Acquired Multidrug Antifungal Resistance in *Candida lusitaniae* during Therapy

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*Candida lusitaniae* is usually susceptible to echinocandins. Beta-1,3-glucan synthase encoded by FKS genes is the target of echinocandins. A few missense mutations in the *C. lusitaniae* FKS1 hot spot 1 (HS1) have been reported. We report here the rapid emergence of antifungal resistance in *C. lusitaniae* isolated during therapy with amphotericin B (AMB), caspofungin (CAS), and azoles for treatment of persistent candidemia in an immunocompromised child with severe enterocolitis and visceral adenoviral disease. As documented from restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) analysis, the five *C. lusitaniae* isolates examined were related to each other. From antifungal susceptibility and molecular analyses, 5 different profiles (P) were obtained. These profiles included the following: profile 1 (P1) (CAS MIC [µg/mL], 0.5; fluconazole [FLC] MIC, 0.25), determined while the patient was being treated with liposomal AMB for 3 months; P2 (FLC MIC [µg/mL], 0.25; CAS MIC, 4), while the patient was being treated with CAS for 2 weeks; P3 (CAS MIC [µg/mL], 0.5; FLC MIC, 32), while the patient was being treated with azoles and CAS initially followed by azoles alone for a week; P4 (CAS MIC [µg/mL], 8; FLC MIC, 8), while the patient was being treated with both drugs for 3 weeks; and P5 (AMB MIC [µg/mL], 0.125; CAS MIC, 8), while the patient was being treated with AMB and FLC for 2 weeks. CAS resistance was associated with resistance not only to micafungin and anidulafungin but also to AMB. Analysis of CAS resistance revealed 3 novel FKS1 mutations in CAS-resistant isolates (S638Y in P2; S631Y in P4; S638P in P5). While S638Y and -P are within HS1, S631Y is in close proximity to this domain. 

**MATERIALS AND METHODS**

**Strains and media.** *C. lusitaniae* strains were grown in complete yeast extract-peptone-dextrose (YPD) medium (1% Bacto peptone [Difco Laboratories, Basel, Switzerland], 0.5% yeast extract [Difco]) with 2% (wt/vol) glucose (Fluka, Buchs, Switzerland). Saccharomyces cerevisiae was grown on YEPD medium for isolate precultures and on yeast nitrogen base (YNB) agar (Difco) with 2% (wt/vol) glucose. Species identification was performed using matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry (MS) Microflex LT systems (Bruker Daltonics GmbH, Leipzig, Germany) and with analysis of data using FlexControl (version 3.0) software (Bruker Daltonics) as described in reference 15.

**Susceptibility assays.** Determinations of drug MICs for *C. lusitaniae* clinical isolates according to EUCAST guidelines were performed in RPMI 1640 medium (Sigma-Aldrich, Switzerland) with 2% glucose and in flat-well microtiter plates. RPMI 1640 buffered at pH 7.0 with MOPS buffer was used as the medium in all experiments.

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(morpholinepropanesulfonic acid) was used for MIC tests of azoles, 5-fluorocytosine (5-FC), candins, and AMB. Cells were diluted to a density of 0.5 × 10^5 to 2 × 10^5 cells/ml. All compounds were dissolved to obtain final concentrations ranging from 128 μg/ml to 0.0162 μg/ml. Plates were incubated at 35°C for 24 h, and readings were carried out in a microplate reader at 540 nm. The MIC was defined as the drug concentration at which the optical density was ≤50% of that of the drug-free culture. Quality controls included S. cerevisiae strain ATCC 928. Antifungal agents used in this study were provided as pure substances by pharmaceutical companies (CAS, Merck; micafungin [MICA], Astellas; anidulafungin [ANI] and FLC, Pfizer). AMB deoxycholate (fugine) was obtained from Bristol-Myers Squibb (Cham, Switzerland).

RFLP and RAPD analysis. The recovered C. lusitaniae isolates were subjected to restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) analysis as described elsewhere (16). Genomic DNA was isolated by glass bead extraction from each isolate as previously described (17) and was subjected to EcoR1 and MspI digestion. RAPD analysis was performed with primer OPE-18 (GG ACTCGAGA) as previously recommended (16). Gel electrophoresis was carried out with 0.8% agarose followed by ethidium bromide staining. Additional software (ImageJ) (18) was used to corroborate our findings from the RFLP analysis (see Fig. S1 in the supplemental material).

FKS1 sequencing. Primers were used to amplify FKS1 alleles encoding β-glucan synthase from C. lusitaniae isolates (19). These primers were designed to amplify conserved HS1 (hot spot region 1) and HS2 regions (for HS1, MDO002 [GCGTTGGTGGTTGGTTTTA] and MDO003 [T CGGAATCTCTTGGGAAGAA]; for HS2, MDO004 [TGGCTGGTATGGGTGAAAG] and MDO005 [CCAGAACCCTCCAAGATGGAG]). Sequencing procedures were performed with the same primers and are described elsewhere (20). Sequence alignments were performed with Geneious software (Biomatters Ltd., New Zealand).

qRT-PCR. Quantitative reverse transcription-PCR (qRT-PCR) was performed as described elsewhere (21). Total RNA was extracted from log-phase cultures with an RNase-free Pretect minikit (Qiagen) by a procedure involving mechanical disruption of the cells with glass beads and an RNase-free DNase treatment step as previously described (22). Gene expression levels were determined by real-time qRT-PCR in a StepOne real-time PCR system (Applied Biosystems) using a Mesa Blue quantitative PCR (qPCR) Mastermix Plus for Sybr assay kit (Eurogentec). Each reaction was run in triplicate on three separate occasions. Expression levels were normalized to ACT1 expression. Primers for C. lusitaniae ATP-binding cassette (ABC) and major facilitator superfamily (MFS) transporter genes were designed with Primer3Plus. The primers were chosen on the basis of the available genome sequences (Broad Institute). Primers are listed in Table S1 in the supplemental material. Gene names were given according to the work of Reboutier et al. (23). Primers ABC15-F and ABC15-R as well as primers ABC9-F and ABC9-R were selected from the C. lusitaniae genomes of the closest homologs of the C. albicans CDR1 gene. MFS7-R and MFS7-F as well as ABC12-R and ABC12-F were selected since the corresponding genes were previously shown to be differentially expressed in several C. lusitaniae isolates (23).

Construction of FKS1 mutants. In order to introduce specific mutations in FKS1 for testing their effect on echinocandin susceptibility, the model yeast S. cerevisiae was used in combination with the clustered regularly interspaced short palindromic repeat (CRISPR)-Cas9 genome editing system. Briefly, a 20-nucleotide (nt) guide sequence adjacent to a protospacer-adjacent motif (PAM) sequence was selected within the region of FKS1 HS1. This region was selected using the online CHOPCHOP selection tool (24) (https://chopchop.ucsf.edu) and is situated between positions 1892 and 1914 with respect to first ATG codon. The guide sequence was flanked by pMEL10 sequences (25) to allow homologous recombination in S. cerevisiae. The guide-pMEL10 sequence was produced by complementary assembly of primers FKS_crisp_R and FKS_crisp_F (see Table S2 in the supplemental material), pMEL10 was prepared by inverse PCR with primers p426 CRISPR rv and p426 CRISPR fw (see Table S2 in the supplemental material) followed by DpnI digestion as described by Mans et al. (25). Three different repair fragments were produced, with each containing the desired FKS1 mutation with overlapping primers for the FKS1 mutations S636Y, S643P, and S643Y. Primer pairs using left and right primers are indicated in Table S2 in the supplemental material and were used for PCR amplification to produce 120-bp repair fragments. Genome editing was performed by combining pMEL10, the guide-pMEL10 fragment, and each of the repair fragments and by transformation into S. cerevisiae IMX581 (25). Transformation of S. cerevisiae was performed as described previously (25), and selection was carried out in YNB agar lacking uracil. Verifications of introduced mutations were performed by PCR amplification with primers FKS1verif left and FKS1verif right (see Table S2 in the supplemental material) of the HS1 region and by sequence analysis as described above. Derivatives from IMX581 are described in Table S3 in the supplemental material.

β-glucan measurements. Patient blood samples were drawn, and sera were stored at −80°C and subjected to batch analysis with duplicate testing by Fungitell on an ELX8081I microplate reader (Associates of Cape Cod, East Falmouth, MA) per the manufacturer’s package insert. Samples with β-glucan (BG) values above the upper limit of quantification (500 pg/ml) were diluted (58 of 921; 6%). The mean BG values of duplicates were used for data analysis.

Galactomannan assay. The Bio-Rad Platelet Aspergillus antigen (Ag) assay was used to measure galactomannan levels. This immunoenzymatic sandwich microplate assay enabled the detection of Aspergillus galactomannan antigen in serum and bronchoalveolar lavage fluid samples through the use of rat EBA-2 monoclonal antibodies. Results are reported in standard international units (provided as index values with limits of 0.25 to 0.5), which refer to the absorbance (optical density) of specimens determined with a spectrophotometer set at 450 nm.

TDM. Therapeutic drug monitoring (TDM) was performed according to published procedures with multiplex ultraperformance liquid chromatography-tandem mass spectrometry methods that enable simultaneous quantification in plasma of azoles and candins (26).

Nucleotide sequence accession numbers. C. lusitaniae sequences were deposited in GenBank under accession no. JF304613 and JF304615. Sequences from isolates P1 to P5 were deposited in GenBank under accession numbers KM383792 to KM383795 and KP100692.

RESULTS
Case report. A 3-year-old female with hematologic and central nervous system (CNS) relapse of acute myeloid leukemia (AML) was started on high-dose cytarabine and clofarabine as second-line induction. She remained profoundly neutropenic over the following 4 months until her death. Three weeks after induction chemotherapy, she presented with fever and diarrhea. She had been on prolonged prophylactic treatment with intravenous (i.v.) liposomal AMB (3 mg/kg of body weight/day) and broad-spectrum antibiotics for over a month. Clinical and radiological examination showed severe enterocolitis. As such invasive C. lusitaniae candidiasis was suspected based on the documentation of C. lusitaniae in her stools and a positive mannan assay result (immunoenzyme assay, 500 pg/ml) (27, 28). Blood culture results were, however, negative. Given the lack of validated clinical breakpoint definitions for C. lusitaniae, we used those available for C. albicans (29, 30). Given the profile (P1; see Fig. 1 and Table 2 for susceptibility profiles) of the susceptibility of the recovered C. lusitaniae isolate to echinocandins (CAS, micafungin [MICA], and anidulafungin [ANI]) MICs, 0.5 μg/ml, 0.03 μg/ml, and 0.06 μg/ml, respectively), to azoles (FLC, MIC, 0.25 μg/ml) and to AMB (MIC, 0.06 μg/ml), she was started on CAS at 100 mg/m2/day, which resulted in clinical improvement. While she was on CAS for 2 weeks with measured plasma levels of 4.3 mg/liter, she presented...
with *C. lusitaniae* candidemia (isolate P2; see Fig. 1), which was resistant to all echinocandins (CAS, MICA, and ANI MICs, 4 μg/ml, 16 μg/ml, and 2 μg/ml, respectively) and AMB (MIC, 2 μg/ml) but not to azoles (FLC MIC, 0.25 μg/ml) or 5-fluorocytosine (5-FC) (MIC, 0.5 μg/ml). At the same time, she presented with visceral adenovirus disease (subtype 41 F) with high viral loads in her blood (5-FC MIC, 0.5 μg/ml) but not to azoles (FLC MIC, 0.25 μg/ml, 16 μg/ml). While she was on combined therapy for a week, she presented with a new onset of fever, a maculopapular rash, profuse diarrhea, and candidemia. Surprisingly, her new isolate (P3; see Fig. 1) was susceptible to echinocandins (CAS, MICA, and ANI MICs, 8 μg/ml, 16 μg/ml, and 4 μg/ml, respectively), FLC (MIC, 8 μg/ml), and 5-FC (MIC, 32 μg/ml) but susceptible to AMB (MIC, 0.06 μg/ml). CAS was therefore replaced by AMB (5 mg/kg/day). FLC was replaced by voriconazole (VORI) (9 mg/kg twice a day [b.i.d.]; plasma level, 1.5 mg/liter), as probable invasive pulmonary aspergillosis (32) was suspected based on new pulmonary infiltrates on a repeat CT and a positive blood galactomannan assay result (enzyme-linked immunosorbent assay [ELISA], 6.54 pg/ml) (32). Although AMB monotherapy would have covered both fungal infections, combined therapy was preferred because of the severity of both infections. Further disease evolution was marked by persistent fever and diarrhea associated with persistent adenovirus viremia (10⁶ cp/ml). A week later, she underwent allogeneic hematopoietic stem cell transplantation (HSCT) after receiving conditioning chemotherapy with busulfan, anti-thymocyte globulins (ATG), and fludarabine. While she was on AMB and VORI at subtherapeutic (0.7 μg/liter) levels for almost 2 weeks, her blood cultures were again found to be positive for *C. lusitaniae*. At that point, she was continued on the same antifungal regimen. An isolate (P5) with a susceptibility profile similar to that of P2 (CAS, AMB, and FLC MICs, 8 μg/ml, 2.0 μg/ml, and 0.125 μg/ml, respectively) was recovered during this period. All her lines were changed, an endovascular source was ruled out, and a repeat CT scan still evidenced severe enterocolitis. The further evolution of her disease state was marked with progressive fulminant hepatitis, renal dysfunction, and death, mainly attributed to drug toxicities and disseminated adenoviral infection. The latter was corroborated by persistently high-level viremia (>5 × 10⁶ cp/ml) despite her having received 11 doses of intravenous cidofovir (5 mg/kg) but no administration of adeno-

![Graph](http://aac.asm.org/)
virus-specific cytotoxic T lymphocytes. Invasive candidiasis and pulmonary aspergillosis probably also contributed to her death. Autopsy was refused by the family.

Molecular analysis of \textit{C. lusitaniae} strains. The RFLP and RAPD profiles of recovered isolates P1 to P5 were identical (Fig. 2; see also Fig. S1 in the supplemental material) and thus suggest that the strains originated from the same parent. Molecular analyses of candin resistance revealed two novel \textit{FKS1} mutations in resistant isolates (S638Y in P2 and S631Y in P4). These mutations correspond to positions S638 and S645 in \textit{FKS1} of \textit{C. albicans}; the latter position is known to be involved in CAS resistance (S645F, -P, and -Y) (Fig. 3) (8, 33). Interestingly, the last recovered isolate, P5, with a drug susceptibility profile similar to that of P2, exhibited the \textit{FKS1} S638P substitution (corresponding to S645P in \textit{C. albicans}), which was different from that exhibited by P2 (S638Y). Thus, P2 and P5 are of distinct genotypes. No mutations were observed in HS2 of \textit{FKS1} (data not shown). As summarized in Table 1, CAS resistance was associated with cross-resistance to other candins (MICA MICs, 8 to 16 $\mu$g/ml; ANI MICs, 2 to 4 $\mu$g/ml) and, surprisingly, with resistance to AMB (MIC, 2 $\mu$g/ml) in P2.

Since mutation S631Y in \textit{C. lusitaniae} and the equivalent mutation, Ser638, in \textit{C. albicans} have not yet been reported to be involved in candin resistance, we performed site-directed mutagenesis analysis in \textit{FKS1} from \textit{S. cerevisiae} at the equivalent position (Ser636) to produce a S636Y variant. \textit{FKS1} variants at position Ser643 (S643Y and S643P), which is equivalent to position Ser638 in \textit{C. lusitaniae}, were also produced as comparisons. A recent genome editing system (CRISPR-Cas) was used for this purpose (25) and thus introduced the desired mutations at the \textit{S. cerevisiae} genomic \textit{FKS1} locus. The resulting isolates exhibited resistance to all three candins compared to the wild type (Table 2). The \textit{FKS1} S636Y mutation showed CAS, MICA, and ANI MICs that had increased by 32-, 32-, and 16-fold, respectively, compared...
to the wild type (Table 2). The mutations S643Y and S643P increased candin MICs from 32- to 256-fold compared to the wild type (Table 2); thus, the data suggest that they have a greater impact on candin resistance than S636Y. In any case, position Ser636 (Ser631 in C. lusitaniae) can be added as another novel site relevant for candin resistance.

Azole resistance in C. albicans is mediated by several mechanisms, among which transport-related mechanisms involving either major facilitator superfamily (MFS) genes or ATP-binding cassette (ABC) transporter genes are the most frequently reported (34). Few studies have explored azole resistance and the involvement of major drug transporters. Among these, a recent report by Reboutier et al. (23) suggested upregulation of MFS7 in documented FLC-resistant isolates. As illustrated in Fig. 4, we corroborated the overexpression of MFS7 in FLC-resistant C. lusitaniae isolates P3 and P4 (50- and 32-fold compared to P1, respectively). No expression variations in azole resistance genes belonging to the ABC transporter family or to ERG11 (target of azoles) were identified among our C. lusitaniae isolates (Fig. 4). Interestingly, FLC resistance in P3 and P4 correlated with an elevated VORI MIC (0.25 μg/ml, compared with 0.008 μg/ml for P1, P2, and P5) but also with resistance to 5-FC (MIC, 32 μg/ml).

In contrast to our findings, other studies reported 5-FC/azole cross-resistance correlating with mutations of FCY1 and FCY2 genes encoding cytosine deaminase and purine-cytosine permease involved in 5-FC transport and metabolism (35). These mutations were documented following simultaneous use of 5-FC and azoles in susceptibility assays, thus suggesting a different mechanism for 5-FC-azole cross-resistance. Indeed, no mutations in 5-FC resistance genes (FCY1 and FCY2) were detected in the P3 and P4 isolates (data not shown). Analysis of FUR1, encoding uracil phosphoribosyl transferase, was not conducted as isolates P1 to P5 were not resistant to 5-fluorouracil, which is commonly reported among FUR1-deficient isolates (data not shown) (36).

### Table 2 Candin MICs of S. cerevisiae FKS1 mutants

<table>
<thead>
<tr>
<th>Isolate</th>
<th>MIC (μg/ml)</th>
<th>ANI</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>S. cerevisiae wild-type IMX581</strong></td>
<td>0.03</td>
<td>0.03</td>
</tr>
<tr>
<td>DSY4762 (FKS1&lt;sup&gt;Δ645&lt;/sup&gt;)</td>
<td>1 (32)</td>
<td>0.5 (32)</td>
</tr>
<tr>
<td>DSY4763 (FKS1&lt;sup&gt;Δ645&lt;/sup&gt;)</td>
<td>2 (64)</td>
<td>4 (256)</td>
</tr>
<tr>
<td>DSY4764 (FKS1&lt;sup&gt;Δ645&lt;/sup&gt;)</td>
<td>8 (256)</td>
<td>4 (256)</td>
</tr>
</tbody>
</table>

a MIC assays were performed according to the EUCAST protocol but at 30°C and with YEPD medium. Numbers in parentheses represent relative fold increases in MICs compared to the MIC value of the wild type.

### Discussion

This clinical report describes acquired resistance of C. lusitaniae to all common antifungals in a profoundly neutropenic host with severe enterocolitis. When simultaneous combinations of resistance to 2 or more different drug classes occur, which was the case in the present study, the phenotype is referred to as multidrug resistance (MDR). While MDR is not a common phenotype among fungal pathogens, it was reported earlier in C. glabrata, with simultaneous acquisition of resistance to echinocandins and to azoles and separate acquisition of resistance to 5-FC (37). In the United States, a significant proportion (30% to 40%) of echinocandin-resistant isolates are also resistant to azoles (38, 39). While a recent study (40) suggested an association between MDR and the use of echinocandins and azoles, another investigation (41) described MDR to echinocandins, azoles, and amphotericin B in C. glabrata isolates recovered from a neutropenic patient with prolonged fever.

Antifungal resistance is a growing concern worldwide (42–44); however, less is known about the mechanism of resistance to echinocandins in C. lusitaniae. In the present paper, resistance to CAS was correlated to the identification of 3 novel FKS1 mutations (S638Y, S638P, and S631Y). Among these, FKS1 mutations S638Y and P corresponded to C. albicans and S. cerevisiae positions Ser645 and Ser643, respectively, which are commonly attributed to echinocandin resistance (45), whereas the remaining FKS1 S631Y mutation corresponded to position Ser638 in C. albicans.
and position Ser636 in *S. cerevisiae*. Here we confirmed that a mutation at this position can alter candin susceptibility in *S. cerevisiae*. The FKS1 S636Y mutation yields lower candid MICS than S643Y and S643P; thus, one may conclude that Ser636 is less effective for candin resistance development. In evaluating the relative increases of candid MICS in *C. lusitaniae* and *S. cerevisiae* compared to their respective wild types (Table 1 and 2), the trends seen with the two yeast species are globally similar, with the exception of the relative MICA MIC increases (32-fold versus 256-fold and 256-fold for *C. lusitaniae* and *S. cerevisiae*, respectively). Such differences might be due to differences in the intrinsic FKS1 structures of the two species. Similarly to our case report, a recent study (19) documented isolates of *C. lusitaniae* exhibiting missense mutation S645F in FKS1, which resulted in increased MICS of several echinocandins (CAS, MICA, and ANI) following CAS exposure. In contrast to our report, those isolates were not cross-resistant to other classes of antifungal drugs (5-FC, FLC, and AMB).

Overexpression of a major facilitator gene (MFS7) was documented among our FLC-resistant isolates. MFS transporter upregulation in *Candida* spp. is associated with mutations in the MRR1 transcriptional activator in *C. albicans* (46). Similar mutations could be suspected in *C. lusitaniae* strains. In the present report, FLC resistance was coupled to 5-FC resistance, despite the lack of 5-FC exposure, thus suggesting that the 5-FC resistance resulted from FLC resistance. Given that MFS7 upregulation results in FLC efﬂux in FLC-resistant strains, a similar mechanism might be involved in 5-FC-resistant strains. While this hypothesis remains speculative, mutations responsible for FLC/5-FC cross-resistance differ from the usual nonsense and missense mutations in FCY2 and FCY1 reported in *C. lusitaniae* strains and should thus be further explored (35).

Cross-resistance to AMB resistance and candidias occurred without ongoing exposure to AMB (see Fig. 1). The molecular basis of AMB resistance in *C. lusitaniae* has not yet been well documented. While some studies attributed AMB resistance to a rapidly switching phenotype occurring at a frequency of $10^{-2}$ to $10^{-4}$ (47), other studies attributed it to cell wall reorganization (48). FKS mutations documented in *C. lusitaniae* P2 could have induced cell wall stress, which then could result in AMB resistance. Even if CAS resistance was not associated with AMB resistance in P4 (FKS1 mutation S631Y), this hypothesis should be further investigated. Such issues could now be addressed using the genetic tools that have become accessible for use in *C. lusitaniae* studies.

The present report illustrates rapid selection of resistant mutants under conditions of drug pressure. The sequential administration of specific agents resulted in the emergence of isolates resistant to a specific molecule as illustrated in Fig. 1. Treatments using CAS and azole and their combination were followed within days by the selection of CAS- and/or azole-resistant isolates. This rapid emergence can be facilitated by the haploid nature of *C. lusitaniae*. It is also possible that several resistant populations with P1 to P5 profiles may have coexisted in the patient and that dominant resistance profiles were selected and emerged under conditions of exposure to a specific antifungal agent. As such, the colon may have been a colonizing reservoir which then seeded infection and different resistance phenotypes. Long-lasting neutropenia and enterocolitis certainly also contributed to this mechanism. Rapid emergence of multidrug-resistant mutants under conditions of combined therapy reinforces the idea of a need for limiting dual therapy to exceptional situations.

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