Site-Directed Mutagenesis of the 1,3-β-Glucan Synthase Catalytic Subunit of *Pneumocystis jirovecii* and Susceptibility Assays Suggest Its Sensitivity to Caspofungin

A. Luraschi,a S. Richard,a P. M. Hausera

Institute of Microbiology, Lausanne University Hospital, Lausanne, Switzerland

**ABSTRACT** The echinocandin caspofungin inhibits the catalytic subunit Gsc1 of the enzymatic complex synthesizing 1,3-β-glucan, an essential compound of the fungal wall. Studies with rodents showed that caspofungin is effective against *Pneumocystis* asci. However, its efficacy against asci of *Pneumocystis jirovecii*, the species infecting exclusively humans, remains controversial. The aim of this study was to assess the sensitivity to caspofungin of the *P. jirovecii* Gsc1 subunit, as well as of those of *Pneumocystis carinii* and *Pneumocystis murina* infecting, respectively, rats and mice. In the absence of an established in vitro culture method for *Pneumocystis* species, we used functional complementation of the *Saccharomyces cerevisiae* gsc1 deletant. In the fungal pathogen *Candida albicans*, mutations leading to amino acid substitutions in Gsc1 confer resistance to caspofungin. We introduced the corresponding mutations into the *Pneumocystis gsc1* genes using site-directed mutagenesis. In spot dilution tests, the sensitivity to caspofungin of the complemented strains decreased with the number of mutations introduced, suggesting that the wild-type enzymes are sensitive. The MICs of caspofungin determined by Etest and YeastOne for strains complemented with *Pneumocystis* enzymes (respectively, 0.125 and 0.12 µg/ml) were identical to those upon complementation with the enzyme of *C. albicans*, for which caspofungin presents low MICs. However, they were lower than the MICs upon complementation with the enzyme of the resistant species *Candida parapsilosis* (0.19 and 0.25 µg/ml). Sensitivity levels of Gsc1 enzymes of the three *Pneumocystis* species were similar. Our results suggest that *P. jirovecii* is sensitive to caspofungin during infections, as are *P. carinii* and *P. murina*.

**KEYWORDS** echinocandins, drug sensitivity, drug resistance, heterologous functional complementation, *Pneumocystis*

The *Pneumocystis* genus comprises fungal species that colonize the lungs of mammals (1–4). Each of them displays strict host specificity for a single mammalian species. The species infecting humans is *Pneumocystis jirovecii*, an opportunistic pathogen that can cause fatal pneumonia (*Pneumocystis pneumonia* [PCP]) if not treated. The most effective drug against *P. jirovecii* is currently co-trimoxazole, a combination of sulfamethoxazole and trimethoprim, two inhibitors of enzymes that are involved in the folic acid biosynthesis pathway. However, potential resistance is emerging due to the selection of strains carrying specific mutations in the active site of the targets of both molecules (5–9). Moreover, co-trimoxazole can cause important side effects in some patients, such as intolerance and toxicity. For these reasons, it is crucial to find new drugs to treat PCP.

Echinocandins constitute an alternative class of antifungal drugs to consider for the treatment of PCP. This class includes caspofungin (CAS), anidulafungin, and micafungin. They are cyclic hexapeptides with fatty acyl side chains and act as noncompetitive inhibitors of the catalytic subunit Gsc1 of the 1,3-β-glucan synthase enzymatic complex.

Received 4 June 2018 Returned for modification 2 August 2018 Accepted 11 September 2018 Accepted manuscript posted online 24 September 2018 Citation Luraschi A, Richard S, Hauser PM. 2018. Site-directed mutagenesis of the 1,3-β-glucan synthase catalytic subunit of *Pneumocystis jirovecii* and susceptibility assays suggest its sensitivity to caspofungin. Antimicrob Agents Chemother 62:e01159-18. https://doi.org/10.1128/AAC.01159-18. Copyright © 2018 American Society for Microbiology. All Rights Reserved. Address correspondence to P. M. Hauser, Philippe.Hauser@chu.ch.
(10). The decrease of the β-glucan synthesis results in the loss of cell integrity and rigidity, which can lead to cell lysis. β-Glucan molecules are components of the cell wall that are homopolymers of β-1,3-linked d-glucose with β-1,6-linked d-glucose side chains present in minority. The Gsc1 protein of Pneumocystis carinii, the species infecting rats, was first reported to be inhibited by the compound L-733,560, a molecule structurally close to echinocandins (11) but never used clinically. More recently, Cushion et al. (12) reported the efficacy of the echinocandins (caspofungin, micafungin, and anidulafungin) in reducing cysts number within the lungs of the host. Recently, we identified and functionally ascertained the function of the Gsc1 subunit of P. jirovecii using complementation of the orthologous gene of Saccharomyces cerevisiae (13). The presence of a unique gsc1 gene in the genome of P. jirovecii, as in that of P. carinii, further suggests that the Gsc1 subunit is a potential interesting drug target to fight PCP.

In S. cerevisiae, the 1,3-β-glucan synthase catalytic subunit is encoded by two different genes, GSC1 and GSC2. A third paralog, GSC3, is also present, but it is involved only during sporulation. The two subunits GSC1 and GSC2 are functionally redundant, but their expression is differentially regulated. The expression of GSC1 is constitutive and responsible for cell wall synthesis during the vegetative growth, while that of GSC2 is induced by glucose deprivation or pheromones and is also involved in cell wall synthesis during sporulation. The GSC1 and GSC2 genes have an essential overlapping function; i.e., only disruption of both genes is lethal. Importantly, the GSC2 gene can replace the function of the GSC1 gene during vegetative growth in the case of loss by mutation or deletion (14). The S. cerevisiae strain with a deletion of the GSC1 gene shows a reduced and impaired growth in the presence of CAS (15) or anidulafungin (16) but not of micafungin (16). On the other hand, the S. cerevisiae wild type (WT) shows normal growth in the presence of low doses of CAS and anidulafungin, but its growth is severely impaired in the presence of micafungin. These observations showed that the S. cerevisiae Gsc1 and Gsc2 subunits have different sensitivities to each echinocandin despite the fact that their identity at the amino acid sequence level is as high as 87% over the whole protein, with 81% and 94% identity at the level of 1,3-β-glucan synthase domains 1 and 2, respectively (Table 1). To our knowledge, the polymorphisms responsible for these different sensitivities have not been determined so far.

Spontaneous mutants resistant to echinocandins were initially isolated in S. cerevisiae and Candida albicans (17–19). Rare clinical isolates of C. albicans were also found to be resistant (20, 21). A specific change of a serine in position 645 to a proline (S645P) was identified in all spontaneous and most clinical resistant C. albicans isolates (21). It is localized within a highly conserved region of the Gsc1 protein in which other mutations conferring resistance to CAS were also identified in C. albicans (21). This “hot spot no. 1” of mutations starts at residue 641 and ends at residue 649 of C. albicans Gsc1. A second but less relevant hot spot of mutations conferring resistance has been identified in another region of the enzyme, from residue 1357 to residue 1364. The S645P substitution has been most frequently observed, a phenylalanine-to-serine substitution in position 641 (F641S) being the second most frequent substitution (22).

### TABLE 1 Sequence identity of Gsc proteins to their orthologs and paralogs

<table>
<thead>
<tr>
<th>Protein</th>
<th>Ortholog or paralog</th>
<th>% identity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Whole protein</td>
<td>1,3-β-Glucan synthase domain 1</td>
</tr>
<tr>
<td>P. jirovecii Gsc1</td>
<td>P. carinii Gsc1</td>
<td>90</td>
</tr>
<tr>
<td>P. murina Gsc1</td>
<td>91</td>
<td>95</td>
</tr>
<tr>
<td>S. cerevisiae Gsc1</td>
<td>S. cerevisiae Gsc2</td>
<td>87</td>
</tr>
<tr>
<td>S. cerevisiae Gsc3</td>
<td>S. cerevisiae Gsc3</td>
<td>51</td>
</tr>
</tbody>
</table>

---

*aAlignment of P. jirovecii Gsc1 to its ortholog or paralog is shown in Fig. S1.
*bAlignment of S. cerevisiae Gsc1 to its ortholog or paralog is shown in Fig. S2.*
The mutation corresponding to the *C. albicans* S645P substitution introduced by site-directed mutagenesis was found to confer reduced susceptibility to CAS *in vitro* to the mold *Aspergillus fumigatus* (23, 24).

Although demonstrated to reduce efficiently the asci during *P. carinii* and *P. murina* infections (10, 12, 25–28), the efficacy of CAS against *P. jirovecii* remains controversial. Indeed, clinical reports documented the clearance of PCP treated with CAS alone (29–31) or used in combination with co-trimoxazole (32–36) or clindamycin (37). However, failures of CAS treatment were also described (38, 39). Despite the generally high conservation of active sites among orthologous enzymes, one cannot exclude that the sensitivity to CAS may vary among *P. jirovecii* and the two *Pneumocystis* species infecting rodents because these species are relatively distant from each other (20% mean divergence at nucleotide level in genomic coding sequences [40]). The *P. jirovecii* Gsc1 subunit bears 90% and 91% identity with those of *P. carinii* and *P. murina*, respectively (Table 1). At the level of 1,3-β-glucan synthase domains 1 and 2, i.e., the active sites, the identities are from 94% to 97%. These values are comparable to those between the Gsc1 and Gsc2 subunits of *S. cerevisiae* (see above), which present drastically different sensitivities to the different echinocandins.

The aim of the present study was to determine if the Gsc1 subunit of *P. jirovecii* is sensitive to the echinocandin CAS, as are those of *P. carinii* and *P. murina*. To investigate the issue, we analyzed the level of sensitivity of *S. cerevisiae* strains functionally complemented by the expression of the wild-type or mutated enzymes of the three *Pneumocystis* species.

(The present work was submitted by A. Luraschi as a partial fulfillment of a Ph.D. degree at the Faculty of Biology and Medicine of the University of Lausanne.)

**RESULTS**

Functional ascertainment of the *P. carinii* and *P. murina gsc1* genes by complementation of the *S. cerevisiae gsc1* deletant. We identified a single Gsc1 protein within the *P. murina* proteome by a homology search using the Gsc1 protein of *P. carinii* as the query sequence. To ascertain the function of the *P. carinii* and *P. murina gsc1* genes, recombinant plasmids expressing them were introduced into the *S. cerevisiae gsc1* deletant. The identities of the Gsc1 proteins studied relatively to that of *S. cerevisiae* are given in Table S3 in the supplemental material. Serial dilutions of the transformed strains were spotted onto medium containing or not containing CAS (spot dilution test [Fig. 1]). The deletion of the GSC1 gene in *S. cerevisiae* causes a paradoxical effect, i.e., an increased susceptibility to CAS, though the target of CAS is absent (15). This is due to the replacement of Gsc1 by Gsc2, an enzyme that is more sensitive to CAS (14). On the other hand, this replacement allows the growth of the deletant strain on medium without CAS. On medium supplemented with CAS, a complete restoration of the wild-type growth was observed in the presence of the control *S. cerevisiae GSC1* gene, but not in the presence of the empty vector (Fig. 1, *S. cerevisiae gsc1* deletant + empty plasmid). A partial restoration was observed in the presence of the *P. carinii* or *P. murina* gene, as we previously reported for *P. jirovecii* and reproduced here (Fig. 1). These observations demonstrated that the expression of *P. carinii* and *P. murina gsc1* genes rescued the function of the deleted *S. cerevisiae GSC1* gene, demonstrating their function. In order to investigate the sensitivity to CAS of the three *Pneumocystis* enzymes, we used site-directed mutagenesis to introduce mutations that correspond to those conferring resistance in other fungi.

**Sensitivity to CAS of the *S. cerevisiae* strains complemented with the *Pneumocystis* Gsc1 mutated proteins.** Mutants resistant to echinocandins carrying mutations F641S and S645P within hot spot no. 1 of Gsc1 have been described for the pathogenic fungus *C. albicans* (17–21). The sequences of this hot spot of mutations of the *P. jirovecii*, *P. carinii*, and *P. murina* Gsc1 protein were aligned with those of *C. albicans*, *S. cerevisiae*, and *C. parapsilosis* (Fig. 2; alignment of the complete proteins is shown in Fig. S1). This alignment identified the positions in the three *Pneumocystis gsc1* genes corresponding to the *C. albicans* F641S and S645P substitutions. Site-directed mutagen-
esis was used to introduce one or two mutations for the corresponding substitutions within the gsc1 gene of P. jirovecii, P. carinii, or P. murina (the polymorphisms introduced at the nucleotide sequence level are described in Table S1).

The partial restoration of the wild-type growth on CAS observed with the P. jirovecii Gsc1 enzyme increased in the presence of one mutation (Fig. 1, compare the S718P mutant to the wild type) and increased more upon introduction of the two mutations simultaneously (compare the S718P and F714S double mutant to the S718P mutant and the wild type). Similarly, the partial restoration with the P. carinii or P. murina enzyme increased in the presence of a single mutation (compare the S715P and S719P mutants to their respective wild types). This increase of complementation efficiency corresponds to a decrease of sensitivity to CAS. This demonstrated that the three wild-type Pneumocystis enzymes present a certain level of sensitivity to CAS.

**MIC assessment using Etest and Sensititre YeastOne.** We determined the MICs of CAS for the S. cerevisiae WT and complemented gsc1 deletant strains. To assess the sensitivity of the two methods, we also analyzed C. albicans and C. parapsilosis, as well as C. albicans and C. parapsilosis, as well as C. albicans and C. parapsilosis, as well as C. albicans and C. parapsilosis, as well as C. albicans and C. parapsilosis, as well as C. albicans and C. parapsilosis, as well as C. albicans and C. parapsilosis, as well as C. albicans and C. parapsilosis, as well as C. albicans and C. parapsilosis, as well as C. albicans and C. parapsilosis, as well as C. albicans and C. parapsilosis, as well as C. albicans and C. parapsilosis, as well as C. albicans and C. parapsilosis, as well as C. albicans and C. parapsilosis, as well as C. albicans and C. parapsilosis, as well as C. albicans and C. parapsilosis, as well as C. albicans and C. parapsilosis, as well as C. albicans and C. parapsilosis, as well as C. albicans and C. parapsilosis, as well as C. albicans and C. parapsilosis, as well as C. albicans and C. parapsilosis, as well as C. albicans and C. parapsilosis, as well as C. albicans and C. parapsilosis, as well as C. albicans and C. parapsilosis, as well as C. albicans and C. parapsilosis, as well as C. albicans and C. parapsilosis, as well as C. albicans and C. parapsilosis, as well as C. albicans and C. parapsilosis, as well as C. albicans and C. parapsilosis, as well as C. albicans and C. parapsilosis, as well as C. albicans and C. parapsilosis, as well as C. albicans and C. parapsilosis, as well as C. albicans and C. parapsilosis, as well as C. albicans and C. parapsilosis, as well as C. albicans and C. parapsilosis, as well as C. albicans and C. parapsilosis, as well as C. albicans and C. parapsilosis, as well as C. al
as the *S. cerevisiae gsc1* deletant complemented with the *GSC1* gene of these two *Candida* species. CAS presents low MICs for the former *Candida* species, whereas it presents high MICs for the latter (41, 42). The natural high MICs for *C. parapsilosis* are due to a polymorphism at the end of the hot spot no. 1 that has not been observed in *C. albicans* so far (43) (proline to serine in position 660 [Fig. 2]). According to Espinel-Ingroff et al. (41) and Canton et al. (42), the MICs of CAS for *C. parapsilosis* that we obtained were below the epidemiological cutoff values for both Etest and YeastOne methods (0.5 versus 4 and 0.5 versus 2 μg/ml, respectively). The MICs for *C. albicans* we obtained were also below the epidemiological cutoff values of wild-type isolates (0.38 versus 0.5 and 0.12 versus 0.25 μg/ml, respectively). In agreement with the spot dilution results described here, we observed using both Etest and YeastOne a decreased MIC for the *S. cerevisiae gsc1* deletant compared to that for the WT (respectively, 0.125 and 0.12 versus 0.250 and 0.25 μg/ml [Table 2]; the Etest results are shown in Fig. 53). All *S. cerevisiae* strains complemented with the *Pneumocystis* wild-type or mutated genes had MICs identical to those of the *gsc1* deletant (0.125 μg/ml for Etest and 0.12 μg/ml for YeastOne). The increase of MIC of CAS conferred by the mutations introduced was not detected using Etest or YeastOne. Thus, these methods are less sensitive than the spot dilution test, since the latter always allowed detection of this decrease in several experiments. The MICs for *C. albicans* whole cells using Etest and YeastOne were similar to those for the *S. cerevisiae* WT strain (0.380 and 0.12 μg/ml versus 0.250 and 0.25 μg/ml), whereas, consistent with its reported high MICs, *C. parapsilosis* had higher MICs also in our hands using both methods (0.500 and 0.50 μg/ml). The increased MICs for *C. parapsilosis* were also detected using both methods upon heterologous expression of its Gsc1 subunit in *S. cerevisiae* (respectively, 0.190 and 0.25 μg/ml, versus 0.125 and 0.12 μg/ml for *C. albicans* Gsc1), despite the fact that MICs were systematically lower using heterologous expression than whole cells. Using the heterologous expression system, the wild-type *Pneumocystis* Gsc1 subunits had MICs identical to those of *C. albicans* Gsc1 (0.125 and 0.12 μg/ml), whereas *C. parapsilosis* Gsc1 presented higher MICs (0.190 and 0.25 μg/ml). These observations suggested that the sensitivity to CAS of the three *Pneumocystis* enzymes is similar to that of *C. albicans*, which presents low MICs for CAS.

**TABLE 2** MICs of caspofungin (CAS) for the *S. cerevisiae* WT and functionally complemented *gsc1* deletant strains, as well as for *Candida* species

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC (μg/ml)</th>
<th>E-test</th>
<th>YeastOne</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. cerevisiae</em> WT + empty plasmid</td>
<td>0.250</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td><em>S. cerevisiae</em> WT + empty plasmid</td>
<td>0.125</td>
<td>0.12</td>
<td></td>
</tr>
<tr>
<td><em>S. cerevisiae</em> WT + empty plasmid</td>
<td>0.125</td>
<td>0.12</td>
<td></td>
</tr>
<tr>
<td><em>C. albicans</em> WT</td>
<td>0.380</td>
<td>0.12</td>
<td></td>
</tr>
<tr>
<td><em>C. parapsilosis</em></td>
<td>0.500</td>
<td>0.50</td>
<td></td>
</tr>
</tbody>
</table>

*One isolate among three of each complemented strain was chosen randomly for analysis. One out of two experiments that gave similar results is reported here. Although obtained using various methods that have various sensitivities, the MICs previously published correspond roughly to ours for the *S. cerevisiae* WT (0.25 versus 0.03 to 0.4 μg/ml [15, 16, 21]) and *gsc1* deletant (0.12 to 0.125 versus 0.0015 to 0.1 μg/ml [15, 16, 21]), as well as for *C. albicans* (0.12 to 0.380 versus 0.12 to 0.25 μg/ml [22, 41]) and *C. parapsilosis* (0.50 versus 0.25 to 8 μg/ml [41–43]).

Downloaded from https://journals.asm.org/journal/aac on 24 October 2021 by 54.201.197.22.
DISCUSSION

Because of the absence of an in vitro culture method, testing for sensitivity to CAS cannot be performed directly on whole Pneumocystis cells. A study reported the effects of echinocandins against P. murina and P. carinii using suspension and biofilm culture methods (44). Unfortunately, these methods are not established for P. jirovecii. Consequently, we studied the Gsc1 enzymes of three Pneumocystis species in the heterologous system of expression of S. cerevisiae. We used site-directed mutagenesis to introduce into the Pneumocystis enzymes the substitutions corresponding to those conferring resistance to CAS in C. albicans. This revealed that despite the divergence among their active sites, the three Pneumocystis Gsc1 enzymes present low MICs for CAS, and this to similar levels. Because CAS has been demonstrated to be effective in reducing P. carinii and P. murina asci during infections (12, 27), this observation suggested that CAS could also be effective against P. jirovecii. Moreover, MIC determination showed that the level of sensitivity of Pneumocystis Gsc1 was similar to that of the C. albicans enzyme, suggesting that the sensitivity of the Pneumocystis enzymes is at a level that is usable clinically. It is of course difficult to translate our results obtained at the enzyme level to the whole-cell level. Nevertheless, Gsc1 is a cell surface enzyme that is easily reachable by drugs and thus more likely to behave similarly among the three Pneumocystis species. A structural difference of the cell wall could cause various sensitivities to CAS of the Gsc1 subunit among the three Pneumocystis species. However, there is presently no obvious reason to think that the wall of P. jirovecii is different from those of P. carinii and P. murina. The efficacy of echinocandins, and specifically of CAS, to treat P. jirovecii infections remains controversial, and accordingly, the American drug and European medical agencies do not advise their use for that purpose. Our results bring new arguments in favor of the use of this class of antifungals for the treatment of PCP, suggesting the need to implement clinical trials in humans. Finally, our results support the high relevance of the animal models as tools to understand the effect of CAS on the human pathogen P. jirovecii.

Studies with animal models showed that echinocandins provoke the disappearance of P. carinii and P. murina asci but not of the trophic forms, probably because the latter cells have no or little cell wall made of 1,3-β-glucan (12). Thus, the treatment did not eradicate the infection, and its cessation resulted in the repopulation in asci from the remaining trophic cells. Consequently, it is likely that CAS is useful only in combination with another therapy targeting trophic forms, or both cellular forms, such as cotrimoxazole. CAS inhibited efficiently the dissemination of the pathogen in animal models (12), which is consistent with the fact that asci are believed to be the transmission particles (12, 44).

In conclusion, our results demonstrate that the Gsc1 enzyme of the human pathogen P. jirovecii is sensitive to caspofungin, similar to the enzymes of the animal pathogens P. carinii and P. murina. This suggests that echinocandins might be a good alternative to treat PCP in humans when used in combination with an established treatment. The use of echinocandins to fight Pneumocystis infections deserves further investigation.

MATERIALS AND METHODS

Strains and growth conditions. Y05251 is an S. cerevisiae haploid strain in which the 1,3-β-glucan synthase catalytic subunit gene GSC1 (also called FKS1) was deleted (MATa his3Δ0 leu2Δ0 met15Δ0 ura3Δ0 YLR432c:kanMX4). It was obtained from Euroscarf (European S. cerevisiae Archive for Functional Analysis [http://www.euroscarf.de]). The strain, called the gsc1 deletant here, exhibits an impaired growth in the presence of low doses of CAS (14). The parental strain of the gsc1 deletant is BY4741 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0) and was also obtained from Euroscarf (here referred to as the wild type [WT]). The latter was used as a control in the sensitivity tests and in MIC assays. Strains were grown on complete yeast extract-peptone-dextrose (YPD) medium (1% [wt/vol] Difco yeast extract, 2% Difco peptone, 2% glucose).

Single colonies of Candida albicans (ATCC 10231) and Candida parapsilosis (sensu stricto, i.e., group I of the C. parapsilosis complex; ATCC 22019) were streaked on Sabouraud medium (0.5% [wt/vol] casein peptone, 0.5% meat extract peptone, 2% glucose) and then grown on minimal solid yeast nitrogen base (YNB) medium (0.67% [wt/vol] yeast nitrogen base, 2% glucose, 2% Gibco agar) supplemented with a
complete supplement mixture (CSM; MP Biomedicals). C. _albicans_ and _C. parapsilosis_ were chosen because CAS presents, respectively, low and high MICs for them.

**Cloning of the fungal gsc1 genes.** To identify the _P. murina_ gsc1 gene, the _P. carinii_ Gsc1 protein (Q9HEZ4) was used as query sequence in BLAST search against _P. murina_ proteome at http://blast.ncbi.nlm.nih.gov/Blast.cgi. A single putative ortholog was detected (locus tag PNEG_03180). The _P. murina_ gene sequence encoding the Gsc1 protein was then retrieved from the European Nucleotide Archive (http://www.ebi.ac.uk/ena). The cloning of the _P. jirovecii_ and _S. cerevisiae_ gsc1 genes was previously described (13). Since the _P. carinii_ and _P. murina_ gsc1 genes include three introns, their CDNAS were synthesized and cloned into the p416GPD vector (45) by GeneCust Europe (Ellange, Luxembourg). Their sizes without introns are, respectively, 5,835 bp and 5,847 bp.

To perform a control of sensitivity of our heterologous expression model, the GSC1 genes of _C. albicans_ (GenBank accession number D88815) and _C. parapsilosis_ (European Nucleotide Archive accession number EU221325) were amplified by PCR from yeast genomic DNA extracted as described previously (46). The detailed procedures for PCR amplification using the proofreading high-fidelity Expand polymerase (Roche Diagnostics) and cloning were described previously (47). Their sizes are, respectively, 5,694 and 5,730 bp. PCR primers and conditions are listed in Tables S1 and S2. Because these primers were intended for oriented cloning, they were designed to create unique restriction sites at ends of the PCR products. After the PCRs, the products were sequenced using a QiAquick gel extraction kit (Qiagen, Basel, Switzerland). For cloning each _Candida_ GSC1 gene into the p416GPD expression vector, the double restriction description in Table S1 were used.

**Site-directed mutagenesis.** The Gsc1 protein sequences of _C. albicans_ (UniProt identifier O13428), _S. cerevisiae_ (P38631), _P. jirovecii_ (LOPD34, locus tag PNEJ1_001061), _P. carinii_ (Q9HEZ4), _P. murina_ (M7P3D9, locus tag PNEG_03180), and _C. parapsilosis_ (A9YLC3) were aligned using T-Coffee (48). This alignment allowed determination of the positions within the _Pneumocystis_ genes corresponding to the mutations F641S and S645P conferring resistance to CAS on _C. albicans_ (Fig. 2; alignment of the complete proteins is shown in Fig. S1). To perform site-directed mutagenesis, two different kits were used. The QuikChange II XL site-directed mutagenesis kit (Agilent Technologies) was used to create the mutation in the _P. jirovecii_ gsc1 gene leading to the change of the serine at position 718 of the Gsc1 protein to a proline (S718P). The Q5 site-directed mutagenesis kit (New England Biolabs) was used to introduce the F714S/S718P double substitution in _P. jirovecii_, the S715P substitution in _P. carinii_, and the S719P substitution in _P. murina_. Mutagenesis was performed according to the manufacturers’ instructions. Mini-preparations of plasmid DNA were subsequently carried out (49). In order to verify the presence of the desired mutations, an internal segment of the _gsc1_ genes was amplified and subsequently sequenced. Primers for mutagenic reactions and PCR amplifications are listed in Table S1. Mutagenesis amplification reactions and PCR conditions are described in Table S2. Sequencing of both strands was performed using the two primers used for amplification, as well as the BigDye Terminator DNA sequencing kit and ABI PRISM 3100 automated sequencer (both from PerkinElmer Biosystems).

**Transformation of the _S. cerevisiae_ gsc1 deletant.** Transformation with plasmids containing the _P. jirovecii_ gsc1 or the _S. cerevisiae_ GSC1 gene were previously described (13). The _S. cerevisiae_ GSC1 gene could not be cloned in the p416GPD plasmid because of restriction sites issues, but it was cloned into p415GPD (leu marker instead of ura). The recombinant p416GPD plasmids containing the _Pneumocystis_ mutated gsc1 alleles, as well as the _C. albicans_ or _C. parapsilosis_ GSC1 gene, were introduced into the gsc1 deletant by transformation for uracil prototrophy using the one-step method (50). Transformants were selected on solid YNB medium supplemented with CSM (MP Biomedicals) lacking uracil. In order to be used as controls in the sensitivity tests and in the MIC assays, the gsc1 deletant and the WT were transformed with the empty p416GPD plasmid. Three transformants of each constructed strain were randomly chosen and purified by growth on the same selective medium.

**Test of complementation and susceptibility to caspofungin.** Before studying the sensitivity to echinocandins, we had to assess the function of the _P. carinii_ and _P. murina_ gsc1 genes, as we previously did for the _P. jirovecii_ gsc1 gene (13). Functional complementation of the _gsc1_ deletant was proven by the spot dilution test on YNB selective medium lacking uracil and supplemented or not with 150 μg/ml of CAS (Fluka Chemie AG). CAS appeared sensitive to temperature variation in our study. Consequently, the medium was cooled down to 54°C before addition of CAS. In addition, petri dishes containing CAS were stored at room temperature but not at 4°C. The concentration of CAS used in our experiments was selected after several trials. To that aim, transformant isolates carrying the _P. carinii_ gsc1 or _P. murina_ gsc1 gene were grown overnight in YNB selective medium supplemented with CSM lacking uracil to avoid the loss of the plasmid. Cells were then diluted at an optical density at 540 nm (OD540) of 0.1 in 0.9% (wt/vol) NaCl (ca. 7.5 × 105 cells/ml). Four serial 10-fold dilutions in 0.9% NaCl were prepared, and 3 μl of each dilution was spotted onto the medium. Spots were observed after 3 to 4 days of incubation at 30°C. The same procedure was used to assess the functionality and sensitivity to CAS of the strains complemented with the mutated gsc1 alleles. The sensitivity to micafungin could not be studied because the _gsc1_ deletant had no phenotype to complement on this drug, i.e., the Gsc2 subunit is resistant. Anidulafungin could also not be studied because, for underdetermined reasons, the results were not reproducible in our hands.

**MIC assessment using Etest.** Assays were performed according to the manufacturer’s instructions. Each strain was grown overnight in YNB selective medium plus CSM lacking uracil, or leucine for the _S. cerevisiae_ GSC1 gene, and then adjusted in 0.9% NaCl to an OD540 of 0.2 (~1.5 × 106 cells/ml). One hundred microliters of this dilution was spread on fresh YNB solid medium plus CSM lacking uracil or leucine. A single strip of Etest caspofungin (bioMerieux) was then applied on each petri dish. MICs were
read after 2 days of incubation at 30°C, or at 35°C for the Candida species. The MIC was defined as the concentration at which no growth was observed on both sides of the Etest strip.

MIC assessment using Sensititre YeastOne. Assays were performed according to the manufacturer’s instructions, except that the incubation time was increased from 24 to 48 h for all S. cerevisiae strains because of their low growth rate. Each strain was grown overnight in YNB selective medium and then adjusted in 0.9% NaCl to an OD of 0.2 (ca. 1.5 × 10^6 cells/ml). Twenty microliters of this dilution was then diluted into 11 ml of YeastOne inoculum broth in order to obtain ca. 3 × 10^5 cells/ml. One hundred microliters was then transferred into each well of a YeastOne plate (Thermo Fisher Scientific). Plates were observed and MICs determined after 24 or 48 h of incubation at 30°C, or at 35°C for the Candida species. The MIC was defined as the first well in which no pellet of cells was observable.

SUPPLEMENTAL MATERIAL
Supplemental material for this article may be found at https://doi.org/10.1128/AAC.01159-18.

SUPPLEMENTAL FILE 1, PDF file, 0.5 MB.

ACKNOWLEDGMENTS
This work was supported by Swiss National Science Foundation grant 310030_165825. We thank Michel Monod for his critical reading of the manuscript.

REFERENCES


