**Phenotypic Switching in *Candida lusitaniae* on Copper Sulfate Indicator Agar: Association with Amphotericin B Resistance and Filamentation**

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*Candida lusitaniae* is an opportunistic yeast pathogen that has the ability to develop resistance to amphotericin B (AmB). The mechanism(s) for this resistance is not well understood, although there are data supporting mutations in sterol pathways and other data supporting phenotypic switching (PS). The goal of this study was to determine whether *C. lusitaniae* has a PS system and to characterize any phenotypes, including any changes in AmB MICs. When 10⁴ CFU of an AmB-resistant (MIC of 16 to 32 μg/ml) clinical strain was plated on yeast-peptone-dextrose (YPD) agar with 1 mM CuSO₄, three colony colors were observed: light brown (LB) > dark brown (DB) > white (W), similar to the result for *Candida glabrata*. Switching did occur with high AmB resistance (MIC of 256 μg/ml) being associated with W, whereas LB and DB colonies had MICs of 2 to 8 μg/ml and 2 to 16 μg/ml, respectively. Filamentation (pseudohyphae) was associated with DB colonies. All phenotypes occurred spontaneously with greater frequency (~10⁻² to 10⁻⁴) than spontaneous mutations, and all phenotypes were reversible, fulfilling the two PS criteria. High AmB MICs were always associated with W colonies but not with all W colonies. Detection of PS on YPD-CuSO₄ is also similar to that in *Candida glabrata*, and we hypothesize that this is due to similarities in metallothionine gene expression. Phenotypic switching represents a key strategy in *C. lusitaniae* that confers a selective advantage during environmental challenges, including the ability to switch to AmB resistance.

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*Candida lusitaniae* is a yeast species that is infrequently found as a human commensal (skin and respiratory, gastrointestinal, and genitourinary tracts), with less than 1% of yeasts recovered from hospitalized patients being *C. lusitaniae* (14). Similarly, *C. lusitaniae* is an infrequent cause of infection in highly compromised patient populations, comprising just 2% (29/1,465) of cases of candidemia at The Johns Hopkins Hospital from 1997 to 2004 (unpublished data).

*Candida lusitaniae* is different from most medically important *Candida* species, because it can readily develop in vivo resistance to amphotericin B (AmB) during therapy. Although this phenomenon has been known and noted for more than 25 years (24), the genetic mechanism(s) for this acquired resistance is not well understood. There are data supporting the presence of gene mutations in the ergosterol biosynthetic pathway, thereby blocking production of the binding target, i.e., ergosterol (4, 17). A mutation mechanism(s) is credible since *C. lusitaniae* is a haploid yeast and nonlethal mutations can have a direct effect on gene expression. Other proposed mechanisms involve biosynthetic pathways, enzyme expression, and cell wall and cell membrane structures (6). There also are limited data suggesting that phenotypic switching (PS) may be responsible for changes in gene expression in *C. lusitaniae*, including those able to affect resistance to AmB and other antifungals (5, 27).

Phenotypic switching is an attractive explanation for such phenotypic variations in *C. lusitaniae*, since switching has been documented for other pathogenic yeasts, including *Candida albicans* (3, 12, 15, 16, 19, 20, 21, 23, 25, 26), *Candida glabrata* (2, 10, 22), *Trichosporon asahii* (9), and *Cryptococcus neoformans* (7). This mechanism permits an organism to adapt to environmental changes through selective gene expression, including changes in the fungal response to antifungal agents.

The goal of this study was to determine whether *C. lusitaniae* has a PS system similar to those of other medically important yeasts, and if so, to determine whether changes in AmB susceptibility are associated with phenotypic switching. To prove that a PS system is active, two important criteria have to be fulfilled. First, the organism must have the ability to alternate between two or more phenotypes at frequencies greater than the frequency of changes due to random mutations. Second, these phenotypic changes must be reversible (2, 10, 15, 18, 19, 27). The strategy was to test a clinical strain of *C. lusitaniae* on media that permit discrimination of switching phenotypes for other medically important yeasts, such as *Candida albicans*, *C. glabrata*, and *C. neoformans*. For this study, a resistant clinical isolate (AmB MIC range, 16 to 32 μg/ml) was selected, with the rationale that a resistant strain would be a strong candidate for possession of a PS system.

**MATERIALS AND METHODS**

**Organisms tested.** Cells of *C. lusitaniae* were maintained on Sabouraud’s dextrose agar (SDA) and at ~70°C in glycerol. A clinical strain of *C. lusitaniae* (JHH Clu-3B) was used in this study. This strain had a MIC of 16 to 32 μg/ml, as determined by the agar dilution susceptibility assay described below.

**Media.** Three media were used to ascertain whether *C. lusitaniae* produced different colony switching phenotypes. These included SDA (Becton Dickinson, Sparks, MD), zinc-supplemented Lee’s medium (18), and yeast extract-peptone-d-glucose (YPD) agar containing 1 mM CuSO₄ (YPD-CuSO₄) (11). None of the screening media contained AmB.
Screening assay for phenotypic switching. Yeast cells used for the screening assay were initially grown on SDA for 48 h at 37°C, followed by growth in plain YPD broth, and shaker incubated for 48 h at 35°C. Cells were washed by centrifugation and resuspended to match a 0.5 McFarland turbidity standard (previously determined to be approximately 1.0 $\times$ 10$^6$ cells/ml). Using appropriate standard dilutions, a total of 1 $\times$ 10$^4$ to 1 $\times$ 10$^5$ C. lusitaniae cells were plated (100 to 300 CFU/plate) by thoroughly streaking a 1-ml suspension of cells onto the surfaces of test agars in 150- by 15-mm plastic petri dishes. Plates were incubated for 5 days at 30°C. The number of colonies was counted and scored for any colony phenotypes (color, size, shape, borders, and texture). Cells taken directly from random colonies of different colony phenotypes on YPD-CuSO$_4$ medium were examined for filamentation versus budding yeast cells and were semiquantitatively scored (0 to 4). Similarly, cells from random colonies from different colony phenotypes were tested for in vitro susceptibility to AmB.

Reversal studies. To assess the reversal of a switch event, cells from random single colonies of different colors or sectors of homogenous color were grown in YPD broth, plated onto YPD-CuSO$_4$ agar as in the original screen, and scored as described above. Cells were plated again to confirm phenotype and reversibility and to detect additional phenotypes.

In vitro susceptibility testing and interpretation. For in vitro susceptibility testing, an agar dilution method (4) was used. Briefly, a suspension of 1 $\times$ 10$^6$ CFU/ml was made, as determined by visual comparison to a 0.5 McFarland standard. After appropriate dilution, a 0.01-ml aliquot (1 $\times$ 10$^4$ CFU) was inoculated with a Steers replicator onto the surfaces of SDA plates containing 0, 1, 2, 4, 8, 16, 32, 64, 128, and 256 $\mu$g/ml of AmB. Lyophilized AmB was dissolved in dimethyl sulfoxide, diluted with saline, and stored frozen at $-20^\circ$C in light-protected vials. Plates were incubated at 37°C and were scored for growth/no growth at 24 and 48 h. The MIC was defined as the lowest concentration that inhibited $\geq$80% of growth at 48 h. Resistance was defined as an MIC of 2.0 $\mu$g/ml or higher.

RESULTS

Media for detection of colony phenotypes. To assess whether different colony phenotypes would be detected on any of the three selected agar media, inocula of 1.0 $\times$ 10$^4$ to 1.0 $\times$ 10$^5$ CFU of C. lusitaniae strain 3B were plated onto three test media, as described in Materials and Methods. No differences in colony morphologies were evident on SDA or on the zinc-supplemented Lee’s medium after 5 days of incubation. All colonies were opaque and creamy white, with similar borders and textures. However, three differently colored colony phenotypes were evident on the YPD-CuSO$_4$ agar. Therefore, YPD-CuSO$_4$ agar was used in subsequent experiments as the indicator agar for phenotypic switching.

Colony color phenotypes on YPD-CuSO$_4$ agar. After 1 $\times$ 10$^5$ wild-type C. lusitaniae cells were plated onto the surface of YPD-CuSO$_4$ agar, all the progeny cells were light brown (LB), with the exception of nine white (W) colonies. A total of 1.8 $\times$ 10$^3$ representative LB phenotype cells were plated again, and LB was dominant, but there were also 120 colonies that were either completely dark brown (DB) or LB with a DB sector. The frequencies of switching were 1 $\times$ 10$^{-3}$ (wild type to W) and 6.6 $\times$ 10$^{-2}$ (LB to DB or with a DB sector) (Fig. 1).

Filamentation among the phenotypes. Cells of random colonies of the three color phenotypes were examined microscop-
ically after the 5-day incubation to assess any differences in cellular morphology, especially filamentation (pseudohyphal formation). Budding yeast cells with no or little (0 to 1+) filamentation were present upon examination of LB and W colonies. This microscopic morphology was consistent with examination of the wild-type C. lusitaniae strain. In contrast, examination of cells from DB colonies revealed pseudohyphae production levels (2+ to 4+) that were more than those in the LB and W colonies and the wild-type strain.

**AmB MICs of the different colony phenotypes.** Cells taken from random colonies of the three different color phenotypes were subcultured to SDA and then tested for the AmB MIC as described above. Cells from LB colonies had an MIC range of 2 to 4 μg/ml, cells from DB colonies had an MIC of 8 μg/ml, and cells from W colonies had an MIC of 256 μg/ml (Fig. 1).

Reversal of colony color phenotypes, filamentation, and AmB susceptibility. When cells from random colonies of the three color phenotypes were replated, progeny colonies maintained their original colony color as the dominant phenotype but also showed variably reversible switching involving all three colors (Fig. 1). Reversal of colony color occurred at frequencies ranging from $1.4 \times 10^{-2}$ to $7.8 \times 10^{-4}$. Filamentation remained associated with the DB phenotype. AmB susceptibility switched also; cells from LB colonies had an MIC range of 2 to 8 μg/ml; cells from DB colonies had an MIC range of 2 to 16 μg/ml. White, high-MIC cells yielded other colony color phenotypes and lower AmB MICs. However, when high-MIC resistance to AmB was demonstrated (256 μg/ml), it was always associated with a W phenotype, but not all W colonies had high AmB MICs. In these instances (LB to W and DB to W), the W colonies had an MIC range of 2 to 8 μg/ml. For W colonies with a high MIC of 256 μg/ml, switching to lower MICs was observed only in association with color phenotype switching at frequencies of $1.8 \times 10^{-3}$ (W to DB) and $2 \times 10^{-4}$ (W to LB). High- to lower-MIC switching with preservation of the W phenotype was not observed.

**DISCUSSION**

PS has been demonstrated and described for pathogenic yeasts, including C. albicans (3, 12, 15, 16, 19, 20, 21, 23, 25, 26), Candida glabrata (2, 10, 22), T. asahii (9), and C. neoformans (7). As a manifestation of apparent developmental programming, PS confers phenotypic variability and environmental adaptation that facilitates the survival and/or pathogenic success of the organism. Candida albicans has been studied most extensively, and PS has been observed in association with classic putative virulence factors, such as pseudohyphal formation, bud-hypha transitioning, and secretion of aspartic proteases. In both C. albicans and C. glabrata, PS has been demonstrated to have a role in mating as well (2, 8, 20, 22). Switching has also been associated with antifungal resistance, including AmB resistance. This has been reported for clinical strains of C. albicans from human immunodeficiency virus-positive individuals (26). In addition, data support a role for PS in AmB drug resistance in C. lusitaniae (5, 27).

In the present study, phenotypic differences in colony color, microscopic morphology (filamentation), and AmB MICs were detected. The frequencies of switching were observed to be from approximately $10^{-2}$ to $10^{-4}$. This was more frequent than was expected for spontaneous random mutations, thereby fulfilling one of the criteria required for PS. The phenotypes were reversible, fulfilling the second criterion for a PS system.

Switching was associated with changes in AmB MIC profiles. MICs of at least 3 dilutions higher than that for the parental strain were detected for cells from W colonies. Since AmB resistance was not consistently observed for all switching events of the W lineage, this suggests that multiple switching events, independent or sequential, are required to confer the switched phenotypes. Possibly related to this last comment and peculiar to the present C. lusitaniae study is the fact that the switching effect on the AmB MIC was demonstrated regardless of the fact that the wild-type strain already manifested significant in vitro resistance to the AmB MIC (16 to 32 μg/ml). A high-MIC W phenotype (256 μg/ml) was associated with a colony color switch, and this high-MIC W phenotype was readily reversible to a lower-MIC LB or DB phenotype. Whether MICs this high would have occurred with an AmB-susceptible, wild-type strain is not known.

High-MIC to lower-MIC switching supports the utility of PS in maintaining an in vivo selective advantage for an organism, by facilitating phenotypes best suited to environmental conditions. This would be a survival mechanism for cells with the high-MIC phenotype if cell wall or membrane alterations conferred an only-transient survival advantage in the presence of AmB at the expense of overall relative fitness compared to that of cells with a normal structure and low or susceptible MICs.

Filamentation was also associated with the switching colony phenotypes. It was associated with the DB phenotype; very little, if any, was seen with the LB or W phenotype. In vivo, pseudohyphal filamentation in C. lusitaniae might be beneficial to the organism as it is for other candidal species, enhancing cellular adhesion, colonization, and/or initiation of infection in a compromised host. Comparative studies of the DB, LB, and W phenotypes and their relative adhesion to human cells, extracellular matrices, and/or hardware would be very interesting.

Yoon et al. (27) observed in vitro, high-frequency switching from an AmB-susceptible phenotype to an AmB-resistant phenotype (on SDA or synthetic dextrose agar with and without AmB). Heterogeneity within individual C. lusitaniae colonies of clonal lineage was present, consisting of apparent “switching lineages” and stable “resistant lineages.” The switching frequencies were in a range consistent with ours (i.e., 1 in $10^{2}$ to $10^{4}$ cells), but reverse switching from AmB resistance to AmB susceptibility occurred at a lower frequency, which was increased by exposure to UV light, heat shock, and whole blood. Unlike in our study, morphological differences between AmB-susceptible and AmB-resistant clonal isolates were observed (susceptible cells were elongated, forming short chains; resistant cells were round to ovoid, budding, and clustered in small aggregates).

Favela et al. (5) reported phenotypic switching for C. lusitaniae among three of seven isolates recovered over the course of an infection in a neonate. Two colony types were noted on SDA, a full-size and a small type. AmB and azole resistances were documented with the final three isolates recovered from the patient, and the resistance was associated with the small-colony type. We did not notice these differences in colony size when we screened for colony phenotypes on SDA. It seems logical to conclude that different strains of C. lusitaniae may
have different PS types. We and Yoon et al. (27) noted differences in cell morphology/filamentation, whereas Favela et al. (5) did not.

Differences in AmB susceptibility for C. lusitaniae have also been reported in association with color variation on CHROMagar Candida (13). Similar observations have been made for C. glabrata and azole susceptibilities (1).

Interestingly, the C. lusitaniae colony phenotypes that we detected on YPD-CuSO 4 agar are strikingly similar to the PS described for C. glabrata by Lachke et al. (10, 11). A core switching system of four color phenotypes on this indicator agar was detected with C. glabrata. These four phenotypes (similar to our three colors) were seen in a graded hierarchy (white > light brown > dark brown > very dark brown) that had an inverse correlation to the level of metallothionein gene expression. We hypothesize that the colored colonies in our study were due to the same metallothionein gene expression. We hypothesize that the colored colonies in our study were due to the same metallothionein gene expression.

We hypothesize that the colored colonies in our study were due to the same metallothionein gene expression. With C. glabrata, core switching occurred in the absence of CuSO 4 as did the maintenance of phenotypes. Lachke et al. proved that PS in C. glabrata was not induced by CuSO 4 media, and we hypothesize that this is also the case for C. lusitaniae. However, unlike in the present study of C. lusitaniae, both budding yeast cells and pseudohyphal tubes were seen in similar proportions for all four switch phenotypes during the colony development of C. glabrata. Overall, the PS observed by Lachke et al. for C. glabrata was seen in the majority of 62 tested clinical isolates, suggesting the general nature of developmental programming in C. glabrata. The likelihood that this is true for C. lusitaniae is suggested by the results of Yoon et al., but screening more C. lusitaniae strains on YPD-CuSO 4 is necessary to confirm this.

This study documents that C. lusitaniae has a PS system by fulfilling the criteria of discrete phenotypes, appropriate frequency, and reversibility of phenotypes when cells are grown on YPD-CuSO 4 agar. The association of high-MIC AmB resistance and filamentation points to the presumed importance of this mechanism in host-fungal interactions. More C. lusitaniae strains need to be screened to determine the extent of PS in C. lusitaniae. In addition, studies correlating phenotypes with putative or known virulence factors would be very important. These studies should be designed to evaluate their adherence to human cells, extracellular matrices and hardware, phagocytosis and intracellular killing, and, ultimately, virulence in an experimental animal model.

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