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Elevated Chitin Content Reduces the Susceptibility of *Candida* Species to Caspofungin

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The echinocandin antifungal drugs inhibit synthesis of the major fungal cell wall polysaccharide β(1,3)-glucan. Echinocandins have good efficacy against *Candida albicans* but reduced activity against other *Candida* species, in particular *Candida parapsilosis* and *Candida guilliermondii*. Treatment of *Candida albicans* with a sub-MIC level of caspofungin has been reported to cause a compensatory increase in chitin content and to select for sporadic echinocandin-resistant *FKS1* point mutants that also have elevated cell wall chitin. Here we show that elevated chitin in response to caspofungin is a common response in various *Candida* species. Activation of chitin synthesis was observed in isolates of *C. albicans*, *Candida tropicalis*, *C. parapsilosis*, and *C. guilliermondii* and in some isolates of *Candida krusei* in response to caspofungin treatment. However, *Candida glabrata* isolates demonstrated no exposure-induced change in chitin content. Furthermore, isolates of *C. albicans*, *C. krusei*, *C. parapsilosis*, and *C. guilliermondii* which were stimulated to have higher chitin levels via activation of the calcineurin and protein kinase C (PKC) signaling pathways had reduced susceptibility to caspofungin. Isolates containing point mutations in the *FKS1* gene generally had higher chitin levels and did not demonstrate a further compensatory increase in chitin content in response to caspofungin treatment. These results highlight the potential of increased chitin synthesis as a potential mechanism of tolerance to caspofungin for the major pathogenic *Candida* species.

The echinocandins are the newest class of antifungal agents and target the fungal cell wall through noncompetitive inhibition of β(1,3)-glucan synthase (1–3). The inhibition of β-glucan synthesis by the echinocandins occurs predominantly through inhibition of Fks [β(1,3)-glucan synthase] subunits (1, 2). Currently, three echinocandins have been approved for clinical use: caspofungin (CSF), micafungin (MCF), and anidulafungin (ANF) (4–7). CSF is the most widely used echinocandin in clinical settings and has fungicidal activity against the majority of *Candida* species, although *Candida parapsilosis* and *Candida guilliermondii* are relatively insensitive to CSF (8–13). The incidence of clinical resistance to the echinocandins is rare, with more than 99% of *Candida* clinical isolates (with the exception of *C. parapsilosis* and *C. guilliermondii*) being inhibited by ≤2 μg ml^{-1} CSF (14–17). However, 5% of nosocomial *C. glabrata* isolates are resistant to caspofungin (18). The CLSI Antifungal Subcommittee has introduced species-specific guidelines that revise the original echinocandin MIC clinical breakpoint (CBP) for susceptibility of ≤2 μg ml^{-1} (13). In these new guidelines, the CBP for resistance has been recommend as ≥1 μg ml^{-1} for CSF, ANF, and MCF for *C. albicans*, *C. tropicalis*, and *C. krusei*. *C. parapsilosis* isolates are classified as resistant if the MIC is ≥8 μg ml^{-1} for CSF, ANF, and MCF, and the CBPs for *C. glabrata* are ≥0.5 μg ml^{-1} for CSF and ANF and ≥0.25 μg ml^{-1} for MCF.

Isolates of different *Candida* species that are resistant to echinocandins, either *in vitro* or *in vivo*, have frequently acquired point mutations in the *FKS1* or *FKS2* target gene (7, 19, 20, 52). In *C. albicans*, these mutations are typically associated with two hot spot regions in the *FKS1* gene, which lie between amino acid residues 641 and 649 (hot spot region 1) and residues 1345 and 1365 (hot spot region 2) (7, 19, 20, 52). The most frequent amino acid substitution resulting in resistance to CSF in *C. albicans* occurs at Ser^{645}, in the first hot spot region (21, 22). In *C. glabrata*, resistance-conferring point mutations have also been identified in *FKS2* that result in amino acid substitutions at positions 659 to 666 and 1375 (23). *C. parapsilosis* is thought to be intrinsically less susceptible to CSF due to an alanine occurring naturally at position 660 in Fks1, which replaces the proline found in other species (24). Similarly, other intrinsically less susceptible fungal species, such as *Neurospora crassa*, *Fusarium solani*, *Fusarium graminearum*, *Fusarium verticillioides*, and *Magnaporthe grisea*, contain a tyrosine at residue 641, replacing the phenylalanine found in Fks1 proteins of susceptible species (25, 26). This suggests that Fks alterations confer resistance to the echinocandins in a wide range of fungal species.

The paradoxical effect of CSF is an *in vitro* phenomenon whereby echinocandins have reduced activity against *Candida* and *Aspergillus* species at concentrations well above the MIC (27–29). Paradoxical growth has been shown to occur most frequently with CSF but has also been observed with the other echinocandins (30). A survey of bloodstream *Candida* isolates, including isolates of *C. albicans*, *C. parapsilosis*, *C. tropicalis*, *C. krusei*, and *C. glabrata*, showed that the frequency of the paradoxical effect is echinocandin specific and also species related (30, 31). The paradoxical effect has also been shown to be growth medium specific and to occur more frequently when biofilms are formed (32–35). The paradoxical effect has been debated as being only an *in vitro* phenomenon because it apparently does not occur in the presence of human serum (36). Paradoxical growth is not associated with point mu-

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TABLE 1 *Candida* sp. clinical isolates used in this project

Isolate	Strain	Site of isolation	Country of origin
C. parapsilosis isolate 1	SCS40113	Blood	Scotland
C. parapsilosis isolate 2	73/116	Anus	England
C. parapsilosis isolate 3	L103	Anus	England
C. parapsilosis isolate 4	81/029	Mouth	England
C. parapsilosis isolate 5	SCS015.50183	Blood	Scotland
C. krusei isolate 1	20479.077	Blood	Slovakia
C. krusei isolate 2	RB1316	Skin biopsy	England
C. krusei isolate 3	RB29-01	Blood	England
C. krusei isolate 4	J990529	Vagina	USA
C. krusei isolate 5	20522.042	Blood	Slovakia
C. krusei isolate 6	L86	Mouth	England
C. glabrata isolate 1	J990055	Sputum	USA
C. glabrata isolate 2	J981302	Vagina	USA
C. glabrata isolate 3	AM2004/0050	Blood	Scotland
C. glabrata isolate 4	BB412783	Blood	Scotland
C. guilliermondii isolate 1	M476/93/6	Animal (seal)	Scotland
C. guilliermondii isolate 2	81/054	Nail	England
C. guilliermondii isolate 3	SCS192139p	Blood	Scotland
C. guilliermondii isolate 4	J960023	Nail	Belgium
C. guilliermondii isolate 5	SCS74555E	Blood	Scotland
C. tropicalis isolate 1	31586/7/04	Central venous pressure catheter tip (femoral)	England
C. tropicalis isolate 2	31580/7/04	Urine	England
C. tropicalis isolate 3	GUI4448	Soil (beach)	Brazil
C. tropicalis isolate 4	B31581/7/04	Urine	England
C. tropicalis isolate 5	L634	Wound	Unknown
C. albicans isolate 1	FJ9	Throat	Australia
C. albicans isolate 2	AM2003/0191	Blood	England
C. albicans isolate 3	AM2003/0069	Vagina	England
C. albicans isolate 4	FC28	Vulva	USA
C. albicans isolate 5	J990102	Vagina	Belgium
C. albicans isolate 6	S20175.016	Blood	Israel

tations in *FKS1* and is therefore more likely due to activation of cell wall salvage mechanisms (7, 37, 38). The cell wall is a dynamic structure which can alter its composition in response to damage by upregulating cell wall biosynthetic genes (7). This results in remodelling of the cell wall to restore cell wall integrity. Often, this cell wall salvage mechanism results in increased chitin synthesis (7, 37, 39). Consequently, treating wild-type *C. albicans* cells with a sub-MIC level of CSF leads to increased chitin synthesis (7). Likewise, one clinical isolate of *C. albicans* which demonstrated the paradoxical effect had a significantly elevated chitin content in the presence of 12.5 μg ml^{-1} CSF (40). Clinical isolates of *C. albicans*, *C. tropicalis*, *C. orthopsilosis*, and *C. parapsilosis* that exhibited paradoxical growth were also shown to have elevated chitin and glucan and thicker cell walls (31). Paradoxical growth of *C. albicans* is eliminated by nikkomycin Z, a chitin synthase inhibitor, and calcineurin inhibitors, which prevent the upregulation of chitin synthesis in response to cell wall stress (36). This suggests that paradoxical growth occurs as a result of increased chitin content, which allows cells to survive otherwise lethal concentrations of CSF. The compensatory increase in chitin content in response to CSF treatment is mediated by the Ca^{2+}-calcineurin, protein kinase C (PKC), and HOG signaling pathways (7, 39). In addition, treating *C. albicans* cells with $CaCl_2$ and calcofluor white (CFW), which activate the Ca^{2+}-calcineurin and PKC pathways, increases chitin content and reduces susceptibility to CSF *in vitro* and *in vivo* (7, 41). Likewise, cell wall mutants of *C. albicans* with increased chitin contents are less susceptible to CSF (42). These findings suggest that *C. albicans* has the ability to adapt to CSF treatment through compensatory elevation of its chitin content.

Because different *Candida* species have varied susceptibilities to CSF, we aimed to determine whether clinical isolates of different *Candida* species were less susceptible to CSF due to a naturally higher cell wall chitin content, the acquisition of *FKS1* point mutations, or a combination of both. The sequences of the *FKS1* hot spot regions of each isolate were analyzed, and the responses of different *Candida* species to varied CSF concentrations, in terms of compensatory chitin synthesis, were also investigated. We show that with the exception of *C. glabrata*, all *Candida* species elevated their chitin content in response to echinocandin treatment and that Fks1 mutations generated stable, high chitin contents in the *Candida* cell wall.

MATERIALS AND METHODS

Strains, media, and growth conditions. The *Candida* species used for investigation were chosen to represent both caspofungin-sensitive species (*C. albicans*, *C. glabrata*, and *C. krusei*) and species that have reduced caspofungin susceptibility *in vitro* (*C. parapsilosis* and *C. guilliermondii*). For each *Candida* species, 4 to 6 clinical isolates were tested (Table 1). The isolates were selected as isolates from different anatomical locations in patients who had no previous exposure to caspofungin. *Candida* strains used in this study are listed in Table 1. *Candida* strains were maintained on Sabouraud dextrose (Sabdex) agar plates (1% mycological peptone [wt/vol], 4% [wt/vol] glucose and 2% [wt/vol] agar). In some experiments, *Candida* isolates were grown in YPD broth (1% yeast extract [wt/vol], 2% mycological peptone [wt/vol], and 2% [wt/vol] glucose) alone or pre-

TABLE 2 Susceptibilities of different *Candida* species to caspofungin[a]

Species	Caspofungin IC_{50} ($\mu g\ ml^{-1}$)					
	Isolate 1	Isolate 2	Isolate 3	Isolate 4	Isolate 5	Isolate 6
C. albicans	0.064	0.064	0.064	0.064	0.064	0.032
C. glabrata	0.032	0.032	0.032	0.032		
C. tropicalis	0.064	0.064	0.064	0.032	0.032	
C. krusei	0.13	0.064	0.032	0.032	0.032	>16
C. parapsilosis	0.13	0.25	0.25	0.13	0.064	
C. guilliermondii	>16	>16	0.064	>16	>16	

[a] As determined by broth microdilution testing. The IC_{50} was defined as the concentration of caspofungin at which growth of the clinical isolates of each *Candida* species was inhibited by 50% during growth in YPD for 24 h at 30°C.

treated by growing in YPD broth containing 0.2 M $CaCl_2$ and 100 $\mu g\ ml^{-1}$ CFW (Sigma-Aldrich, United Kingdom) for 12 h at 30°C with shaking at 200 rpm to stimulate chitin synthesis (7).

Antifungal susceptibility testing. CSF (Merck Research Laboratories, NJ) MICs were determined by broth microdilution testing with YPD medium at 30°C and a starting inoculum of 1×10^6 cells/ml. Drug concentrations ranged from 0.016 $\mu g\ ml^{-1}$ to 16 $\mu g\ ml^{-1}$ CSF. Exponentially growing cultures were diluted to 2×10^6 cells ml^{-1} in 2× YPD, and 100 μl of culture was added to each well. Plates were incubated for 24 h at 30°C. After incubation, optical densities at 405 nm were read in a VERSAmax tunable microplate reader (Molecular Devices, CA).

Calcofluor white susceptibility testing. Susceptibility to CFW was determined by broth microdilution testing. Exponentially grown cultures were diluted to 2×10^6 cells ml^{-1} in 2× YPD, and 100 μl of culture was added to each well. CFW concentrations ranged from 25 $\mu g\ ml^{-1}$ to 400 $\mu g\ ml^{-1}$. Plates were incubated for 24 h at 30°C, and optical densities at 600 nm were read in a VERSAmax tunable microplate reader (Molecular Devices, CA).

Caspofungin susceptibility testing on solid medium. CSF was incorporated into YPD agar plates at 0.032 $\mu g\ ml^{-1}$ to 16 $\mu g\ ml^{-1}$, depending on the 50% inhibitory concentration (IC_{50}) of each isolate. *Candida* cells were serially diluted to generate suspensions containing 1,000 to 1×10^6 cells ml^{-1} in sterile water. Plates were inoculated with 5-μl drops of each cell suspension and incubated for 24 h at 37°C.

Fluorescence microscopy. After washing with sterile water to remove any excess medium, samples were fixed in 10% (vol/vol) neutral buffered formalin (Sigma-Aldrich, United Kingdom) and examined by differential interference contrast (DIC) microscopy. Cells were stained with 25 $\mu g\ ml^{-1}$ CFW (Sigma-Aldrich, United Kingdom) to visualize chitin. All samples were examined by fluorescence microscopy using a Zeiss Axioplan 2 microscope. Images were recorded digitally using an Openlab system (Openlab v 4.04; Improvision, Coventry, United Kingdom) and a Hamamatsu C4742-95 digital camera (Hamamatsu Photonics, Hamamatsu, Japan). Chitin content was measured by quantitative CFW fluorescence of individual yeast cells (7). Mean fluorescence intensities were calculated for 50 individual cells for each condition. In some experiments, the exposure time of fluorescence images was fixed so the intensity of fluorescence relative to a control of known chitin content was determined.

DNA sequence analysis of *FKS1* hot spot regions. *FKS1* hot spot regions were analyzed as described previously. Briefly, genomic DNA was extracted according to the protocol of Hoffman and Winston (53). Hot spot regions were amplified with the following forward and reverse primers (1 μM concentration of each): HS1-F, 5′-TTT ATT CAA ATT CTT GCC-3′; HS2-F, 5′ AAT GCC ATG ATG AGA GGT GG-3′; HS1-R, 5′ GGA ATG CCA TTG TTA TTT CC-3′; HS2-R, 5′ GGT ACA GTT TCT CAT TGG CA-3′. PCRs were performed using Extensor master mix (Abgene) and involved an initial 5-min denaturation step at 94°C followed by 30 cycles of 94°C for 30 s, 53°C for 1 min, and 72°C for 1 min, with a 5-min final extension step at 72°C. PCR products were purified according to the protocol of Rosenthal et al. (43).

Methods for DNA sequence determinations have been described previously (44). Briefly, purified PCR fragments were sequenced on both strands by using a 0.7 μM concentration of each PCR primer. Sequencing reactions were performed with an ABI Prism BigDye Terminator cycle sequencing ready reaction kit (Applied Biosystems) according to the manufacturer's recommendations. Sequences were analyzed with an ABI Prism model 3730xl DNA analyzer (Applied Biosystems) by Geneservice, Oxford, United Kingdom. The sequence of *FKS1*/*GSC1* (orf19.2929) from the *Candida* Genome Database (CGD; http://www.candidagenome.org) was used as a reference (45).

RESULTS

***Candida* species have different susceptibilities to caspofungin.** The relative susceptibilities of *Candida* sp. clinical isolates to CSF were determined using a broth microdilution method. In general, isolates of *C. albicans*, *C. glabrata*, and *C. tropicalis* were the most susceptible to CSF (Table 2). Isolates of *C. krusei* had intermediate susceptibility, and isolates of *C. parapsilosis* and *C. guilliermondii* were relatively insensitive to CSF (Table 2). The IC_{50} range for the susceptible species, *C. albicans*, *C. glabrata*, and *C. tropicalis*, was 0.032 $\mu g\ ml^{-1}$ to 0.064 $\mu g\ ml^{-1}$ CSF (Table 2). The majority of *C. krusei* isolates had IC_{50}s between 0.032 $\mu g\ ml^{-1}$ and 0.13 $\mu g\ ml^{-1}$, similar to the susceptible strains, with the exception of *C. krusei* isolate 6, which was resistant to CSF, with an IC_{50} of ≥16 $\mu g\ ml^{-1}$ CSF (Table 2). The *C. parapsilosis* isolates had significantly higher IC_{50}s than the more susceptible species (Table 2). *C. guilliermondii* isolates are known to be less susceptible to CSF, and indeed, the majority of *C. guilliermondii* isolates had IC_{50}s of ≥16 $\mu g\ ml^{-1}$ CSF (Table 2). Interestingly, *C. guilliermondii* isolate 3 was relatively susceptible to CSF compared to the other *C. guilliermondii* isolates, with an IC_{50} of 0.064 $\mu g\ ml^{-1}$ (Table 2). Therefore, the order of susceptibility of these species to CSF, from most susceptible to most resistant, was as follows: *C. albicans* < *C. glabrata* < *C. krusei* < *C. parapsilosis* < *C. guilliermondii*.

***Candida* species have different sensitivities to the chitin binding agent calcofluor white.** Elevated chitin content can lead to reduced susceptibility to CSF in *C. albicans*, both *in vitro* and *in vivo* (7, 41, 42). Because different *Candida* species are known to have various degrees of susceptibility to CSF, it was of interest to determine the susceptibility of the *Candida* isolates to CFW. CFW is a chitin binding agent which inhibits growth of fungal cells. Typically, cells with a high chitin content are hypersensitive to CFW, whereas cells with low chitin levels are less sensitive; however, other changes in the cell wall that do not involve modifications to the chitin levels may also alter CFW sensitivity (46). Each isolate was tested for susceptibility to CFW by the broth microdilution method. All of the *C. albicans* clinical isolates were inhibited

TABLE 3 Different *Candida* species have different susceptibilities to CFW[a]

Species	Calcofluor white IC_{50} ($\mu g\ ml^{-1}$)					
	Isolate 1	Isolate 2	Isolate 3	Isolate 4	Isolate 5	Isolate 6
C. albicans	100	200	100	100	100	200
C. glabrata	>400	>400	>400	>400		
C. tropicalis	25	50	50	50	50	
C. krusei	>400	>400	>400	>400	>400	50
C. parapsilosis	>400	25	>400	>400	>400	
C. guilliermondii	>400	>400	>400	>400	>400	

[a] Sensitivities of clinical isolates of *Candida* species to CFW were determined by broth microdilution testing in YPD at 30°C for 24 h. The concentration of CFW at which growth of the *Candida* sp. isolates was inhibited by 50% (IC_{50}) was determined.

by intermediate concentrations of CFW, indicating intermediate levels of chitin in the cell wall (Table 3). *C. glabrata* and *C. guilliermondii* isolates were all resistant to CFW (Table 3), suggesting that isolates of *C. glabrata* and *C. guilliermondii* have relatively low chitin contents. Isolates of *C. tropicalis* were very sensitive to CFW (Table 3), suggesting that they may have higher levels of cell wall chitin. The majority of *C. krusei* and *C. parapsilosis* isolates had low sensitivity to CFW, suggesting that they also contain lower levels of chitin, although for both species there were individual isolates which were particularly sensitive to CFW, suggesting that these strains had higher chitin levels (Table 3). In summary, there was a crude but not universal correlation between species type, chitin content, and CFW sensitivity.

***Candida* species have different responses to caspofungin treatment.** To further analyze the chitin contents of the clinical isolates, each strain was stained with 25 $\mu g\ ml^{-1}$ CFW, which can be used as a semiquantitative stain for cell wall chitin content. Isolates were also stained with CFW after treatment with CSF to determine whether CSF treatment activated chitin synthesis in the different *Candida* species, as it does with *C. albicans*. The concentration of CSF used was the IC_{50} presented in Table 2 and varied for individual isolates. Pregrowth of *C. albicans* with 200 mM $CaCl_2$ and 100 $\mu g\ ml^{-1}$ CFW increased cell wall chitin and resulted in cells which were less susceptible to CSF (7). Therefore, each clinical isolate was also pregrown with $CaCl_2$ and CFW to determine whether this would lead to an increase in chitin content. After pregrowth with $CaCl_2$ and CFW, isolates were treated with CSF at the IC_{50} to determine whether this resulted in reduced susceptibility to CSF. The results are summarized below for each species examined.

(i) *C. albicans*. All clinical isolates of *C. albicans* had chitin distributed evenly around the cell wall, with more intense staining in the septal region (Fig. 1A). Likewise, treatment with CSF at the IC_{50} led to a uniform increase in chitin content in the lateral cell wall (Fig. 1B; Table 4). Pregrowth with $CaCl_2$ and CFW also led to increased chitin synthesis in all of the *C. albicans* clinical isolates (Fig. 1C; Table 4). When isolates were pregrown with $CaCl_2$ and CFW and then exposed to CSF at the IC_{50}, there was an even greater elevation of chitin content (Fig. 1D; Table 4), and cells were less susceptible to CSF (Fig. 2).

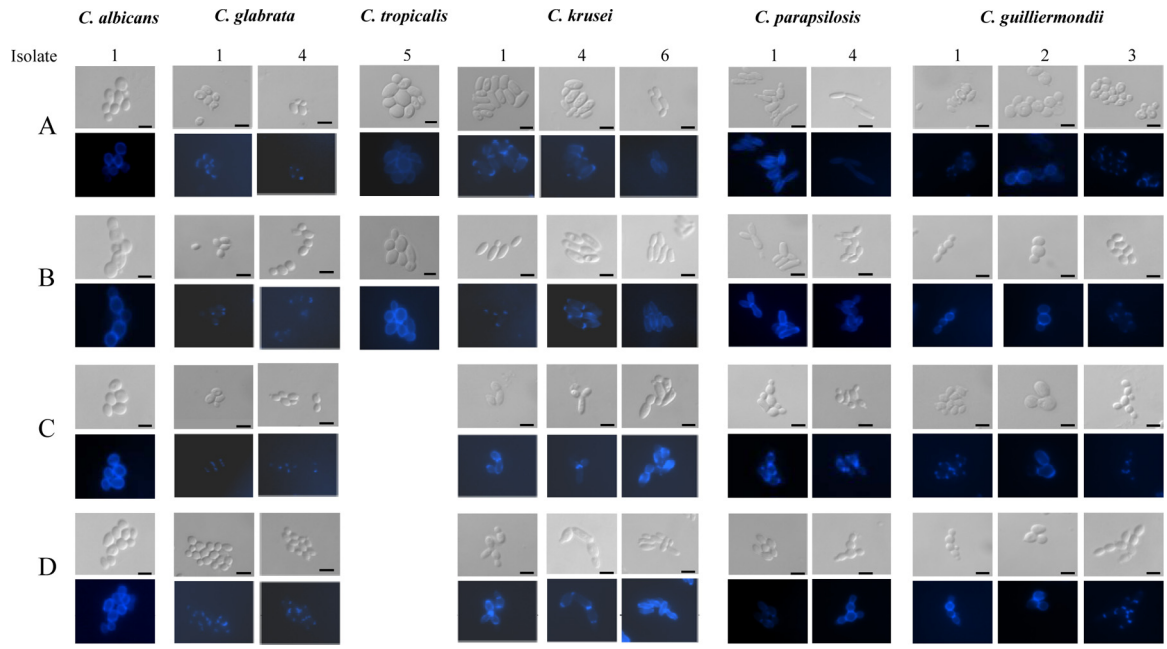

FIG 1 Chitin distribution in *Candida* clinical isolates. DIC microscopy images (top panels) and CFW fluorescence images (bottom panels). Isolates were grown in YPD alone to establish basal chitin levels (A), treated with a sub-MIC level of CSF (B), grown in YPD with 200 mM $CaCl_2$ and 100 $\mu g\ ml^{-1}$ CFW (C), or pregrown with $CaCl_2$ and CFW and then exposed to caspofungin at the IC_{50} (D). Bars, 2 μm.

TABLE 4 Quantification of cell wall chitin contents of *Candida* species, with and without caspofungin and/or $CaCl_2$ and CFW treatment[a]

Candida species	Isolate no.	CFW fluorescence (mean fluorescence intensity)			
		No treatment	Caspofungin treatment	$CaCl_2$ and CFW treatment	$CaCl_2$ and CFW pretreatment and caspofungin treatment
C. albicans	1	605 ± 208	1,402 ± 404*	1,655 ± 328*	2,050 ± 629*
C. glabrata	1	363 ± 119	330 ± 74	405 ± 92	379 ± 98
	4	317 ± 81	344 ± 112	334 ± 76	346 ± 61
C. tropicalis	5	543 ± 166	1,664 ± 249*	NG	NG
C. krusei	1	369 ± 75	393 ± 49	1,282 ± 322*	2,113 ± 384*
	4	375 ± 108	401 ± 76	418 ± 106	408 ± 183
	6	568 ± 149	1,037 ± 169*	1,517 ± 290*	2,275 ± 504*
C. parapsilosis	1	730 ± 172	779 ± 109	1,385 ± 388*	362 ± 120*
	4	260 ± 87	998 ± 218*	1,787 ± 374*	1,876 ± 405*
C. guilliermondii	1	231 ± 92	861 ± 163*	666 ± 107*	1,580 ± 306*
	2	627 ± 146	978 ± 143*	1,115 ± 169*	1,488 ± 304*
	3	300 ± 94	328 ± 96	294 ± 79	329 ± 77

[a] The average relative chitin content of individual cells from isolates of different *Candida* species was determined by measuring the intensity of CFW fluorescence. Measurements were made on untreated control cultures and after growth with caspofungin at the IC_{50} specific for each isolate (as determined in Table 2), after growth with 0.2 M $CaCl_2$ and 100 μg/ml CFW, and for cells pregrown with $CaCl_2$ and CFW and then grown with caspofungin at the IC_{50} for each isolate. Statistical differences are shown for comparison to each untreated isolate (*, $P < 0.05$; Student *t* test), and data are means with standard deviations ($n = 50$). NG, no growth.

(ii) *C. glabrata.* Clinical isolates of *C. glabrata* had little chitin in the cell wall, as assessed by CFW staining and CFW susceptibility. There was some accumulation of chitin concentrated at the poles of the cells. Two representatives are shown in Fig. 1A (isolates 1 and 4). Treating *C. glabrata* isolates with CSF at the IC_{50} had no effect on chitin content (Fig. 1B; Table 4). Similarly, pregrowth with $CaCl_2$ and CFW did not stimulate chitin synthesis (Fig. 1C; Table 4), and exposing these treated cells to CSF did not result in a change in chitin content (Fig. 1D; Table 4). Although this was the general trend for most strains, *C. glabrata* isolate 1 was less susceptible to CSF after pregrowth with $CaCl_2$ and CFW (Fig. 2 and data not shown). Pregrowth with $CaCl_2$ and CFW had no effect on the CSF susceptibility of the other three isolates (Fig. 2 [for isolate 4] and data not shown).

(iii) *C. tropicalis.* Isolates of *C. tropicalis* had relatively high levels of chitin in the cell wall, and chitin was distributed evenly throughout the cell wall (Fig. 1A). Treatment with caspofungin resulted in an increase in chitin content in isolates of *C. tropicalis* (Fig. 1B; Table 4). Correspondingly, *C. tropicalis* isolates appeared to be hypersensitive to $CaCl_2$ and CFW, and consequently, no growth was observed after this treatment (Fig. 2).

(iv) *C. krusei.* The *C. krusei* isolates tested were generally less susceptible to CSF than *C. albicans* and *C. glabrata*. An exception was isolate 6, which was an example of a highly resistant strain (Table 2). The majority of *C. krusei* isolates (83%), including isolates 1 and 4, contained low levels of basal chitin, concentrated predominantly at the poles of the cells (Fig. 1A; Table 4). The exception to this was *C. krusei* isolate 6, which appeared to have a homogeneous distribution of chitin over the cell wall surface (Fig. 1A). Likewise, only *C. krusei* isolate 6 exhibited a substantial increase in chitin content after treatment with CSF at the IC_{50} (Fig. 1B; Table 4). Isolates 1 and 6, which showed an increase in chitin after pregrowth with $CaCl_2$ and CFW (Fig. 1C; Table 4), were less susceptible to CSF (Fig. 2). Pregrowth with $CaCl_2$ and CFW had no effect on the chitin content of *C. krusei* isolate 4 and consequently did not affect its susceptibility to CSF (Fig. 2; Table 4). Isolate 4 alone had unaltered CSF susceptibility after $CaCl_2$ and CFW treatment.

(v) *C. parapsilosis.* The *C. parapsilosis* isolates tested were significantly less susceptible to CSF than most other *Candida* species (Table 2) (14). All of the *C. parapsilosis* clinical isolates tested had a uniform chitin distribution over the cell surface when they were grown without CSF treatment (Fig. 1A). There were some notable differences in the levels of basal chitin in different isolates (Fig. 1A; Table 4). Treatment with CSF at the IC_{50} either had no effect on the chitin content of *C. parapsilosis* (isolate 1) or resulted in a compensatory increase in chitin (e.g., in isolate 4) (Fig. 1B; Table 4). Treatment with $CaCl_2$ and CFW led to an increase in overall chitin content (Fig. 1C; Table 4). After pregrowth with $CaCl_2$ and CFW, the majority of the *C. parapsilosis* isolates, such as isolate 4, exhibited an increase in chitin after treatment with CSF (Fig. 1D; Table 4), and these became less susceptible to CSF than untreated cells (Fig. 2). However, in some cases (for example, isolate 1), pregrowth with $CaCl_2$ and CFW and subsequent treatment with CSF resulted in a decrease in chitin content (Fig. 1D; Table 4). Reflecting this, *C. parapsilosis* isolate 1 was hypersensitive to CSF after pregrowth with $CaCl_2$ and CFW (Fig. 2). Therefore, in the majority of cases, treatment of *C. parapsilosis* with $CaCl_2$ and CFW led to an increase in chitin content and a reduced susceptibility to CSF.

(vi) *C. guilliermondii.* *C. guilliermondii* was the most CSF-resistant species of the *Candida* species tested (Table 2). The cell wall chitin distribution of *C. guilliermondii* isolates varied. Some (for example, isolate 2) had a homogeneous distribution of chitin, whereas other isolates (isolates 1 and 3) had lower chitin levels, with some chitin concentrated at the poles of the cells (Fig. 1A; Table 4). In contrast, treatment with CSF at the IC_{50} resulted in an increase in chitin in the majority of isolates (Table 4). An exception was isolate 3 (Fig. 1B; Table 4). Pregrowth of the *C. guilliermondii* isolates with $CaCl_2$ and CFW resulted in an increase in chitin in all isolates, again with the exception of isolate 3 (Fig. 1C;

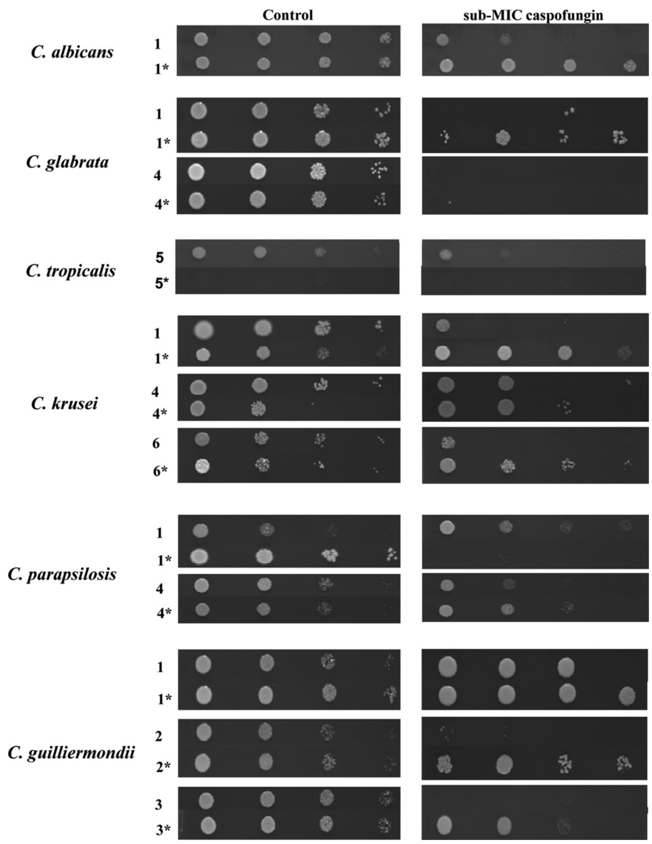

FIG 2 Pregrowth of *Candida* isolates with $CaCl_2$ and CFW reduces susceptibility to caspofungin. Plate dilution sensitivity tests were performed on *Candida* clinical isolates on YPD agar containing caspofungin at the IC_{50} for each isolate. The following CSF concentrations were used: for *C. albicans* isolate 1, 0.064 μg ml^{-1}; for *C. glabrata* isolates 1 and 4, 0.032 μg ml^{-1}; for *C. tropicalis* isolate 5, 0.032 μg ml^{-1}; for *C. krusei* isolate 1, 0.13 μg ml^{-1}; for *C. krusei* isolate 4, 0.032 μg ml^{-1}; for *C. krusei* isolate 6, 16 μg ml^{-1}; for *C. parapsilosis* isolates 1 and 4, 0.13 μg ml^{-1}; for *C. guilliermondii* isolates 1 and 2, 16 μg ml^{-1}; and for *C. guilliermondii* isolate 3, 0.064 μg ml^{-1}. Rows marked with asterisks indicate pregrowth of the inoculum in YPD containing both 200 mM $CaCl_2$ and 100 μg ml^{-1} CFW. Cell numbers per spot were 5,000, 500, 50, and 5 cells, from left to right.

TABLE 5 Summary of results for all *Candida* spp.

Candida species	Elevation of chitin content[a]: Treatment with caspofungin at the IC_{50}	Elevation of chitin content[a]: Treatment with $CaCl_2$ and CFW	Elevation of chitin content[a]: Protection after pretreatment with $CaCl_2$ and CFW
C. albicans	+	+	+
C. glabrata	−	−	+
C. tropicalis	+	NG	NG
C. krusei	−	−	−
C. parapsilosis	+	+	+
C. guilliermondii	+	+	+

[a] NG, no growth.

Table 4). Pregrowth of *C. guilliermondii* isolates with $CaCl_2$ and CFW and subsequent treatment with CSF also led to an increase in chitin in most isolates (Fig. 1D; Table 4). The higher chitin levels appeared to be distributed uniformly rather than concentrated at the poles of cells (Fig. 1D). With the exception of isolate 3, pregrowth with $CaCl_2$ and CFW increased chitin synthesis and led to a reduced susceptibility to CSF in *C. guilliermondii* (Fig. 2).

This analysis revealed that specific *Candida* species displayed a compensatory increase in chitin content in response to CSF treatment, but the response varied between species and isolates of the same species (Table 5). Likewise, treatment with $CaCl_2$ and CFW was capable of eliciting an increase in chitin content, leading to CSF protection in some, but not all, *Candida* species (Table 5).

Sequencing of *FKS1* hot spot regions. The *FKS1* hot spot regions of the *Candida* sp. clinical isolates were sequenced to determine whether reduced susceptibility to CSF was due to point mutations in *FKS1* (Fig. 3). Isolates of *C. albicans*, *C. glabrata*, and *C. krusei* contained no point mutations in *FKS1* (Fig. 3). All of the *C. parapsilosis* isolates contained the P660A substitution (Fig. 3) which is thought to render *C. parapsilosis* intrinsically less susceptible to CSF (24). *C. parapsilosis* isolate 4 contained an additional S645P point mutation (Fig. 3). Isolate 3 of *C. guilliermondii* contained an L646I point mutation in the first *FKS1* hot spot region

```
C. albicans isolates:

                641      649  1345                  1365
Seq_C.alb       FLTLSLRDP     YPFGCYNIAPAVDWIRRYTLS
C.alb_1         FLTLSLRDP     YPFGCYNIAPAVDWIRRYTLS
                *********     *********************

C. glabrata isolates:

                625      633  1328                  1348
Seq_C.glab      FLILSIRDP     YPLGCYNLAPAIDWIRRYTLS
C.glab_1        FLILSIRDP     YPLGCYNLAPAIDWIRRYTLS
C.glab_4        FLILSIRDP     YPLGCYNLAPAIDWIRRYTLS
                *********     *********************

C. tropicalis:

                25        33   730                  750
Seq_C.trop      FLTLSLRDP     YPFGCYNIAPAVDWIRRYTLS
C.trop_5        FLTLSLRDP     YPFGCYNIAPAVDWIRRYTLS
                *********     *********************

C. krusei isolates:

                655      663  1352                  1372
Seq_C.kru       FLILSIRDP     YPLGCYNLAPAIDWIRRYTLS
C.krus_1        FLILSIRDP     YPLGCYNLAPAIDWIRRYTLS
C.krus_4        FLILSIRDP     YPLGCYNLAPAIDWIRRYTLS
C.krus_6        FLILSIRDP     YPLGCYNLAPAIDWIRRYTLS
                *********     *********************

C. parapsilosis isolates:

                652      660  1357                  1377
Seq_C.para      FLTLSLRDA     YPFGCYNIAPAVDWIRRYTLS
C.para_1        FLTLSLRDA     YPFGCYNIAPAVDWIRRYTLS
C.para_4        FLTLPLRDA     YPFGCYNIAPAVDWIRRYTLS
                **** ****     *********************

C. guilliermondii isolates:

                632      640  1335                  1355
Seq_C.guill     FMALSLRDP     YPYGCYNFAPAVDWIRRYTLS
C.guill_1       FMALSLRDP     YPYGCYNFAPAVDWIRRYTLS
C.guill_2       FMALSLRDP     YPYGCYNFAPAVDWIRRYTLS
C.guill_3       FMALSIRDP     YPYGCYNFAPAVDWIRRYTLS
                ***** ***     *********************
```

FIG 3 Sequencing of the *FKS1* hot spot region in *Candida* species. For each species, the sequenced control strain was used as a template to determine whether any of the clinical isolates contained point mutations in *FKS1*. All isolates of *C. parapsilosis* contained a P660A substitution, and isolate 4 contained an additional S645P point mutation (highlighted in bold). *C. guilliermondii* isolate 3 contained an L646I point mutation (highlighted in bold).

(Fig. 3). No point mutations were observed in the second hot spot region of *FKS1* (Fig. 3).

DISCUSSION

Different *Candida* species are known to have various susceptibilities to CSF. *C. albicans* is the most sensitive of these species, with *C. glabrata*, *C. tropicalis* and *C. krusei*, *C. parapsilosis*, and *C. guilliermondii* representing the rank order of susceptibility for the non-*C. albicans* species (8, 10, 14, 47). The clinical isolates of *Candida* species tested in this study followed the same susceptibility trends. The *C. albicans* clinical isolates tested all demonstrated an elevation of chitin content upon treatment with CSF at the IC_{50}. After treatment with $CaCl_2$ and CFW, these isolates showed an increase in chitin content which resulted in reduced CSF susceptibility. Therefore, increased chitin synthesis rescued a range of *C. albicans* clinical isolates from the inhibitory effects of CSF treatment.

Increases in *SLT2* expression and chitin content have been associated with incomplete killing of *C. glabrata* by CSF (48). In contrast, none of the *C. glabrata* isolates tested in this study showed a change in chitin content after treatment with CSF. Similarly, pregrowth with $CaCl_2$ and CFW did not elevate the chitin content of *C. glabrata* isolates. Despite this observation, two isolates of *C. glabrata* showed reduced susceptibility to CSF after pregrowth with $CaCl_2$ and CFW. Because there was no visible change in chitin content in these strains, the pretreatment may have elicited different, as yet uncharacterized responses in these isolates. The difference in results between this work and a previous study (48) may relate to strain differences in the *C. glabrata* isolates that were used. In this work, susceptible clinical isolates of *C. glabrata* were used, whereas the study by Cota et al. employed a genetically modified control strain (48). This study used measurements of CFW fluorescence to determine the chitin contents of isolates, whereas Cota et al. used a biochemical assay to quantify chitin content. Another consideration for differences between the studies may be the concentrations of caspofungin that were used. It is conceivable that treatment with concentrations of caspofungin above the MIC may also have resulted in an increase in chitin content in the *C. glabrata* isolates in this study.

The clinical isolates of *C. krusei* exhibited significantly variable chitin contents, and chitin was distributed either at polar locations or uniformly throughout the cell wall surface. A previous study showed that *C. krusei* isolates from soil sediments in which there were various levels of environmental pollution had significantly different chitin contents and chitin synthase activities (54). Isolates taken from the most polluted areas had the highest chitin contents, whereas isolates with low chitin levels were predominant in areas with less pollution (54). In the present study, the majority of *C. krusei* isolates had a polar distribution of chitin. *C. krusei* isolate 6 had a uniform chitin distribution and was the only isolate to be isolated from an oral infection site. Because an elevated chitin content was the only obvious difference between this isolate and the other *C. krusei* isolates, it is possible that the reduced susceptibility to CSF was a result of the strain's higher basal chitin content. In most cases, an elevation of chitin content rescued *C. krusei* from CSF treatments.

C. parapsilosis isolates had various basal chitin contents. *C. parapsilosis* is known to be significantly less susceptible to CSF, due to a naturally occurring alanine at position 660. The MIC value and Fks1 sequence of this strain corroborated this. Interestingly, *C. parapsilosis* isolates which contained this amino acid substitution had higher chitin contents than those of isolates which contained further additional point mutations. Isolates with the naturally occurring alternative Fks1 sequence showed no change in chitin content upon exposure to CSF. In contrast, isolates which had additional point mutations in *FKS1* had significantly lower basal chitin contents and were the only *C. parapsilosis* isolates to display elevated chitin contents after treatment with CSF. Pregrowth with $CaCl_2$ and CFW increased the chitin content of most *C. parapsilosis* isolates and consequently led to reduced susceptibility to CSF in most cases. However, in a minority of cases, subsequent exposure of these pretreated cells to CSF led, paradoxically, to isolates becoming more sensitive to CSF. This may again be related to the strain chitin content, because pregrowth with $CaCl_2$ and CFW prior to treatment of these isolates with CSF resulted in a significant decrease rather than the normal increase in chitin content. *C. albicans* cells with reduced chitin content have also been shown to have increased sensitivity to CSF (42). There are some indications that activation of chitin synthesis may have a potential role in the reduced susceptibility of *C. parapsilosis* to CSF. Clinical isolates from the *C. parapsilosis* group, which encompasses *C. parapsilosis*, *C. orthopsilosis*, and *C. metapsilosis*, all contain an alanine at position 660. Despite this observation, *C. parapsilosis* has been shown to be less susceptible to CSF and anidulafungin than *C. orthopsilosis* and *C. metapsilosis* (24, 49, 50). Because there are no differences in the Fks1 hot spot sequences between these three species, this difference in susceptibility may be due to differences in cell wall composition or the properties of the signaling pathways that respond to cell wall stress. In support of this, the paradoxical effect, which is thought to be a consequence of elevated chitin content, occurred more frequently in isolates of *C. parapsilosis* than in the other two species (50). Furthermore, isolates of *C. parapsilosis* can switch between four different morphological types. Two of these variants are associated with pseudohypha formation, and two with growth in the yeast form. Interestingly, one main difference between the two yeast colonial forms, termed "smooth" and "crater," is that smooth-phase yeast cells have a significantly lower chitin content, with chitin only at the poles of the cells (51).

The isolates of *C. guilliermondii* used in this study had the highest MIC of CSF and had various basal chitin levels and differing distributions of chitin in the cell wall. Most isolates of *C. guilliermondii* demonstrated an increase in chitin in response to CSF treatment. As with *C. parapsilosis*, the isolate of *C. guilliermondii* which contained a point mutation in *FKS1* did not show a compensatory increase in chitin content in response to CSF treatment. However, in most cases, increasing the chitin content again led to reduced susceptibility to CSF in the *C. guilliermondii* isolates.

The results of this work demonstrate that different *Candida* species have different basal chitin levels and differences in the chitin response to CSF treatment. In some cases, there was considerable variation in chitin content between clinical isolates of the same *Candida* species. One limitation of this study is the lack of paired isogenic sensitive and resistant isolates to look specifically at the impact of *FKS1* point mutations on chitin levels in the different species, as this would have removed the strain-to-strain variation that we observed. The majority of *Candida* species did not have a significantly high basal chitin content, which could explain their marked susceptibility to CSF. Several species responded to the inhibition of β(1,3)-glucan synthesis by CSF by

TABLE 6 Classes of chitin synthase genes in different *Candida* species

Species	No. of chitin synthase genes		
	Class I	Class II	Class IV
C. albicans	2	1	1
C. glabrata	1	1	1
C. krusei	?	?	?
C. tropicalis	2	1	1
C. parapsilosis	3	1	1
C. guilliermondii	2	2	1

stimulating a compensatory increase in chitin synthesis. Isolates of *C. albicans*, *C. parapsilosis*, and *C. guilliermondii* increased their chitin contents after treatment with CSF at the IC_{50}. In contrast, isolates of *C. glabrata* and *C. krusei* did not significantly increase their chitin levels in response to CSF treatment. Interestingly, isolates from the species tested which contained *FKS1* point mutations also did not alter their chitin levels in response to treatment with CSF. In contrast, all *Candida* species, with the exception of *C. glabrata*, could be stimulated by treatment with $CaCl_2$ and CFW to increase their chitin content, which in turn led to reduced susceptibility to CSF. Therefore, the majority of *Candida* species exhibited reduced susceptibility to CSF as a result of increases in chitin content. This may be an indication that isolates from some *Candida* species are better able to tolerate cell wall damage or that they have different abilities to sense cell wall damage and adapt to that damage through elevations of chitin content. All of the *Candida* species contain the same three classes of Chs enzymes, i.e. classes I, II, and IV, although some species have more than one enzyme per class (Table 6). All three classes of Chs enzymes are known to be activated in response to CSF treatment in *C. albicans*. *C. glabrata* has the smallest number of *CHS* genes, with one gene per class (Table 6). All species that increased chitin content in response to CSF treatment had more than one *CHS* gene per chitin synthase class. The *Candida* species with the largest numbers of *CHS* genes (*C. parapsilosis* and *C. guilliermondii*, with 5 each) also tended to have the most marked responses to cell wall damage caused by CSF treatment.

At present, the incidence of resistance to the echinocandins is relatively low, but there has been a growing number of reports of sporadic cases of breakthrough infections (7). The chitin response, however, is fully reversible upon removal of cell wall stress, complicating the evaluation of the true significance of this phenomenon *in vivo*. It is therefore possible that the increasing use of echinocandins may lead to the emergence of further examples of resistant isolates (20). None of the *Candida* species tested here appeared to have a chitin content that was naturally higher than that of *C. albicans*, which could account for their reduced susceptibility to CSF (with the exception of one *C. krusei* isolate). However, the majority of species displayed a compensatory increase in chitin in response to inhibition of β(1,3)-glucan by CSF. Therefore, most *Candida* species demonstrated the potential to adapt to CSF treatment by increasing the synthesis of chitin.

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