrast, the growth of the wild-type strain was impaired in the presence of micafungin at pH 3 and even more severely at pH 2 compared with each control plate. Under acidic conditions, the Δ*slt2* strain was less tolerant to micafungin than the

wild-type strain, while the *SLT2*-overexpressing strain displayed the most growth and the highest tolerance levels.

In addition to micafungin, the susceptibility of the wild-type and mutant strains to caspofungin was examined using the Etest method at various pHs (Fig. 2c). As expected, there were no differences in caspofungin susceptibility between the strains at pH 7 (MIC: 0.125 μg/ml). The growth inhibition zones and MICs of each strain at pH 8 were similar to those observed at pH 7. In the wild-type strain, although the inhibition zones at pH 3 and pH 7 were similar, a larger inhibition zone and decreased MIC were observed at pH 2 compared with the results at pH 7 (MIC of 0.064 μg/ml at pH 2). The increase in caspofungin susceptibility at acidic pH was more prominent in the Δ*slt2* strain (MICs of 0.016 and 0.006 μg/ml at pH 3 and pH 2, respectively). The results obtained with the *SLT2*-overexpressing strains in the wild-type and Δ*slt2* backgrounds were similar and also comparable to those with the wild-type strain at all the pHs tested in this study. Taken together, these results indicate that echinocandin tolerance decreases under acidic conditions in the *C. glabrata* wild-type strain and more severely in the Δ*slt2* strain. In addition, the MIC was also drastically decreased in the Δ*slt2* strain. According to previous studies in *S. cerevisiae*, the upregulation of certain genes involved in cell wall biogenesis and cell wall remodeling occurs in response to a low pH (Kapteyn, *et al.*, 2001, Kawahata, *et al.*, 2006, Chen, *et al.*, 2009). Our results indicate that the cell wall integrity pathway mediated by Slt2 is required for the proliferation of *C. glabrata* when concurrently exposed to low pH stress and echinocandins.

The Slt2-Rlm1 pathway is involved in micafungin tolerance in *C. glabrata*.

In *S. cerevisiae*, Slt2 MAPK has at least two downstream targets: the transcription factors Rlm1 and Swi4-Swi6. As Rlm1 is responsible for most of the transcriptional activation of genes required for cell wall integrity (Garcia, *et al.*, 2004), a *C. glabrata RLM1* ortholog was identified in the genome database and subsequently deleted (GenBank accession no. XP447040, Genolevures ID CAGL0H05621g). The resulting Δ*rlm1* strain displayed modestly impaired growth at 41°C and in the presence of micafungin compared with the wild-type control; however, these phenotypes were intermediate to those observed for the Δ*slt2* strain (Fig. 3). Overexpression of *RLM1* resulted in increased tolerance to elevated temperature and micafungin in both the wild-type and the Δ*rlm1* strains. In the Δ*slt2* background, the overexpression of *RLM1* partially rescued cell growth in the presence of micafungin, but not at elevated temperature, suggesting that Slt2 may be required for Rlm1 to be fully functional in *C. glabrata*. Taken together, the results indicate that the Slt2-Rlm1 pathway plays an important role in the response to cell wall damage and also suggest that an additional downstream effector of Slt2 is involved in cell wall integrity in this fungus.

The chitin synthesis inhibitor nikkomycin Z enhances the fungicidal activity of micafungin against *C. glabrata*.

Recently, Cota et al. suggested that *C. glabrata* is able to resist the fungicidal activity of caspofungin by activating the Slt2 MAPK pathway, which leads to a compensatory increase in the cell wall chitin content (Cota, *et al.*, 2008). Therefore, we hypothesized that, in combination with micafungin, the pharmacological inhibition of chitin synthesis by nikkomycin Z would provide additional antifungal activity against *C. glabrata*. As was observed with caspofungin exposure, *C. glabrata* wild-type cells were not completely killed

by micafungin even though they were exposed to relatively high concentrations of the drug (Fig. 4a). The Δ*slt2* strain displayed increased susceptibility to micafungin in the time-kill assay, which is consistent with the results reported for caspofungin (Cota*, et al.*, 2008). While the wild-type and the *SLT2*-overexpressing strains were able to grow in the presence of 0.01 μg/ml micafungin, the Δ*slt2* strain was rapidly killed under this condition. Although higher micafungin concentrations (0.1 and 1 μg/ml) reduced the number of viable cells for the wild-type and the *SLT2*-overexpressing strains, they were fungicidal for the Δ*slt2* strain.

The effects of the chitin synthesis inhibitor nikkomycin Z on the fungicidal activity of micafungin were examined in *C. glabrata* using an identical time-kill assay (Fig. 4b). Although the growth of the wild-type and *SLT2*-overexpressing strains was not inhibited by nikkomycin Z alone, slightly slower growth was observed in the Δ*slt2* strain. The wild-type cells treated with a combination of nikkomycin Z and micafungin were more rapidly killed than cells exposed to micafungin alone, with a 1.8$\log_{10}$ reduction in CFUs/ml being observed after 24 h. Of particular note, this synergistic effect was not seen in either the Δ*slt2* or the *SLT2*-overexpressing strains. This suggests that nikkomycin Z inhibits the Slt2-mediated compensatory increase of chitin synthesis in response to micafungin-induced β1,3-glucan defects. The synergistic effect of nikkomycin Z and caspofungin have also been reported in other pathogenic fungi, including *C. albicans* and *A. fumigatus* (Stevens, 2000, Chiou*, et al.*, 2001, Ganesan*, et al.*, 2004, Sandovsky-Losica*, et al.*, 2008, Walker*, et al.*, 2008). Although nikkomycin Z has not yet been approved for clinical use, accumulating evidence indicates that the combination of this compound with echinocandins results in synergistic antifungal effects and may provide clues for developing new antifungal strategies.

The *SLT2* expression level affects the survival and dissemination of *C. glabrata* in a mouse model of disseminated candidiasis.

As the involvement of Slt2 in the virulence of *C. glabrata* was unknown, we performed a virulence study using a murine model of disseminated candidiasis. The growth rate of the Δ*slt2* strain at 37°C was comparable to that of the wild-type and the *SLT2*-overexpressing strains (Fig. 1), which is a necessary prerequisite for comparing virulence. As our preliminary experiments showed that CFUs from spleen and liver samples were higher and more consistent than those of the kidney in mice intravenously infected with *C. glabrata*, a finding that is consistent with previous reports (Ju, *et al.*, 2002, Chen, *et al.*, 2007), the CFU data of those two organs were examined in detail (Fig. 5). Fewer CFUs were recovered from mice infected with the Δ*slt2* strain compared with those infected with the wild-type strain (P = 0.0257 in the spleen and P = 0.0232 in the liver). In contrast, mice infected with the *SLT2*-overexpressing strain showed higher CFUs in both organs than those infected with the wild-type strain (P = 0.0007 in the spleen and P = 0.0001 in the liver). These results indicate that the expression level of *SLT2* affects virulence in *C. glabrata*. Increased *SLT2* expression may facilitate the survival of *C. glabrata* cells in host tissues, perhaps due to increased tolerance to stressful conditions that affect cell wall integrity. However, the attenuation of virulence by the deletion of *SLT2* alone was very minor in *C. glabrata*, in contrast to the *mpk1*/*slt2* mutant in *C. neoformans* (Kraus, *et al.*, 2003). The growth defects shown at 37°C, in addition to weakened cell wall integrity, may be largely responsible for the reduced virulence of the *C. neoformans mpk1*/*slt2* mutant (Kraus, *et al.*, 2003). As the loss of Slt2 may facilitate attenuation of fungal virulence when combined with a defect in another signaling

pathway (e.g. calcineurin pathway), the possible physical and genetic interactions between the Slt2 MAPK and other signaling pathways in *C. glabrata* need to be examined in detail.

Concluding remarks

The current report provides evidence that *C. glabrata* Slt2 is required for cell wall integrity based on the growth characteristics of the Δ*slt2* and *SLT2*-overexpressing strains exposed to cell wall-damaging agents. Although the key functions of Slt2p seem to be well conserved in fungi, the observed differences in Δ*slt2* phenotypes between fungal species suggest that the interactions between the Slt2 MAPK and other signaling pathways vary depending on their ecological niches. Although cell wall-integrity pathways have been well characterized in *S. cerevisiae*, they have yet to be examined in detail in most pathogenic fungi including *C. glabrata*. The accumulation of such fundamental data will help in developing more effective treatments using echinocandins in clinical settings.

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Table 1. Strains used in this study.

Strain	Genotype or description	Reference or source
S. cerevisiae		
BY4742	*MATα his3*-Δ1, *leu2*-Δ0, *lys2*-Δ0, *ura3*-Δ0	Open Biosystems (Winzeler, *et al.*, 1999)
SCS-1	*MATα his3*-Δ1, *leu2*-Δ0, *lys2*-Δ0, *ura3*-Δ0, Δ*slt2*::*KanMX4* (derivative of BY4742)	Open Biosystems (Winzeler, *et al.*, 1999)
C. glabrata		
CBS138	Wild-type	(Dujon, *et al.*, 2004)
2001T	Δ*trp1* (a derivative of CBS138)	(Kitada, *et al.*, 1995)
2001HT	Δ*his3*, Δ*trp1* (a derivative of CBS138)	(Kitada, *et al.*, 1995)
TG11	2001T containing pCgACT-P	This study
TG15	2001T containing pCgACT-PS2	This study
TG21	2001T containing pCgACT-PR1	This study
TG151	Δ*slt2*::*HIS3*, Δ*trp1* (made from 2001HT)	This study
TG152	TG151 containing pCgACT-P	This study
TG153	TG151 containing pCgACT-PS2	This study
TG154	TG151 containing pCgACT-PR1	This study
TG181	Δ*rlm1*::*HIS3*, Δ*trp1* (made from 2001HT)	This study
TG182	TG181 containing pCgACT-P	This study
TG183	TG181 containing pCgACT-PR1	This study

Table 2. Primers used in this study

For gene deletion		
Primer name [a]	Target gene	Sequence (5' – 3') (Sequences homologous to flanking regions of the target gene are shown in italics. Sequences shown in bold exist in pBSK-HIS.)
CgSLT 100-F	*SLT2*	*TTGATAAGTTATAACTAAAGATATTGATCCCTTCCATT TATTAACTGTTGAACAGTAGTAAATAAAAAAGGTCTCA TTAACACGCATTGGTATACAACC***TAATACGACTCA CTATAGGGC**
CgSLT 100-R	*SLT2*	*CTCTGTAACTGCATGTGAAAACGGTTAAACGAATTAA TGCATTACCTAATGGTTGTAGAAAAATATATATGTTAT TTACTTTGCATGTCTCTCTATCTC***CGCTCTAGAACT AGTGGATCC**
CgRLM 100-F	*RLM1*	*TGGATTTTGCAACATCGCTGGTCAAATATAAAAATAC CTCAGTTTCTTCGTCAGTCCTTCTGAGAACTACTATTA ATATTCCACTTCTCAAGCCTTGAT***TAATACGACTCA CTATAGGGC**
CgRLM 100-R	*RLM1*	*TTCTTAAATATGAATTATATATTTGTCACCTTTGTGAAG CTCTAGTACAATAAAGAGATTCTTTGAGATTTCAACAG CACCCCTGGTATTTATAGATGG***CGCTCTAGAACTA GTGGATCC**
For gene cloning		
Primer name [a]	Target gene	Sequence (5' – 3') Restriction sites are underlined.
CgSLT2-F1	*SLT2*	ACGCGTCGACATGGACGTTGAGAGACAGACATT
CgSLT2-R1	*SLT2*	ACGCGTCGACCACGGCATCAGTGAAGGCTA
CgRLM1-F2	*RLM1*	CGGGATCCCGATGGGTAGAAGAAAAATCG
CgRLM1-R2	*RLM1*	CGGGATCCCGCAGCACCCCTGGTATTTAT

[a] "F" and "R" indicate forward and reverse primers, respectively.

Table 3. Plasmids used in this study.

Plasmid	Description	Reference or source
pBluescript II SK+	Cloning vector. AmpR	Stratagene
pCgACH	*C. glabrata* centromere-based plasmid containing autonomously replicating sequence and *C. glabrata HIS3*	(Kitada, *et al.*, 1996)
pBSK-HIS	A 1-kb XhoI fragment containing *C. glabrata HIS3* was excised from pCgACH and inserted into the XhoI site of pBluescript II SK+	This study
pGRB2.2	*C. glabrata* centromere based plasmid containing *S. cerevisiae PGK1* promoter, polylinker, *C. glabrata HIS3* 3' UTR and *S. cerevisiae URA3*	(Frieman, *et al.*, 2002)
pCgACT	*C. glabrata* centromere-based plasmid containing autonomously replicating sequence and *C. glabrata TRP1*	(Kitada, *et al.*, 1996)
pCgACT-P	A 1-kb SacI-KpnI fragment containing the *S. cerevisiae PGK1* promoter, polylinker and *C. glabrata HIS3* 3'UTR was excised from pGRB2.2 and inserted into the SacI-KpnI site of pCgACT	This study
pCgACT-PS2	A 1.6-kb SalI PCR fragment (primers CgSLT2-F1/CgSLT2-R1) containing *C. glabrata SLT2* was inserted into the SalI site of pCgACT-P	This study
pCgACT-PR1	A 1.9-kb BamHI PCR fragment (primers CgRLM1-F2/CgRLM1-R2) containing *C. glabrata RLM1* was inserted into the BamHI site of pCgACT-P	This study

Figure legends

Fig. 1. Slt2 is required for cell growth under cell wall-damaging conditions in *C. glabrata*. (a) Growth of *C. glabrata* wild-type, Δ*slt2*, and *SLT2*-overexpressing strains under normal conditions. Logarithmic-phase cells were incubated in MIN broth at 30 and 37°C, and optical density at 600 nm was monitored. (b) Spot dilution assay. Serial 10-fold dilutions of each *C. glabrata* strain were spotted onto MIN plates (pH 5.5) containing either Congo red (CR), Calcofluor white (CFW), or micafungin (MCFG) at the indicated concentrations in the presence and absence of 1 M sorbitol. Plates were incubated for 48 h at 30°C unless otherwise indicated. *C. glabrata* strains: wild-type, 2001T containing an empty vector (strain TG11); Δ*slt2*, a Δ*slt2* strain containing an empty vector (strain TG152); and *SLT2*-overexpression, a Δ*slt2* strain containing a *C. glabrata SLT2*-overexpressing plasmid (strain TG153).

Fig. 2. Effects of environmental pH on micafungin susceptibility in *C. glabrata*. (a) Spot dilution assay of *S. cerevisiae* wild-type (BY4742) and Δ*slt2* (SCS-1) strains. Serial 10-fold dilutions of each strain were spotted onto RPMI 1640 medium with pH ranging from 2 to 8. Plates were incubated at 30°C for 48 h. (b) Spot dilution assay of *C. glabrata* strains at the indicated pH in the presence and absence of 0.01 μg/ml micafungin. *C. glabrata* strains: wild-type, 2001T containing an empty vector (strain TG11); Δ*slt2*, a Δ*slt2* strain containing an empty vector (strain TG152); *SLT2*-overexpression, a Δ*slt2* strain containing a *C. glabrata SLT2*-overexpressing plasmid (strain TG153). (c) Caspofungin susceptibilities of the *C. glabrata* strains were examined by Etest using RPMI 1640 medium at the indicated pH. Plates were incubated at 35°C for 48 h. *C. glabrata* strains: wild-type, strain TG11; Δ*slt2*, strain TG152; *SLT2*-overexpression (OE) in Δ*slt2*, strain TG153; and *SLT2* OE in the wild-type,

strain TG15.

Fig. 3. Effects of *RLM1* deletion and overexpression on cell growth under cell wall-damaging conditions in *C. glabrata*. Serial 10-fold dilutions of each *C. glabrata* strain were spotted onto a MIN plate (pH 5.5) in the presence and absence of either 0.0125 or 0.015 μg/ml micafungin. Plates were incubated for 48 h at 30°C, unless otherwise indicated. *C. glabrata* strains: wild-type (WT), 2001T containing an empty vector (strain TG11); wild-type (WT) + *RLM1*, 2001T containing a *C. glabrata RLM1*-overexpressing plasmid (strain TG21); Δ*slt2*, a Δ*slt2* strain containing an empty vector (strain TG152); Δ*slt2* + *RLM1*, a Δ*slt2* strain containing a *C. glabrata RLM1*-overexpressing plasmid (strain TG154); Δ*rlm1*, a Δ*rlm1* strain containing an empty vector (strain TG182); and Δ*rlm1* + *RLM1*, a Δ*rlm1* strain containing a *C. glabrata RLM1*-overexpressing plasmid (strain TG183).

Fig. 4. Time-kill curves of *C. glabrata* strains exposed to cell wall-damaging agents. (a) Logarithmic-phase cells (5×10^5 CFU/ml) were incubated in RPMI 1640 medium (pH 7) with agitation in the presence or absence of micafungin (MCFG) at the indicated concentrations (μg/ml). (b) Cells were incubated with nikkomycin Z (NKM) (200 μg/ml) or micafungin (1 μg/ml), as indicated. Viability was measured by determining the CFUs after plating appropriate dilutions at each time point. Geometric means and standard deviations for three independent experiments are shown. A 99.9% reduction in the CFU/ml from the starting inoculum was considered "fungicidal". The limit of quantitation was 50 CFU/ml. *C. glabrata* strains: wild type, 2001T containing an empty vector (strain TG11); Δ*slt2*, a Δ*slt2* strain containing an empty vector (strain TG152); and *SLT2*-overexpression, 2001T containing a *C.*

glabrata SLT2-overexpressing plasmid (strain TG15).

Fig. 5. Virulence assay using a mouse model of disseminated candidiasis. Groups of 10 mice were intravenously infected with 8×10^7 cells for each *C. glabrata* strain. Spleens and livers were excised 7 days after injection. Recovered CFUs from each organ are indicated for individual mice in the scatter plots. The geometric mean is shown as a bar. Representative data of two independent experiments are shown. *C. glabrata* strains: wild type, TG11 (wild-type control); Δ*slt2*, TG152 (Δ*slt2* strain containing an empty vector); and *SLT2*-overexpression, TG153 (Δ*slt2* strain containing a *C. glabrata SLT2*-overexpressing plasmid).

Fig. 1.

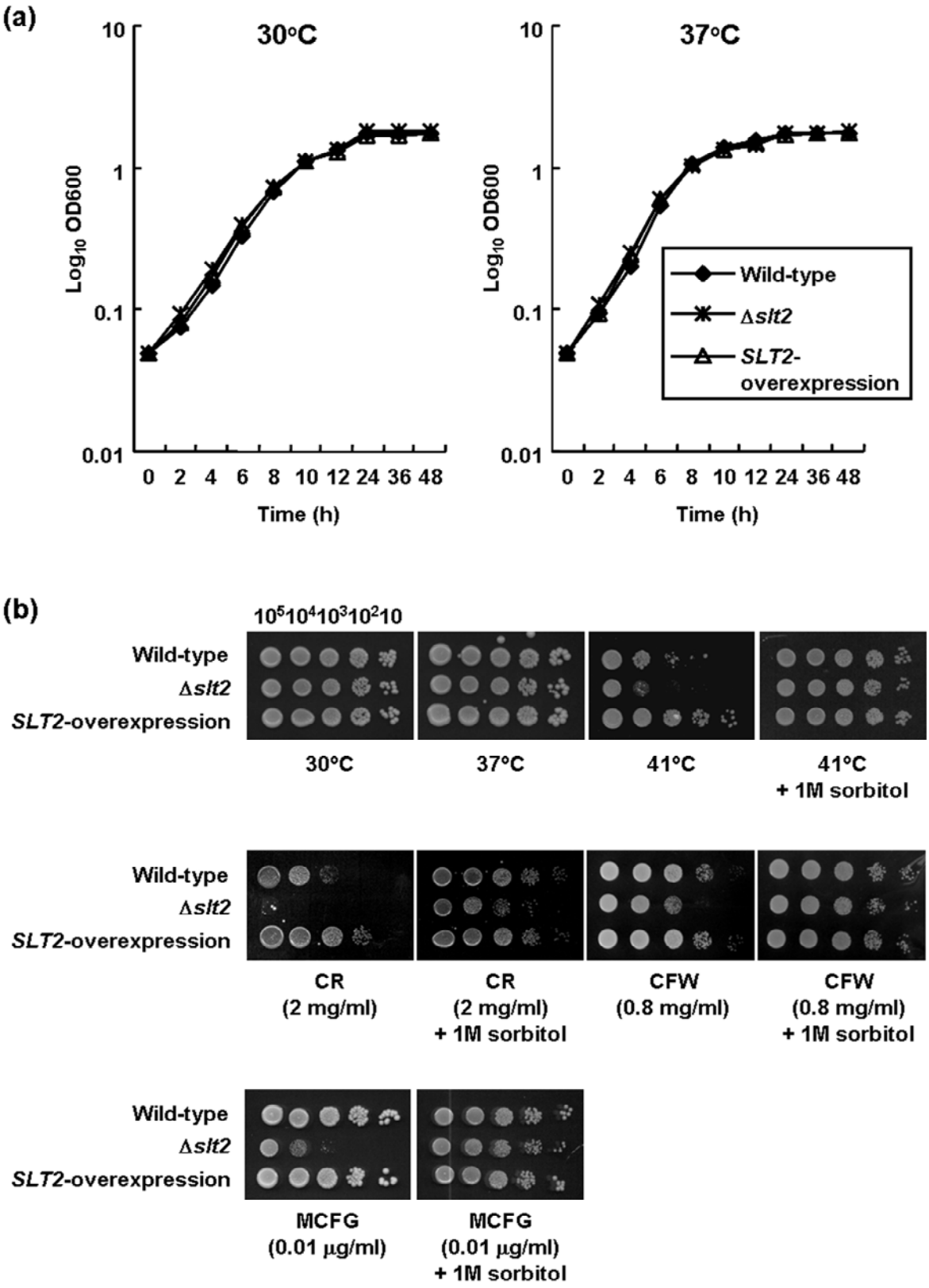
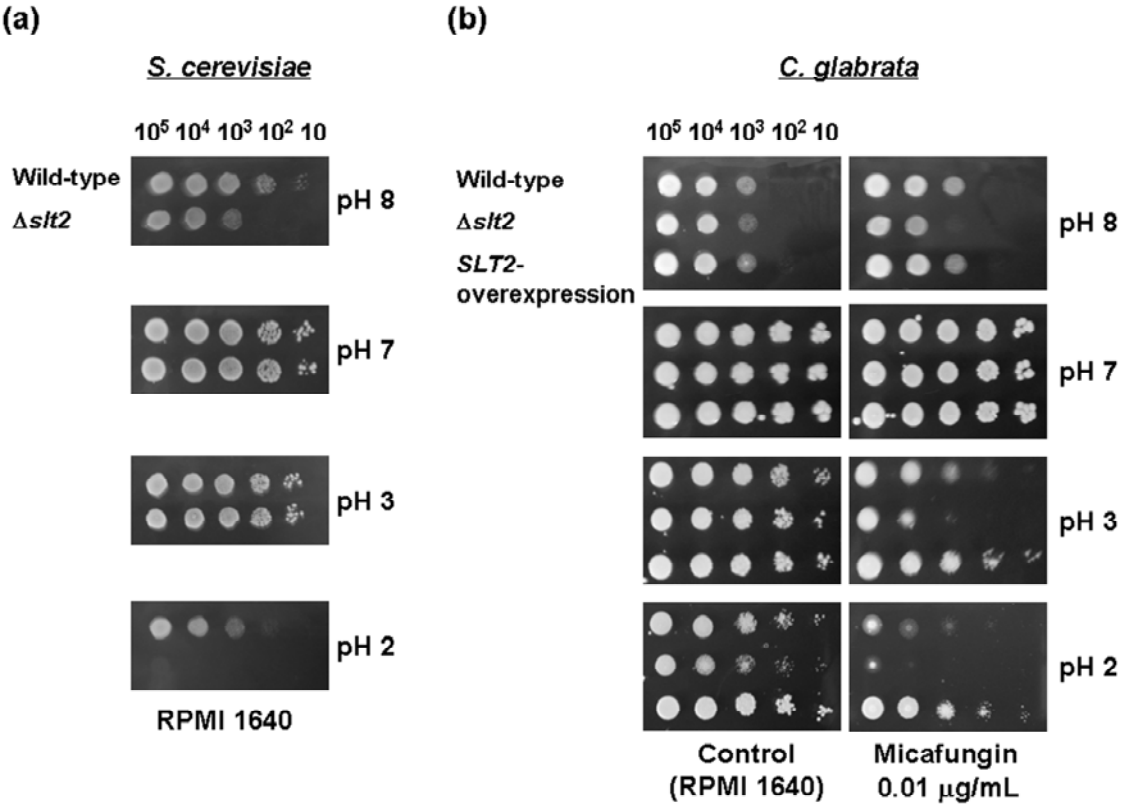

(a)
30°C
37°C
Log10 OD600
10
1
0.1
0.01
0 2 4 6 8 10 12 24 36 48
Time (h)
Wild-type
Δslt2
SLT2-overexpression
(b)
10510410310210
Wild-type
Δslt2
SLT2-overexpression
30°C
37°C
41°C
41°C + 1M sorbitol
CR (2 mg/ml)
CR (2 mg/ml) + 1M sorbitol
CFW (0.8 mg/ml)
CFW (0.8 mg/ml) + 1M sorbitol
MCFG (0.01 μg/ml)
MCFG (0.01 μg/ml) + 1M sorbitol

Fig. 2.


(a)
S. cerevisiae
10⁵ 10⁴ 10³ 10² 10
Wild-type
Δslt2
pH 8
pH 7
pH 3
pH 2
RPMI 1640
(b)
C. glabrata
10⁵ 10⁴ 10³ 10² 10
Wild-type
Δslt2
SLT2-overexpression
pH 8
pH 7
pH 3
pH 2
Control (RPMI 1640)
Micafungin 0.01 μg/mL

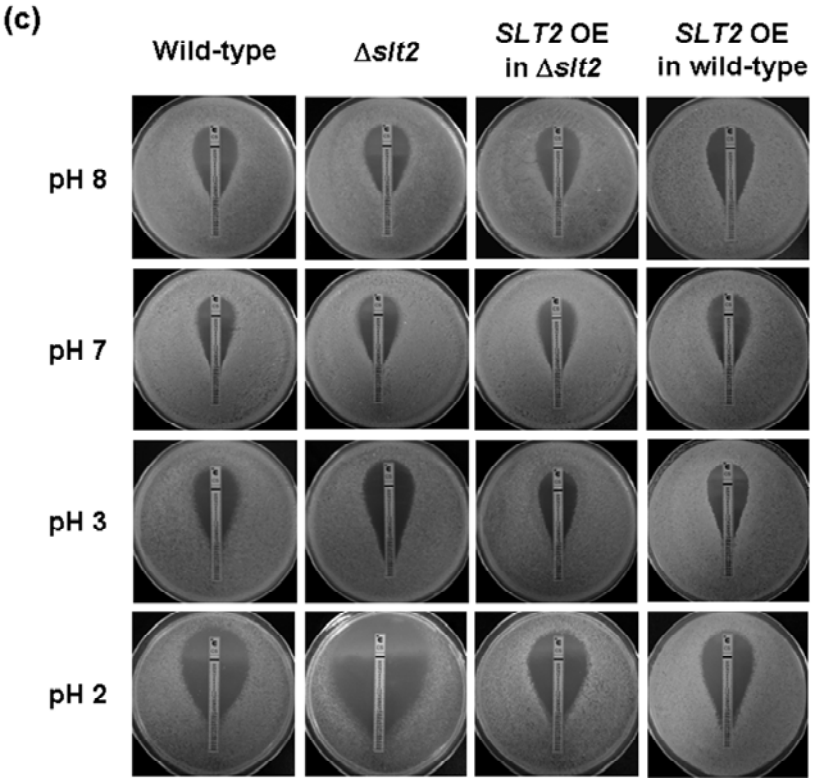

(c)
Wild-type
Δslt2
SLT2 OE in Δslt2
SLT2 OE in wild-type
pH 8
pH 7
pH 3
pH 2

Fig. 3.

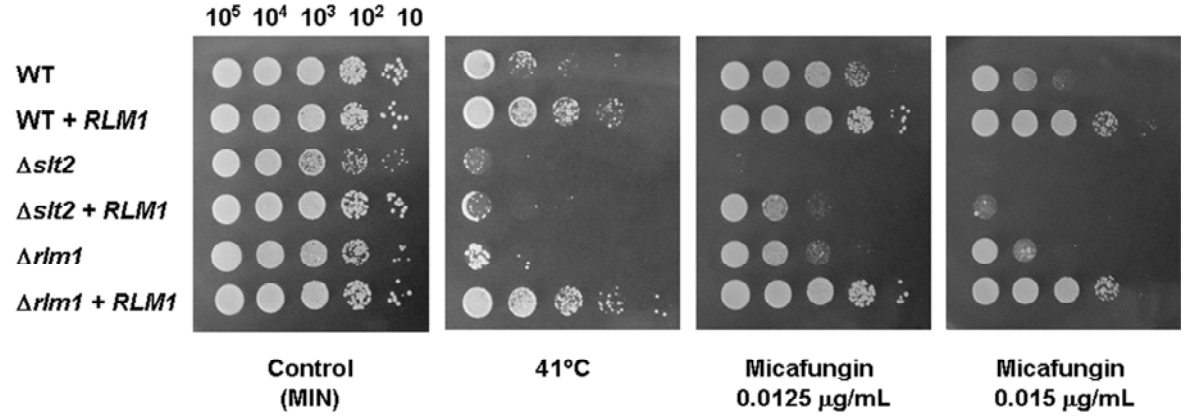

10^5 10^4 10^3 10^2 10
WT
WT + RLM1
Δslt2
Δslt2 + RLM1
Δrlm1
Δrlm1 + RLM1
Control (MIN)
41°C
Micafungin 0.0125 μg/mL
Micafungin 0.015 μg/mL

Fig. 4.

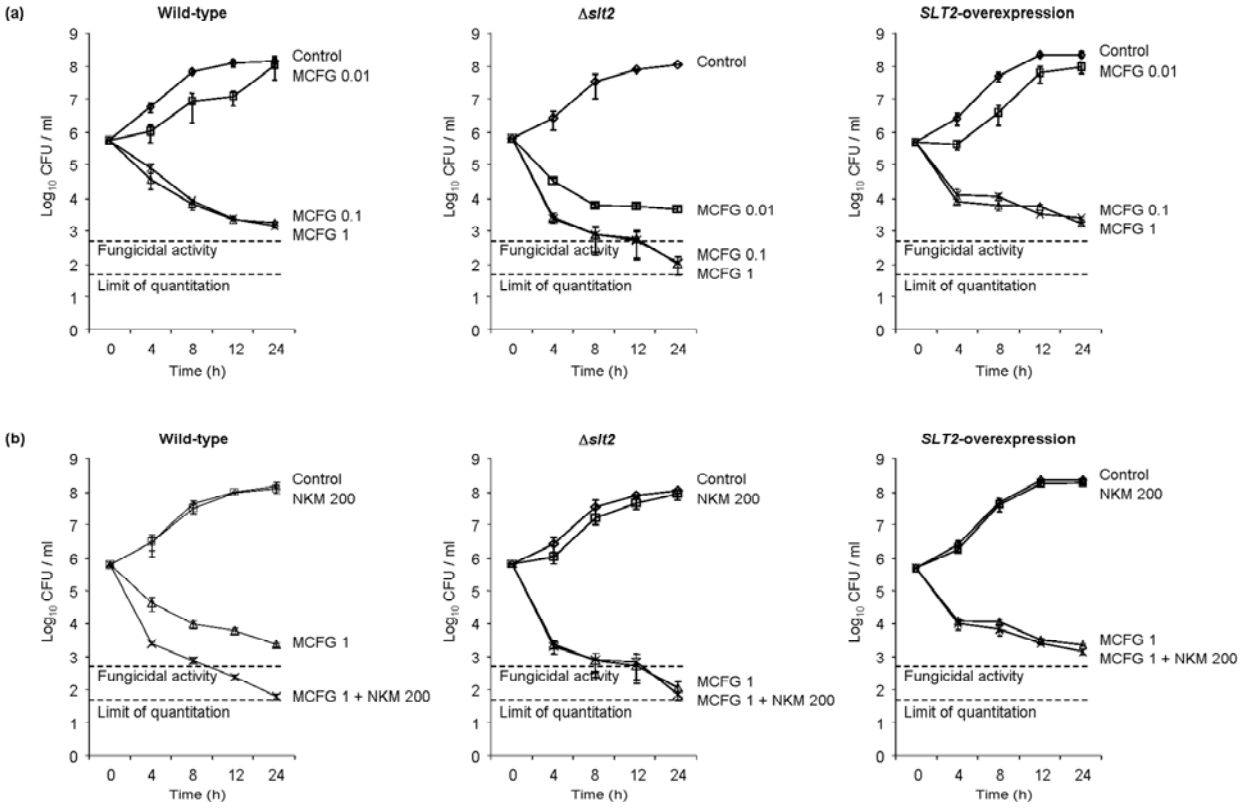
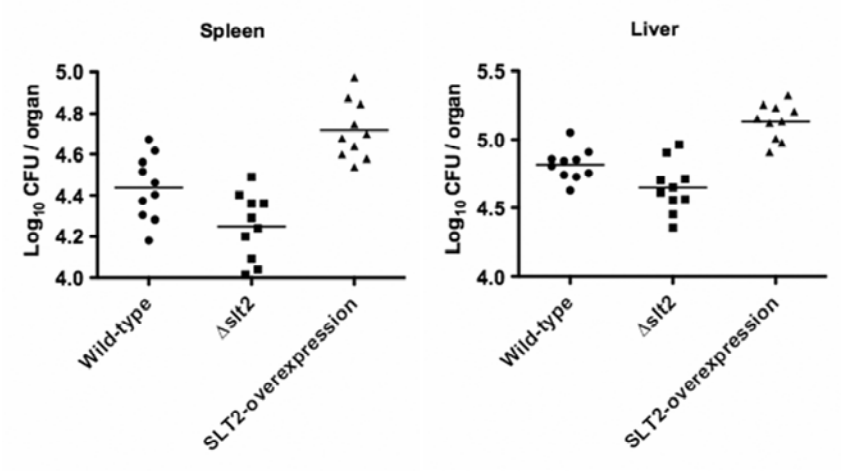

(a)
Wild-type
Δslt2
SLT2-overexpression
Log10 CFU / ml
Time (h)
Control
MCFG 0.01
MCFG 0.1
MCFG 1
Fungicidal activity
Limit of quantitation
(b)
Wild-type
Δslt2
SLT2-overexpression
Control
NKM 200
MCFG 1
MCFG 1 + NKM 200
Fungicidal activity
Limit of quantitation

Fig. 5.


Spleen
Liver
Log10 CFU / organ
5.0
4.8
4.6
4.4
4.2
4.0
5.5
5.0
4.5
4.0
Wild-type
Δslt2
SLT2-overexpression