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Enhanced Efflux Pump Activity in Old *Candida glabrata* Cells

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ABSTRACT We investigated the effect of replicative aging on antifungal resistance in *Candida glabrata*. Our studies demonstrate significantly increased transcription of ABC transporters and efflux pump activity in old versus young *C. glabrata* cells of a fluconazole-sensitive and -resistant strain. In addition, higher tolerance to killing by micafungin and amphotericin B was noted and is associated with higher transcription of glucan synthase gene *FKS1* and lower ergosterol content in older cells.

KEYWORDS *Candida glabrata*, aging, antifungal resistance, drug resistance mechanisms, efflux pumps

Candida glabrata is a formidable pathogen that, analogous to other yeasts, undergoes replicative aging (1). We have reported that growth of older (defined as more generations lived) *C. glabrata* cells was more inhibited by fluconazole (FLC) (1). This finding is relevant because, *in vivo*, the host's neutrophils selectively kill younger cells, which causes old yeast cells to accumulate in the host. Aging *C. glabrata* mother cells have larger cell bodies and thicker cell walls than those of the younger daughter cells (1).

The specific mechanism that underlies the observed age-associated FLC resistance in old *C. glabrata* cells is not known. Azole resistance can have different underlying mechanisms, some of which may act in combination (2). Although mutations of *ERG11* and *PDR1* can mediate azole resistance, these mutations would be inherited by the daughter cells and persist (3–5). Data from *Saccharomyces cerevisiae* indicate that genomic stability rather than mutation rate changes during replicative aging (6). Other studies have reported that *C. glabrata* subpopulations originating from a clonal population can develop heteroresistance to FLC through transient upregulation of efflux pumps (7, 8). Active efflux of azoles is accomplished by membrane transporters, which belong to one of the two superfamilies in fungi: the ATP-binding-cassette superfamily (ABC) and the major facilitator superfamily (MFS) (9–11). Several ABC transporters have been linked to drug resistance in *C. glabrata*, of which Cdr1p and Pdh1p have been extensively investigated (12–16). Previously published transcriptome data comparing young and 14-generation-old *C. glabrata* cells of the FLC-sensitive BG2 strain demonstrated increased expression of the genes *CDR1* (2-fold), *PDR16* (8-fold), and *YBT1* (10-fold), all of which encode ABC transporters associated with FLC resistance (1).

In this study, we investigated these findings further. Young and old *C. glabrata* cells were isolated by first labeling 10^8 exponentially growing cells with biotin and then allowing growth for six generations. Biotin-labeled *C. glabrata* cells were then bound by streptavidin-linked magnetic beads and sorted on a column. A purity of >90% in the samples was verified with fluorescein isothiocyanate-streptavidin biotin stain as described previously (17). The number of generations was calculated from the number of doubling times of the initial population. First, the expression of ABC transporters was quantified in young and old cells of a clinical FLC-resistant *C. glabrata* strain (*Cg*#61 MIC, 64 μg/ml). RNA was extracted from 14-generation-old *Cg*#61 cells, which were isolated by magnetic-bead column separation as described previously (1). The cDNA levels of

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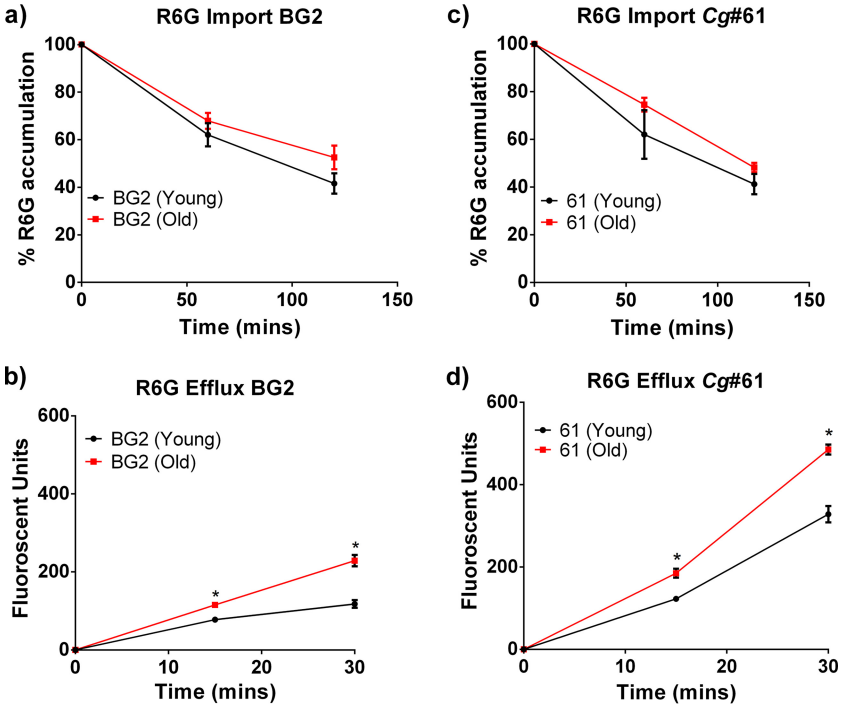

FIG 1 R6G import (a, c) and efflux (b, d) were characterized in old (14 generations) and young (0 to 3 generations) cells of strains BG2 (a, b) and *Cg*#61 (c, d) as described previously (18). The assays were performed in triplicate. Error bars, standard deviations between replicates. All data points were subjected to multiple *t* tests using the Holm-Sidak method. *, $P < 0.05$.

specific ABC transporters were quantified by reverse transcription-quantitative PCR (qRT-PCR) using transporter gene-specific primers (see Table S1 in the supplemental material). Again, significantly increased expression of *CDR1* (49-fold), *PDR16* (4.5-fold), and *YBT1* (76-fold) was documented in older cells (see Fig. S1 in the supplemental material). Next, a functional analysis of efflux activity was performed with the ABC-transporter-specific fluorescent dye rhodamine 6G (R6G) (Sigma-Aldrich) following a previously published protocol (18). Briefly, 10^6 young and 14-generation-old cells of both BG2 and *Cg*#61 cells were starved under deenergized conditions for 2 h in 1× phosphate-buffered saline (PBS)-containing 5 mM 2-deoxyglucose (Sigma-Aldrich). After 2 h, R6G was added to the young and old cells at a final concentration of 1 μM, and the samples were collected every 30 mins for 2 h. The samples were spun down, and R6G import was measured as the decrease in fluorescence of the supernatants over time in a spectrometer (excitation of 525 nm and emission at 555 nm). These data showed that old and young cells of both strains accumulated comparable amounts of R6G at a similar rate (Fig. 1), demonstrating that the thicker cell wall of older cells did not interfere with import. After 2 h of import, the cells were washed with PBS, and 2% glucose was added to initiate the efflux. The samples were collected every 15 mins for a total efflux time of 30 mins and spun down, and R6G efflux was measured as the increase in fluorescence of the supernatant over time. In these assays, old cells of FLC-sensitive and -resistant *C. glabrata* isolates showed significant increases in R6G efflux pump activity compared with that in their younger daughter cells (Fig. 1). As predicted by qRT-PCR data, *Cg*#61 cells exhibited a higher baseline efflux and more pronounced upregulation of efflux with aging. Last, we explored whether the *in vitro* activities of echinocandins and amphotericin B were reduced in older *C. glabrata* cells, because the cellular levels of their specific targets, namely, ergosterol and β-1,3-glucan, were altered by aging (1). However, only the expression of *FKS1* and not that of *FKS2* was altered between the young and old *C. glabrata* cells (1). Specifically, in the older cells, ergosterol content was decreased as a result of redirection of the ergosterol

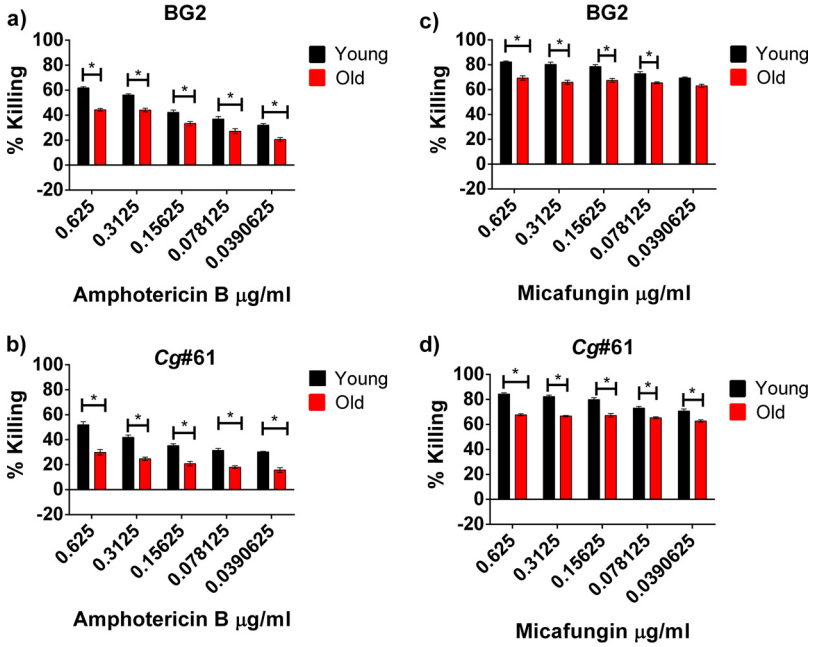

FIG 2 Antifungal killing assay. A total of 10^4 cells/ml of old (14 generations) and young (0 to 3 generations) cells of both BG2 (a, c), and *Cg*#61 (b, d) strains were exposed to amphotericin B (a, b) and micafungin (c, d) for 2 h and incubated at 37°C. After incubation, 100 μl cells from each condition were plated in 25 ml YPD medium and incubated at 37°C for 48 h. CFU were then counted, and the percentages of killings were calculated and plotted. Old and young cells with no exposure to drugs were used as controls. Shaded bars, specific concentration of the individual drugs used in these assays. The assays were performed in triplicate. Error bars, standard deviations between replicates. Multiple *t* tests using the Holm-Sidak method were used to analyze the statistical significance of the data. *, $P < 0.05$.

pathway to upstream or side pathways (1), whereas β-1,3-glucan content was increased as a result of increased glucan synthase (*FKS1*) transcription (1), by 29-fold in BG2 and 9-fold in *Cg*#61 cells (see Fig. S1). Antifungal efficacy was assessed in standard killing assays, where 10^4 cells/ml of young and 14-generation-old *C. glabrata* were exposed to decreasing concentrations of amphotericin B and micafungin for 2 h. Higher tolerance of old BG2 and *Cg*#61 cells was documented for both drugs after plating 100 μl of drug-treated cells on 25 ml yeast extract-peptone-dextrose (YPD) medium (Fig. 2).

Our data indicate that enhanced tolerance to different antifungal drug classes can be observed in two different *C. glabrata* strains, when aged to 14 generations. Enhanced FLC and amphotericin B resistance was also found in older *Cryptococcus neoformans* cells, which accumulate in spinal fluid during chronic infection (19). Similarly, enhanced caspofungin resistance of old *Candida albicans* cells has been described (20).

Although the changes in drug tolerances between young and old *Candida* cells seem modest (Fig. 2), differential killing or growth inhibition of young and old cells would magnify increased accumulation of older cells over time in drug-treated patients, and such persistence may potentially contribute to therapeutic failure. This may be most relevant when static drugs such as FLC are used. Our data suggest that enhanced FLC resistance in older *C. glabrata* cells is the result of enhanced efflux pump activities of ABC transporters. MFS transporters were not assessed but may contribute to enhanced FLC efflux in that some were found to be upregulated in aging cells. Why ABC transporters are overexpressed in older *C. glabrata* cells remains unknown, and no studies have linked increased levels of the transcription factor Pdr1p and increased ABC transporter transcription. Interestingly, in *S. cerevisiae*, ABC transporter Pdr5p is inherited asymmetrically by the daughter cells, and therefore they exhibit enhanced efflux activity compared with that in old *S. cerevisiae* cells (21). Gene amplification secondary to age-induced genomic instability must also be taken into account. Aneuploidy by the

gain of small chromosomes and segmental aneuploidy have been described in resistant *C. glabrata* isolates, especially in the left arm of chromosome F, which encodes the ABC transporters *AUS1* and *PDH1* (22). Whether higher levels of the target enzyme Fks1p contribute to enhanced tolerance to micafungin needs further investigation. Mutations in *FKS1* and *FKS2* are predominantly implicated in micafungin resistance (23). However, in resistant *C. glabrata* strains, a decrease in enzyme function was associated with a relative increase in *FKS1* expression (24). Additionally, in *C. albicans* biofilms, overexpression of *FKS1* was associated with resistance (25). Last, amphotericin B resistance in *Candida lusitaniae* is associated with elevated *ERG6* levels and lower ergosterol (26), so the observed resistance to killing was expected. Our data are the first to show that efflux pump activity is upregulated by cell aging. Further research is required to determine whether this transient phenotypic resistance as a result of aging followed by selection of old *C. glabrata* cells contributes to treatment failure. Note that it would not be diagnosed by standard antifungal testing because that is performed with young *in vitro*-grown *Candida* populations. This research is important because drug resistance related to ABC transporters is clinically relevant in pathogenic fungi.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.02227-17.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

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