Title
Balancing Positive and Negative Selection: \textit{In Vivo} Evolution of Candida lusitaniae \textit{MRR1}.

Permalink
https://escholarship.org/uc/item/5127b34r

Journal
mBio, 12(2)

ISSN
2150-7511

Authors
Demers, Elora G
Stajich, Jason E
Ashare, Alix
\textit{et al.}

Publication Date
2021-03-30

DOI
10.1128/mbio.03328-20

Peer reviewed
Balancing Positive and Negative Selection: *In Vivo* Evolution of *Candida lusitaniae* MRR1

Elora G. Demers,a Jason E. Stajich,b Alix Ashare,c Patricia Occhipinti,a Deborah A. Hogram

aDepartment of Microbiology and Immunology, Geisel School of Medicine at Dartmouth, Hanover, New Hampshire, USA
bDepartment of Microbiology & Plant Pathology and Institute for Integrative Genome Biology, University of California—Riverside, Riverside, California, USA
cDartmouth-Hitchcock Medical Center, Section of Pulmonary and Critical Care Medicine, Lebanon, New Hampshire, USA

**ABSTRACT** The evolution of pathogens in response to selective pressures present during chronic infections can influence their persistence and virulence and the outcomes of antimicrobial therapy. Because subpopulations within an infection can be spatially separated and the host environment can fluctuate, an appreciation of the pathways under selection may be most easily revealed through the analysis of numerous isolates from single infections. Here, we continued our analysis of a set of clonally derived *Clavispora* (*Candida*) *lusitaniae* isolates from a single chronic lung infection with a striking enrichment in the number of alleles of MRR1. Genetic and genomic analyses found evidence for repeated acquisition of gain-of-function mutations that conferred constitutive Mrr1 activity. In the same population, there were multiple alleles with both gain-of-function mutations and secondary suppressor mutations that either attenuated or abolished the constitutive activity, suggesting the presence of counteracting selective pressures. Our studies demonstrated trade-offs between high Mrr1 activity, which confers resistance to the antifungal fluconazole, host factors, and bacterial products through its regulation of MDR1, and resistance to hydrogen peroxide, a reactive oxygen species produced in the neutrophilic environment associated with this infection. This inverse correlation between high Mrr1 activity and hydrogen peroxide resistance was observed in multiple *Candida* species and in serially collected populations from this individual over 3 years. These data lead us to propose that dynamic or variable selective pressures can be reflected in population genomics and that these dynamics can complicate the drug resistance profile of the population.

**IMPORTANCE** Understanding microbial evolution within patients is critical for managing chronic infections and understanding host-pathogen interactions. Here, our analysis of multiple MRR1 alleles in isolates from a single *Clavispora* (*Candida*) *lusitaniae* infection revealed the selection for both high and low Mrr1 activity. Our studies reveal trade-offs between high Mrr1 activity, which confers resistance to the commonly used antifungal fluconazole, host antimicrobial peptides, and bacterial products, and resistance to hydrogen peroxide. This work suggests that spatial or temporal differences within chronic infections can support a large amount of dynamic and parallel evolution and that Mrr1 activity is under both positive and negative selective pressure to balance different traits that are important for microbial survival.

**KEYWORDS** *Candida lusitaniae*, Mrr1, evolution, drug resistance, fluconazole, yeast, hydrogen peroxide, chronic infection, cystic fibrosis, *Candida albicans*, *Candida auris*

Understanding the positive and negative selective pressures that shape drug resistance profiles in microbial populations is critical for combating the development of antimicrobial resistance, an ever-increasing problem in clinical settings. Increased drug resistance in bacteria and fungi has been associated with clinically and agriculturally
used antimicrobial agents (reviewed in references 1–3); however, drug resistance elements may also be selected for based on their ability to protect against factors produced by other microbes or plant, animal, and insect hosts (4, 5). Based on the analysis of bacterial isolates, such as Burkholderia dolosa or Pseudomonas aeruginosa, from single patients and across cohorts of patients, it is clear that in vivo factors can lead to the repeated selection for subpopulations with the same genes or pathways mutated (6–8). Similar studies have further shown that individual pathways can be upregulated and then downregulated in the same phylogenetic lineages. For example, suppressor mutations within P. aeruginosa algU frequently arise in strains already harboring mutations in the gene encoding the AlgU repressor MucA, which causes high AlgU signaling (9). Less is known about the negative selective pressures acting against sustained microbial resistance.

In the study by Demers et al. (10), we described a set of 20 recently diverged Clavispora (Candida) lusitaniae isolates obtained from the lung infection of a single individual with cystic fibrosis (CF). C. lusitaniae is among the emerging non-albicans Candida spp. that cause life-threatening disseminated infections in immunocompromised (11–13) and immunocompetent (14, 15) individuals. C. lusitaniae is notorious for its rapid development of resistance to antifungal drugs, including amphotericin B, azoles, and echinocandins (13, 16–19), which is interesting in light of its close phylogenetic relationship to Candida auris (20), a species in which multidrug-resistant isolates have caused hospital-associated outbreaks (21, 22). Our previous analyses of heterogeneity in fluconazole (FLZ) resistance among these isolates identified numerous distinct alleles of MRR1 (CLUG_00542) (10). Multiple alleles encoded gain-of-function (GOF) mutations causing constitutive Mrr1 activity, which, as in other Candida species, increased expression of MDR1 and Mdr1-dependent multidrug efflux pump activity (10, 23–30). At the time that these isolates were recovered, the patient had no history of antifungal treatment, suggesting that selection for constitutively active Mrr1 variants may have been driven by the need for resistance to other host- or microbe-produced compounds. Within this study, however, we found multiple lineages with recently evolved MRR1 alleles that rendered cells more sensitive to FLZ than even mrr1Δ strains. Here, we address the perplexing question of why this population had recently diverged MRR1 alleles that encoded both high and low Mrr1 activity. To do so, we expressed both native and synthesized MRR1 alleles that represent intermediates during MRR1 evolution in a common genetic background and tested the effects of these alleles on growth under in vivo relevant conditions. We concluded that multiple C. lusitaniae MRR1 alleles conferring low Mrr1 activity resulted from an initial mutation that caused constitutive Mrr1 activity followed by a second mutation that either suppressed constitutive activation or inactivated the protein. Constitutive Mrr1 activity caused increased sensitivity to a variety of biologically relevant compounds, including hydrogen peroxide (H2O2), and suppression of constitutive Mrr1 activity rescued growth under some of these conditions. Monitoring populations from this subject’s respiratory samples over time supports the model that there were opposing selective pressures in vivo that selected for and against constitutive Mrr1 activity, as reflected by the trade-off between FLZ and H2O2 resistance. These data provide insight into the persistence of a heterogeneous fungal population and underscores the complexity and parallelism of evolution that is possible in the human lung during a chronic infection.

RESULTS

Naturally evolved C. lusitaniae MRR1 alleles confer altered Mrr1 activity and FLZ resistance. Each of the 20 closely related C. lusitaniae isolates from a single individual contained at least one nonsynonymous single nucleotide polymorphism (SNP) or single nucleotide insertion or deletion (indel) in MRR1 relative to the deduced MRR1 sequence of their most recent common ancestor (MRR1ancestral), the sequence that resulted upon removing any of the SNPs that varied across the population (Fig. 1A) (10). While we did not identify any isolates with the MRR1ancestor sequence in samples from this subject, we did find an isolate from another patient (B_L06) that contained
the same sequence as the "MRR1ancstral" allele. To determine the impact of specific mutations in MRR1 on Mrr1 activity, we expressed different MRR1 alleles in a common genetic background in which the native MRR1 had been deleted (U04 mrr1Δ). Deletion of MRR1 in the FLZ-resistant strain U04 reduced the FLZ MIC from 32 μg/ml to 4 μg/ml (10), and the decrease in MIC was complemented by restoring the native MRR1Y813C

**FIG 1** Constitutively active and low-activity Mrr1 variants naturally evolved in a single C. lusitaniae population. (A) Maximum likelihood-based phylogeny constructed from SNPs identified in the whole-genome sequences of 20 C. lusitaniae isolates; modified from Demers et al. (10). Select branchpoints are marked with the Mrr1 variants (text colored to match branches) present in subsequent isolates. Mrr1 variants are identified by amino acid changes that resulted in early termination, denoted with "(t)," at N176 and L927, respectively. Gray star at the root of the tree denotes the "ancestral" MRR1 sequence, which lacks any of the mutations listed. U04 and U05, which are used in panels B and C, are highlighted. FLZ MICs (μg/ml) as determined in reference 10 are listed. (B) FLZ MICs for unaltered, mrr1Δ and MRR1 complemented strains in the FLZ-resistant U04 (native allele MRR1Y813C) strain background. (C) Same as in panel B, but in the FLZ-sensitive U05 strain background (native allele MRR1L1191H1Q1197). Strains containing the same MRR1 allele in panels B and C are represented by circles of the same color. Data shown represent at least four independent assays on different days. Each sample was statistically compared to every other sample; the same lowercase letters indicate samples that are not significantly different, and different lowercase letters indicate significant differences (P < 0.0001 [B] or P < 0.001 [C]) as determined by one-way ANOVA with Tukey’s multiple-comparison test of log2-transformed values. (D) Differentially expressed genes between strains harboring the constitutively active Mrr1-Y813C variant (U04 and U04 mrr1Δ+MRR1Y813C) and those lacking MRR1 (U04 mrr1Δ) or harboring low-activity variants (U04 mrr1Δ+MRR1ancstral and U04 mrr1Δ+MRR1L1191H1Q1197*) when grown in liquid YPD medium; statistical cutoffs used were FDR of <0.05 and fold change of ≥2 (see Table S1 in the supplemental material). Normalized counts per million (CPM) from RNA-Seq are scaled by row (gene) with hierarchical clustering by Euclidean distance. Complemented strains are denoted by their respective MRR1 allele. Predicted C. albicans homologs are listed next to C. lusitaniae gene names (Table S1).
allele (Fig. 1B). We previously published that FLZ resistance in isolates from Fig. 1A correlated with expression of MDR1 (10), also known as MFS7 (19), and here show that deletion of MDR1 similarly reduced the FLZ MIC of unaltered U04 and U04 mrr1Δ+MRR1Y813C (8- to 16-fold) (see Fig. S1A in the supplemental material). Complementation of U04 mrr1Δ with the MRR1ancestral allele resulted in a FLZ MIC of 1 μg/ml, 4-fold lower than the FLZ MIC of U04 mrr1Δ, suggesting that Mrr1ancestral had a function that reduced the FLZ MIC (Fig. 1B). Expression of an MRR1 allele from a closely related FLZ-sensitive isolate (MRR1L1191H+Q1197*) reduced the FLZ MIC to 0.5 μg/ml (Fig. 1B). Similar relationships between the MRR1 allele and FLZ MIC were observed when the MRR1ancestral, MRR1Y813C, and MRR1L1191H+Q1197* alleles were expressed in a mrr1Δ derivative of the FLZ-sensitive strain U05 (Fig. 1C); thus, further analyses were performed in the U04 background alone.

RNA-sequencing (RNA-seq) analysis validated the previously published result that MDR1 expression paralleled FLZ MIC (Fig. 1D and Table S1) (10). Comparison of gene expression profiles for U04 (MRR1Y813C), U04 mrr1Δ, and U04 mrr1Δ+MRR1Y813C found that mrr1Δ was fully complemented upon return of MRR1Y813C to the native locus (Fig. 1D and S2A) and confirmed that Mrr1 appears to both positively and negatively regulate a subset of genes (10, 29). Furthermore, a correlation analysis found that gene expression in U04 mrr1Δ+MRR1ancestral and U04 mrr1Δ+MRR1L1191H+Q1197* was similar but distinct from that of the mrr1Δ strain (Fig. 1D and S2A). A linear model comparing these strains identified 41 genes with at least a 2-fold change in expression and corrected P value of <0.05. Comparison of nonisogenic C. lusitaniae strains similarly identified 22 of the genes in Table S1 as putatively Mrr1 regulated, including MRR1 itself (10, 29). Eighteen genes were homologs or had similar predicted functions as genes previously published as regulated by Candida albicans Mrr1 (24), including MDR1, FLU1, and multiple putative methylglyoxal reductases encoded by GRP2-like genes, such as MGD1 and MGD2 (Fig. 1D and Table S1). Other genes within the Mrr1 regulon are discussed further below. These data indicate that the Mrr1-ancestral and Mrr1-L1191H+Q1197* variants had low basal activity, while Mrr1-Y813C was constitutively active.

The unexpected finding that FLZ MIC was higher upon deletion of MRR1 than U04 expressing MRR1ancestral or U05 was also observed in distantly related C. lusitaniae strains, ATCC 42720 and DH2383, with FLZ MICs of ~1 to 2 μg/ml (Fig. 1B and Fig. S1B). In both cases, deletion of MRR1 led to a 2- to 4-fold increase in FLZ MIC (Fig. S1B). The elevated FLZ MIC in mrr1Δ strains was MDR1 dependent, as the FLZ MIC was even lower in U04 mrr1Δ mdr1Δ than in U04 mrr1Δ (Fig. S1A). The increase was not due to introduction of the selectable marker NAT1, which encodes a nourseothricin acetyltransferase (31), as expression of NAT1 from an intergenic site in the FLZ-sensitive U05 strain did not alter the FLZ MIC (Fig. S1C). These data led us to hypothesize that some Mrr1 variants (Mrr1-Y813C) caused constitutively high MDR1 expression, while other Mrr1 variants (both Mrr1-ancestral and the recently diverged Mrr1-L1191H+Q1197*) repressed the expression of at least some Mrr1-controlled genes, such as MDR1. Indeed, the RNA-Seq analysis identified six genes, including MDR1, that while positively regulated when Mrr1 was constitutively active, were more highly expressed in U04 mrr1Δ than in those strains encoding low-activity Mrr1 variants (Fig. 1D and S2B). These data suggest that, for a small subset of Mrr1-regulated genes, including MDR1, low-activity Mrr1 variants may directly or indirectly inhibit expression. Cap1 (Clug_02670), another transcription factor known to regulate MDR1 in C. albicans (32, 33), was not responsible for the increase in MDR1 expression in the absence of MRR1, as deletion of CAP1 from U05 mrr1Δ did not alter the FLZ MIC of this strain (4 to 8 μg/ml, n = 3).

Premature termination had varied effects on Mrr1 activity and inducibility in clinical isolates. Each of the 20 sequenced C. lusitaniae isolates contained MRR1 alleles with either one or two nonsynonymous mutations relative to MRR1ancestral (Fig. 1A), and we found that C. lusitaniae isolates with two mutations in MRR1 had a significantly lower average FLZ MIC than isolates with a single MRR1 mutation (Fig. 2A). Interestingly, six of
the seven MRR1 alleles in the “two-mutation” set had premature stop codons, resulting in the loss of 34 to 906 amino acids (Fig. 2B). There were two instances in which the same mutation was found in combination with different nonsense mutations (e.g., MRR1 Y1126N1 P1174P (t) or MRR1 Y1126N1 S359* and MRR1 R1066S1 L927 (K912N (t)) or MRR1 R1066S1 Y1061* (common mutations in bold) (Fig. 1A). This suggested a complex evolutionary history for these alleles.

To better understand the effects of mutations in MRR1 on Mrr1 activity, we analyzed the effects of a chemical inducer of Mrr1 activity, benomyl (24, 34, 35), on MDR1 expression. Benomyl strongly induced MDR1 expression in an Mrr1-dependent manner in the FLZ-sensitive strain ATCC 42720 (Fig. 2C) and, to a lesser extent, in the FLZ-resistant strain U04, which had high basal MDR1 expression (Fig. 2D). Quantitative reverse transcription-PCR (qRT-PCR) analysis of MDR1 expression and induction by benomyl in
this collection of clinical isolates with different Mrr1 variants found that the two isolates with the lowest basal MDR1 expression and lowest FLZ MIC (U05 and U07) had the greatest induction by benomyl (34- and 27-fold, respectively) (Fig. 2D), suggesting that despite the loss of 68 or 89 amino acids, respectively, they encoded functional but low-activity Mrr1 variants. Three isolates, L11, L12, and U06, had intermediate FLZ MICs and MDR1 expression levels but did not show benomyl induction, similar to mrr1Δ strains (Fig. 2C and D). These strains all encoded Mrr1 variants lacking greater than 200 amino acids, suggesting that these mutations caused a loss of Mrr1 function. Isolate L16, which encoded two mutations in Mrr1 but only lacked 34 amino acids from the C terminus, phenocopied strains with a single mutation in MRR1, such as U04, L10, L17, and U08, suggesting either removal of a C-terminal regulatory region or that the mutation not causing premature termination increased Mrr1 activity (Fig. 2D).

Premature stop codons repeatedly arose in constitutively active Mrr1 variants and caused either a loss of constitutive Mrr1 activity or a complete loss of Mrr1 function. In light of the mixed effects that these two-mutation MRR1 alleles had on Mrr1 activity, we sought to determine the individual effects of mutations within each allele with a focus on the two strains with the lowest basal MDR1 expression and the strongest induction of MDR1 in response to benomyl, MRR1-Y1126N*Q1197* (in U05) (Fig. 3A) and MRR1-Y1126N*P1174P(t) (in U07) (Fig. 3B). We found that the MRR1-Y1126N*Q1197* mutation caused a 32-fold increase in FLZ MIC (Fig. 3C) and 22-fold increase in MDR1 expression (Fig. 3D) compared to that for MRR1 ancestral, indicating that, like the Mrr1-Y813C variant, Mrr1-L1191H was constitutively active. In contrast, MRR1-Q1197*, which caused the loss of 68 amino acids from the C terminus of Mrr1, did not significantly alter the FLZ MIC compared to that for the MRR1 ancestral allele, suggesting that it was neither a constitutively activating nor null mutation (Fig. 3C). The combination of mutations in MRR1-Y1126N*Q1197* resulted in a 128-fold decrease in FLZ MIC (Fig. 3C) and 38-fold decrease in MDR1 expression (Fig. 3D) compared to that for a strain expressing MRR1 ancestral, phenotypes that mirrored the strain expressing MRR1 ancestral.

MRR1-Y1126N*P1174P(t) (from U07) and MRR1-Y1126N*S359* (from the closely related U06) (Fig. 1A), were similarly analyzed (Fig. 3B). Expression of MRR1-Y1126N in U04 mrr1Δ caused high FLZ resistance (MIC of 32 to 64 μg/ml) (Fig. 3C) and MDR1 expression (Fig. 3D), similar to that for strains with MRR1 Y813C or MRR1 L1191H. Addition of the frameshift-inducing indel at P1174, which causes a premature stop codon at N1176 removing 89 amino acids, yielding MRR1-Y1126N*P1174P(t), caused a 128-fold decrease in FLZ MIC and >100-fold decrease in MDR1 expression relative to that for the strain expressing MRR1 ancestral again leading to a strain that phenocopied one expressing MRR1 ancestral (Fig. 3C and D). The addition of the indel at P1174 to an allele with a different constitutively active variant, creating MRR1-Y1126N*P1174P(t), similarly caused 256- and >100-fold decreases in FLZ MIC and MDR1 expression, respectively, relative to that for a strain expressing the MRR1 Y813C allele (Fig. 3C and D). Further RNA-Seq analysis of U04 mrr1Δ+MRR1 Y813C+P1174P(t) showed decreased expression of many genes positively regulated by Mrr1 (Fig. 1D). In contrast, addition of a SNP causing an early stop codon at S359 to the allele with the activating Y1126N mutation (MRR1-Y1126N*S359*) yielded a strain that phenocopied U04 mrr1Δ, indicating this variant, lacking 906 amino acids from the C terminus, was inactive (Fig. 3C and D). Together, these data show that the Y1126N mutation, which arose first, caused constitutive Mrr1 activity that was subsequently suppressed by premature stop codons that either restored low activity (P1174P(t)) or eliminated activity (S359*).

In addition to the differences in basal activity, the individual mutations alone and in combination affected chemical inducibility by benomyl. Levels of MDR1 were strongly induced by benomyl in U04 mrr1Δ+MRR1 ancestral (40-fold increase) but not in the U04 parental strain with high Mrr1 activity or its mrr1Δ derivative (Fig. 3D). The three constitutively active variants (Mrr1-Y813C, Mrr1-L1191H, and Mrr1-Y1126N) showed only a 2- to 3-fold increase in MDR1 expression with benomyl (Fig. 3D), similar to what was observed for FLZ-resistant clinical isolates (Fig. 2D). Surprisingly, addition of mutations that caused premature stop codons within the last 100 amino acids of Mrr1 to the
Premature stop codons repeatedly arose in constitutively active Mrr1 variants resulting in reduced Mrr1 activity but, in some cases, restored Mrr1 inducibility. Schematic of inferred evolution of MRR1 alleles in the L1191H (A) and Y1126N (B) lineages. (C) FLZ MICs for U04, U04 mrr1Δ and MRR1 complemented strains in the U04 mrr1Δ background. Means ± SDs from three independent assays on different days shown. Each sample was statistically compared to every other sample; the same lowercase letters indicate samples that are not significantly different, and different lowercase letters indicate significant differences (P < 0.01) as determined by one-way ANOVA with Tukey’s multiple-comparison test of log2-transformed values. (D) MDR1 expression normalized to ACT1 from culture grown in YPD (bars, left y axis). Means ± SDs from three independent assays on different days; data from each day were normalized to the expression of U04 mrr1Δ MRR1 ancestral. Each sample was statistically compared to every other sample; the same lowercase letters indicate samples that are not significantly different, and different lowercase letters indicate significant differences (b to d, P < 0.05; all other pairwise comparisons, P < 0.01) as determined by one-way ANOVA with Tukey’s multiple-comparison testing of log2-transformed values. (E) MDR1 expression following exposure to 50 μg/ml benomyl (squares, right y axis); full data presented with statistics in Fig. S3D. (C and D) FLZ MICs and MDR1/ACT1 expression data are colored to match; the sample names are shown on the x axis of panel D. Comparison of mean basal MDR1 (E), MGD1 (F), or FLU1 (G) expression from panel D and Fig. S3B or C, respectively, excluding strains lacking functional MRR1, and mean ± SD log2-transformed FC of the induction following benomyl exposure from Fig. S3D to F; circles colored to match those in panel C. Goodness of fit r² value for nonlinear regression shown.
constitutively active variants restored inducibility by benomyl (Fig. 3D). In fact, there was a significant inverse correlation between basal MDR1 expression and fold induction by benomyl (Fig. 3E).

As in C. albicans, C. lusitaniae Mrr1 regulates the expression of the methylglyoxal reductase encoded by MGD1 (CLU_01281 or GRP2) (10, 24, 29, 36, 37) and the multidrug efflux pump encoded by FLU1 (CLU_05825) (10, 29, 38, 39) (Table S1 and Fig. S3A). As with MDR1, expression of both MGD1 and FLU1 was significantly higher in strains encoding constitutively active variants than in a strain encoding the Mrr1 ancestral variant, and the absence of the C terminus in strains with activating mutations caused a significant decrease in basal expression (Fig. S3B and S3C). Benomyl induction of MGD1, like that of MDR1 (Fig. S3D), was restored upon loss of the C terminus from constitutively active Mrr1 variants, further supporting the strong negative correlation between basal expression and benomyl inducibility (Fig. 2F and Fig. S3E).

Expression of FLU1, however, was not induced by benomyl in any strain, suggesting that FLU1 regulation by Mrr1 differs from MGD1 and MDR1 regulation (Fig. 2G and Fig. S3F). Furthermore, MDR1 and MGD1 were both derepressed in the absence of Mrr1 (Fig. 1D and Fig. S2B), while FLU1 was not and was only weakly differentially expressed between strains with constitutive and low Mrr1 activity (<2-fold) (Fig. S3C). Together these data indicate the C terminus of Mrr1 is required for constitutive expression of multiple Mrr1-regulated genes but not for benomyl induction of the Mrr1-regulated genes tested (Fig. S3A). Combined with the Mrr1 activity across clinical isolates (Fig. 2D), these data indicate that in some strains with constitutively active Mrr1 variants, there was repeated selection for mutations to decrease Mrr1 activity, resulting in a mixed population containing constitutively active, prematurely terminated but inducible and loss-of-function (LOF) Mrr1 variants.

Constitutive Mrr1 activity negatively impacts H2O2 resistance. We next sought to understand why mutations that reduce Mrr1 activity might repeatedly arise in this chronic infection. Previous studies have shown that overexpression of drug efflux pumps in drug-resistant microbes can cause a fitness defect due to the energetic cost of constitutive pump production and activity in the absence of a selective substrate (40–42). Deletion of MDR1 from U04 mrr1Δ+ MRR1Y813C, which constitutively expresses MDR1, however, did not alter the growth kinetics (Fig. 4A). In the absence of an obvious growth defect, we considered factors present in the CF lung, which has been characterized as a highly inflamed environment containing elevated levels of neutrophils, macrophages, and oxidative stress (reviewed in references 43 and 44). Although little is known about fungus-dominated chronic lung infections in CF, such as the infection from which these isolates were obtained, an analysis of cytokines within the bronchoalveolar lavage (BAL) fluid from the patient these isolates originated from showed proinflammatory cytokines (interleukin 8 [IL-8] and IL-1β) present were consistent with the neutrophilic environment seen in other patients with CF (Fig. 4B) (44).

In light of these findings, we investigated the effects of Mrr1 activity on reactive oxygen species (ROS) stress generated by H2O2, a stress strongly associated with high neutrophil counts. In a serial dilution assay, we found that isogenic strains harboring constitutively active Mrr1 variants, while resistant to FLZ and diamide (Fig. S4A), had increased sensitivity to 4 mM H2O2 compared to that of one harboring the Mrr1 ancestral variant (Fig. 4C). Diamide was used to illustrate relative Mrr1 activity instead of FLZ, because serial dilution assays on rich medium (yeast-peptone-glucose [YPD]) containing FLZ are not always representative of FLZ MIC, which are assessed in defined medium (Fig. S4B). Secondary mutations resulting in either a phenotype associated with low Mrr1 activity that is inducible or a complete loss of Mrr1 activity both restored H2O2 resistance to levels similar to that of strains harboring the Mrr1 ancestral variant (Fig. 4C; Fig. S4A). The effects of Mrr1 activity on H2O2 sensitivity were similar among isogenic strains in both the U04 and U05 backgrounds (Fig. 4C and Fig. S4B). Surprisingly, deletion of MDR1 from strains harboring the constitutively active Mrr1-Y813C variant partially rescued growth (Fig. S4A); however, the absence of MDR1 did
Constitutive Mrr1 activity decreases resistance to H$_2$O$_2$ in multiple Candida species. (A) Growth curve of U04 mrr1Δ + MRR1$^{Y913C}$ (teal) and U04 mrr1Δ + MRR1$^{Y913C}$ mdr1Δ (black) grown at 37°C in YNB medium supplemented with the indicated carbon source: glucose (circles), amino acids (triangles), or glycerol (squares). Means from representative data acquired in triplicates shown. (B) Quantification of cytokines IL-8 and IL-1β in BAL fluid from the CF patient with (red) or seven patients without (black) C. lusitaniae in their lungs. Two-way ANOVA with Sidak’s multiple-comparison testing found no significant differences. (C) Serial dilution assays of C. lusitaniae, C. albicans, and C. dubliniensis strains on YPD or YPD supplemented with the indicated concentration of diamide or H$_2$O$_2$. Strain names in bold font were shown to contain GOF mutations in Mrr1 resulting in increased FLZ resistance (Fig. 3C and references 39, 45, and 46). Plates were imaged after 24 or 48 h of growth at 37°C, as indicated. (D) Percent growth in well-aerated 5 ml YPD plus 1 mM H$_2$O$_2$ was calculated relative to that of the vehicle only control after 22 to 24 h growth at 37°C. These data represent six independent assays performed on different days. Significance determined by paired t test; **, P < 0.01.
not completely explain the differences, as strains lacking MRR1 had increased H\textsubscript{2}O\textsubscript{2} resistance despite elevated MDR1 expression (Fig. 4C). Additionally, the double mutant U04 mrr1\textDelta mdr1\textDelta did not have increased resistance to H\textsubscript{2}O\textsubscript{2} compared to that of U04 mrr1\textDelta (Fig. S4A) suggesting this may be a complex response. A secondary assay quantifying growth after \(~24\) h in liquid cultures containing 1 mM H\textsubscript{2}O\textsubscript{2}, though variable day to day, confirmed there was a reproducible difference in growth between strains harboring the low-activity Mrr1 ancestral and constitutively active Mrr1-Y813C variants (Fig. 4D). To determine if this phenomenon was unique to C. lusitaniae, we examined a set of isogenic C. albicans isolates (39) and in vivo- or in vitro-evolved Candida dubilienisis isolates (25) expressing MRR1 alleles containing GOF mutations. We found that for all C. albicans and C. dubilienisis strain sets tested, strains with high Mrr1 activity, which had increased FLZ (39, 45, 46) and diamide resistance, were more sensitive to H\textsubscript{2}O\textsubscript{2} than strains with low Mrr1 activity or lacking MRR1 (Fig. 4C). As in C. lusitaniae, deletion of MDR1 restored growth on H\textsubscript{2}O\textsubscript{2} in C. albicans (Fig. S4C). These data show that the Mrr1 activity-driven trade-off between FLZ and H\textsubscript{2}O\textsubscript{2} resistance is conserved across multiple Candida species.

A screen of isogenic C. lusitaniae strains for growth in various concentrations of 48 chemical compounds resuspended from the Biolog Phenotype MicroArray microplates (Fig. S5) supported our findings that constitutive Mrr1 activity can increase sensitivity to oxidative stress. When comparing strains harboring either the low-activity Mrr1 ancestral variant or the constitutively active Mrr1-Y813C variant, with either MDR1 intact or removed, we found there were minimal differences in growth in the medium used to resuspend the Biolog compounds (Fig. S5A), and many conditions caused less than a 25% difference in growth. Unsurprisingly, constitutive Mrr1 activity conferred Mdr1-dependent resistance to 12 compounds, including three triazoles (FLZ, propiconazole, and myclobutanil) (Fig. S5B and C). High Mrr1 activity also led to Mdr1-independent resistance to four additional compounds, including two additional azoles (3-amino-1,2,4-triazole and miconazole nitrate) (Fig. S5B and C). Eight compounds caused a largely Mdr1-independent decrease in growth in strains harboring the constitutively active Mrr1-Y813C variant: 6-azauracil, berberine, BAPTA, lithium chloride, aminacrine, sodium metasilicate, pentamidine isethionate, and potassium chromate (Fig. S5D). Though diverse, these compounds have been reported to have varied effects on microbial metabolism or respiration (47–53) and/or alter DNA/RNA integrity (54–59) directly or indirectly through oxidative damage. Interestingly, berberine and calcium inhibitors, such as BAPTA, have previously been shown to alter growth of antifungal-resistant Candida species (60–62). Strains lacking MRR1 or harboring the low-activity Mrr1-L1191H+Q1197* variant were not as sensitive to some of these compounds, supporting our findings that secondary mutations causing a decrease or loss of Mrr1 activity can restore resistance in some oxidative stress environments (Fig. S5D).

To gain insight into the mechanisms that lead to differences in oxidative stress resistance between strains with different levels of Mrr1 activity, we compared the gene expression profiles after a 30-min exposure to 0.5 mM H\textsubscript{2}O\textsubscript{2}, a partially inhibitory concentration. H\textsubscript{2}O\textsubscript{2} exposure had broad strain-independent effects on the transcriptome, altering expression of 786 genes (fold change [FC] \(\geq 2\), false-discovery rate [FDR] < 0.05), including increased expression of CLUG_04072, a homolog of C. albicans catalase (CAT1), which was previously shown to be important for the resistance of C. lusitaniae to H\textsubscript{2}O\textsubscript{2} (63) (see Fig. S6A and Table S2). While there were subtle differences in the H\textsubscript{2}O\textsubscript{2} response between strains expressing the constitutively active Mrr1-Y813C variant compared to U04 mrr1\textDelta MRR1ancestral, there were no clear patterns that would explain the difference in H\textsubscript{2}O\textsubscript{2} resistance (Fig. S6B). The majority of differences in gene expression were seen in the magnitude of induction of Mrr1-regulated genes by H\textsubscript{2}O\textsubscript{2}, a known inducer of Mrr1 in other species (24, 32) (Fig. S6B and C; Table S2). We analyzed the expression of homologs of oxidative stress response (OSR) genes previously characterized in C. albicans (Ca) and Saccharomyces cerevisiae (Sc) and found that there was not a significant Mrr1-dependent difference in basal or H\textsubscript{2}O\textsubscript{2}-induced expression of these genes (Fig. S6A). Genes
assessed included the OSR transcription factor encoded by CaCAP1 or ScYAP1, superoxide dismutase (SOD2, SOD4, and SOD6), enzymes involved in the thioredoxin (TSA1, TRX1, and TRR1) and glutathione (GPX and GSH1) systems, catalases, and OSR genes involved in carbohydrate metabolism and the DNA-damage response (64, 65). Further analysis is required to better understand the link between constitutive Mrr1 activity and H2O2 sensitivity; however, these data highlight that the sensitivity is not due to failure to induce an oxidative stress response but is more likely a consequence of Mrr1-regulated genes, such as MDR1.

Phenotype dynamics in chronic infection populations over time. In light of the evidence for complex evolution of MRR1 and the potentially advantageous phenotypes associated with both high and low Mrr1 activity, we sought to better understand the fraction of isolates with these Mrr1-associated traits over time. For this analysis we used arrayed C. lusitaniae populations isolated from one BAL procedure sample or from sputum collected from the same subject over 3 years, with the first time point approximately 6 months after the first clinical culture report of the high levels of “non-albicans Candida” as shown in Fig. 5A. When screening isolates for growth on FLZ (8 μg/ml) or H2O2 (4 mM), we found an inverse correlation between growth on FLZ and growth on H2O2 (Fig. 5A). It was uncommon for isolates to be uninhibited under both conditions.
conditions. Isolates from the early samples were predominately sensitive to FLZ (10) and resistant to H₂O₂. During and soon after the course of FLZ therapy (Sp1.5 and Sp2, respectively), however, there was an increase in isolates that were FLZ resistant and H₂O₂ sensitive (Fig. 5A). Subsequent samples from 2 years after the FLZ therapy was completed varied in the proportion of isolates that grew better on H₂O₂ and FLZ. Thus, over the course of 3 years, we repeatedly identified isolates that were either FLZ resistant and H₂O₂ sensitive, or vice versa, but not typically resistant to both, further supporting a trade-off between these phenotypes (Fig. 5B).

** DISCUSSION **

A population of *C. lusitaniae* isolates first described by Demers et al. (10) contained an unexpectedly large number of nonsynonymous mutations in the gene encoding the transcription factor Mrr1, suggesting that Mrr1 activity was under strong selective pressure *in vivo*. These MRR1 alleles contained either one or two nonsynonymous SNPs or indels (Fig. 1A), and isolates with one mutation had on average higher FLZ resistance than those with two nonsynonymous MRR1 mutations (Fig. 2A). Deconstruction of MRR1 alleles with two mutations revealed an evolutionary path in which an activating mutation arose first (e.g., Y1126N) and was followed by suppressing mutations, in the form of premature stop codons, that either restored low basal activity with retention of inducibility [e.g., P1174P(t)], or abolished Mrr1 activity altogether (S359*) (Fig. 3 and see Fig. S3 in the supplemental material). Our findings from the deconstruction of the MRR1Y1126N+P1174P(t) and MRR1Y1126N+S359* alleles (common mutation in bold) led us to propose that similar evolutionary processes likely occurred in lineages containing the L1191H and R1066S mutations (Fig. 1A). It is interesting to note that suppressor mutations only arose in alleles encoding activating mutations affecting the C terminus (Y1126N, L1191H, and R1066S) (see Fig. 2B) and not in alleles with activating mutations affecting the central regulatory domain (e.g., Y813C). This suggests that there may be functional differences between these two types of activating mutations that require future exploration or that these lineages experienced different local environments. Interestingly, a *Candida parapsilosis* strain was recently found to contain a central domain mutation and a premature stop codon (Mrr1P295L1Q1074*) similar to the alleles described above, suggesting that selection for and against elevated Mrr1 activity may also occur in other *Candida* species; however, it is not currently known how these individual mutations impact Mrr1 activity and FLZ resistance (66). While previous studies have identified GOF and LOF mutations in genes such as MRR1, TAC1, PDR1, UPC2, ERG3 and ERG11 that cause increased antifungal resistance (reviewed in references 67 and 68), few instances of sequential mutations in the same gene that cause first an increase then a decrease in activity have been described. Interestingly, multiple studies have shown that alteration in gene copy number, which can be achieved by partial to whole chromosome aneuploidy, can be selected for during stressful conditions and lost upon cessation of the stress, allowing for reversion to a "wild-type" phenotype (reviewed in references 68 and 69). These studies intriguingly parallel our own in that they show both positive and negative selection on a particular phenotype and can result in complex populations with mixed phenotypes. Demers et al. (10) characterized the aneuploidies present in the 20 clinical isolates utilized in these studies; thus far, gene copy number variation has not been determined to be a major driver of antifungal resistance in this *C. lusitaniae* population.

The RNA-Seq analysis of isogenic strains expressing different MRR1 alleles was consistent with prior studies and showed that *C. lusitaniae* Mrr1 both positively and negatively regulates gene expression (Fig. 1D), although further analysis is required to determine which genes are direct targets of Mrr1. Adding to previous studies in *C. lusitaniae* (10, 29) and *C. albicans* (24), we found that Mrr1 positively regulates 41 genes with a fold change of ≥2 and 102 genes with a fold change of ≥1.5 (FDR < 0.05).

Mrr1-induced genes include multiple MFS and ABC transporters (i.e., *MDR1, FLU1, CDR1*), methylglyoxal reductases (37), putative alcohol dehydrogenases, and a variety
of other putative metabolic genes (Table S1). Constitutively active Mrr1 also appears to repress expression of 42 genes (fold change $\geq 1.5$, FDR < 0.05), including multiple iron and/or copper transporters and reductases and sugar transporters (Table S1). These data combined with those from Biermann et al., who showed that *C. lusitaniae* Mrr1 is induced by the spontaneously formed stress signal methylglyoxal (37), imply that Mrr1 may play a larger role in a generalized metabolic or stress response beyond what has been previously studied in response to FLZ and xenobiotic stressors. We propose that potential metabolic differences may account for slight strain-to-strain variability in FLZ MIC when expressing the same MRR1 variants, such as the subtle difference seen in the U04 and U05 backgrounds when expressing MRR1$^{\text{ancestral}}$ and MRR1$^{L1191H=Q1197*}$ (Fig. 1B and C).

The analysis of isogenic and nonisogenic strains showed that the C-terminal region of *C. lusitaniae* Mrr1 is necessary for constitutive Mrr1 activity but not required for induction of Mrr1-regulated genes, such as MDR1 and MGD1, in response to benomyl (Fig. 2D, 3, and S3). Mutations resulting in the loss of $\geq200$ amino acids, however, caused strains to phenocopy mrr1$\Delta$ strains (Fig. 2D and 3C and D). These data are consistent with previous studies showing C-terminal truncations prior to amino acid 944 in *C. albicans* MRR1, homologous to position 1116 in *C. lusitaniae* MRR1, caused a complete loss of *C. albicans* Mrr1 activity (34). Surprisingly, LOF Mrr1 variants and mrr1$\Delta$ strains showed intermediate expression of a subset of the most strongly differentially regulated genes compared to that of strains with low-activity Mrr1 (Fig. S2B), which has not been observed in other *Candida* species (24, 26, 28). Elevated MDR1 expression in strains lacking functional Mrr1 caused the unexpectedly high FLZ resistance (Fig. S1A). Though not specifically noted, a slight increase in FLZ resistance was also reported by Kannan et al. (29) upon deletion of MRR1 from their FLZ-sensitive *C. lusitaniae* isolate P1, supporting our conclusion that this phenomenon spans diverse *C. lusitaniae* isolates. Additional studies are required to determine if this phenomenon is unique to *C. lusitaniae* or is more broadly shared among non-albicans *Candida* species closely related to *C. lusitaniae*, such as *C. auris* (20, 70). Furthermore, while we have shown that the increase in MDR1 expression in mrr1$\Delta$ strains is Cap1 independent, additional analyses are required to determine the involvement of other coregulators of the Mrr1 regulon previously described in *C. albicans* (32, 71, 72) and determine if these relationships are conserved in *C. lusitaniae*.

The repeated acquisition of a second mutation in alleles encoding constitutively active Mrr1 variants raised the question as to why, if constitutive Mrr1 was initially selected for, would it later be selected against in vivo. From previous studies, it is clear that constitutive Mrr1 activity can be beneficial under a variety of biologically relevant conditions, including exposure to azoles (10, 24), bacterium-produced toxins, including phenazines (10), and host-produced antifungal peptides, including histatin 5 (10, 39). However, little is known about why constitutively active Mrr1 variants would be selected against if there is not a growth defect (Fig. 4A and Fig. S5A). Here, we explored conditions relevant to a chronic lung infection, such as the one these isolates originated from (Fig. 4B). Chronic lung infections are typically an inflamed environment containing a high number of polymorphonuclear leukocytes (PMNs) that produce proteases, myeloperoxidases, and ROS (73, 74), which are important components of the immune system used to kill fungi (reviewed in reference 75). In a screen of diverse chemical compounds, we found that strains with constitutive Mrr1 activity were more strongly inhibited by multiple compounds that have previously been shown to cause damage through oxidative stress (Fig. S5B to D). When we specifically interrogated H$_2$O$_2$ resistance, we found that *C. lusitaniae* strains harboring constitutively active Mrr1 variants were more sensitive than strains harboring low-activity Mrr1 variants (Mrr1 ancestral and Mrr1 variants lacking <100 amino acids from the C terminus) or lacking a functional Mrr1 (Fig. 4A and S4). Sensitivity to H$_2$O$_2$, and the compounds from the Biolog plates was at least partially dependent on Mdr1, though other Mrr1-regulated genes, such as those involved in metabolism, may also contribute to the decreased
growth under conditions of oxidative stress (Fig. S4B, S5, and S6). Interestingly, the trade-off between FLZ and H$_2$O$_2$ resistance was conserved in other Candida species and among a time series of C. lusitaniae isolates (Fig. 4C and 5A).

As outlined in the model in Fig. 5B, together, these data highlight that changing environments within complex and dynamic chronic infections could contribute to the development of heterogeneous fungal populations. Though it appears that the initial selection on the ancestral version of MrR1 was driven by the need for increased MrR1 activity, over time, either these selective pressures were removed or other pressures became dominant, resulting in a second mutation in some alleles. In most cases, this secondary wave of mutations caused a decrease or loss of MrR1 activity that further contributed toward a population with mixed levels of FLZ resistance (Fig. 5B). Although the exact selective pressures at play in this instance are unknown, these data highlight the importance of understanding how microbes evolve in vivo, as complex environments, even in the absence of clinically used antifungals, can shape the microbial population and lead to antimicrobial resistance.

MATERIALS AND METHODS

**Strains and growth conditions.** Candida strains used in this study are listed in Table S3 in the supplemental material. All strains were stored as frozen stocks with 25% glycerol at −80°C and subcultured on YPD (1% yeast extract, 2% peptone, 2% glucose, 1.5% agar) plates at 30°C. Strains were regularly grown in YPD liquid medium at 30°C on a roller drum. Cells were grown in YNB (0.67% yeast nitrogen base medium with ammonium sulfate [RPI Corp.]) liquid supplemented with either 2% glucose, 2% glycerol, or 2% Casamino Acids and in RPMI 1640 (Sigma; containing l-glutamine, 165 mM morpholinepropanesulfonic acid [MOPS], 2% glucose) liquid where noted. Medium was supplemented with 8 μg/ml FLZ (stock 4 mg/ml in dimethyl sulfoxide [DMSO]), 1 mM diamide (stock 58 mM in water), or 1 to 6 mM H$_2$O$_2$ (30% [wt/vol] in water, −9.8 M) where noted. Escherichia coli strains were grown in LB with either 100 μg/ml carbenicillin or 15 μg/ml gentamicin as necessary to obtain plasmids. BAL fluid and sputum were obtained in accordance with institutional review board protocols as described in reference 76.

**DNA for gene knockout constructs.** Gene replacement constructs for knocking out MRR1 (CLUG_00542, as annotated in reference 10), MDR1 (CLUG_01938/9, as annotated in reference 10), and CAP1 (CLUG_02670) were generated by fusion PCR as described by Grahl et al. (63). All primers (IDT) used are listed in Table S4. Briefly, 0.5 to 1.0 kb of the 5′ and 3′ regions flanking the gene was amplified from U04 DNA, isolated using the MasterPure yeast DNA purification kit (Epicentre). The codon-optimized nourseothricin (NAT1 [77]) or hygromycin (HygB) resistance cassette was amplified from plasmids pNAT (78) and pYM70 (79), respectively, using the Zyppey plasmid miniprep kit (Zymo Research). Nested primers within the amplified flanking regions were used to stitch the flanks and resistance cassette together. PCR products for transformation were purified and concentrated with the Zymo DNA Clean & Concentrator kit (Zymo Research) with a final elution in molecular biology-grade water (Corning).

**DNA for insertion of NAT1 at neutral site in C. lusitaniae genome.** The approximately 4,000-bp genomic region between CLUG_03302 and CLUG_03303 on chromosome 4, which was not predicted to contain any genes or promoter regions, was targeted as a potentially neutral insertion site. To create plasmid pDH3261 containing NAT1 flanked by homology to this region of chromosome 4, approximately 1.0 kb of the flanking regions (positions 228,652 to 229,651 and 229,701 to 230,691) was amplified from U05 genomic DNA (pDNA). All primers (IDT) used are listed in Table S4. NAT1 was amplified from pNAT (78). PCR products were purified, concentrated, and then assembled with the vector (pRS426 [80]) linearized with KpnI-HF and SalI-HF (New England BioLabs) using the NEBuilder HiFi DNA assembly cloning kit (New England BioLabs). Assemblies were transformed into high-efficiency NEL 5-alpha competent E. coli (New England BioLabs). The NAT1 insertion construct was isolated from DH3261 by digestion with KpnI-HF and SalI-HF (New England BioLabs).

**Plasmids for complementation of MRR1.** Plasmids for complementing MRR1 were created as described by Biermann et al. (37). For naturally occurring MRR1 alleles, we amplified (i) the MRR1 gene and terminator with −1,150 bp upstream for homology from the appropriate strain’s genomic DNA, (ii) the selective marker, HygB, from pYM70 (79), and (iii) −950 bp downstream of MRR1 for homology from genomic U05 (identical sequence for all relevant strains) using primers (IDT) listed in Table S4. PCR products were cleaned up using the Zymo DNA Clean & Concentrator kit (Zymo Research) and assembled into pMQ30 using the S. cerevisiae recombination technique as previously described (81). Plasmids created in S. cerevisiae were isolated using a yeast plasmid miniprep kit (Zymo Research) and transformed into high-efficiency NEL 5-alpha competent E. coli (New England BioLabs). E. coli containing pMQ30 derived plasmids were selected for on LB containing 15 μg/ml gentamicin. Plasmids from E. coli were isolated using a Zyppey plasmid miniprep kit (Zymo Research) and subsequently verified by Sanger sequencing at the Dartmouth College Genomics and Molecular Biology Shared Resources Core. Plasmids pMQ30(MRR1) complementation plasmids were linearized with NotI-HF (New England BioLabs), cleaned up with the Zymo DNA Clean & Concentrator kit (Zymo Research), and eluted in molecular biology-grade water (Corning) before transformation of ~2 μg into C. lusitaniae strain U04 mrr1Δ or U05 mrr1Δ, as described below.
The MRR1\textsuperscript{ancestral} allele sequence was amplified from gDNA of a closely related C. lusitaniae isolate (8, 10) that has the same MRR1 sequence but lacked any of the nonsynonymous mutations that varied among the population of C. lusitaniae isolates described here (Fig. 1A). This MRR1 sequence contains multiple synonymous and nonsynonymous mutations in comparison with that of the reference strain, ATCC 42720 (82). Additional MRR1 alleles were amplified from gDNA from U04 (MRR1\textsuperscript{Y813C}), U05 (MRR1\textsuperscript{L1191H-Q1197*}), U02 (MRR1\textsuperscript{L1191H-Q1197*+Y717M}) and U06 (MRR1\textsuperscript{S359T+Y712A}). While making the pMQ30\textsuperscript{mrr1\textsuperscript{ ancestral}-S359T+Y712A} plasmid, one clone was identified that lacked the S359\textsuperscript{T} mutation, resulting in the pMQ30\textsuperscript{mrr1\textsuperscript{ ancestral}} plasmid. To create additional MRR1 alleles that were not identified within any C. lusitaniae isolates, pieces of MRR1 were selectively removed and repaired with DNA either containing or lacking the desired mutation. Because the L1191H and Q1197\textsuperscript{*} mutations were close together, an alternate strategy was used to separate these mutations. DNA fragments synthesized by IDT containing either the L1191H or Q1197\textsuperscript{*} mutations alone (sequences in Table S4) were amplified then assembled with pMQ10\textsuperscript{mrr1\textsuperscript{ ancestral}-L1191H-Q1197*} (linearized with PvuI-HF) using the NEBuilder HiFi DNA assembly cloning kit (New England BioLabs). To remove an unexpected nonsynonymous mutation in pMQ30\textsuperscript{MRR1\textsuperscript{ ancestral}-Q1197*}, this plasmid was digesting with EcoNI and repaired with a piece of DNA amplified from U04 mrr1\textsuperscript{Δ}+MRR1\textsuperscript{ ancestral} lacking the unwanted mutation.

**Strain construction.** Mutants were constructed as previously described by Grahl et al. using an expression-free ribonucleoprotein CRISPR-Cas9 method (63). Briefly, 1 to 2 μg of DNA for gene knockout constructs generated by PCR or 2 μg of digested plasmid, purified, and concentrated with a final elution in molecular biology-grade water (Corning) was used per transformation. Plasmids containing complementation and knockout constructs and resulting strains are listed in Table S3 and CRISPR RNAs (crRNAs; IDT) are listed in Table S4. Transforms were selected on agar containing 200 μg/ml nourseothricin or 600 μg/ml hygromycin B.

**Drug susceptibility assays.** MIC was determined using a broth microdilution method as previously described (83) with slight modifications (10). Briefly, 2 × 10^5 cells were added to a 2-fold dilution series of FLZ prepared in RPMI 1640 medium, testing concentrations from 64 to 0.125 μg/ml, and then incubated at 35°C for 24 h. The MIC was defined as the drug concentration that abolished visible growth compared to that of a drug-free control.

**Quantitative RT-PCR.** Overnight cultures were back diluted to an optical density of 0.60 mm (OD\textsubscript{600}) of ~0.1 and grown for 6 h in YPD liquid medium at 30°C. Then, 50 μg/ml of benomyl (stock 10 mg/ml in DMSO) or an equivalent volume of DMSO was added for experiments assessing the induction of Mrr1 activity; 7.5 μg RNA (harvested using the MasterPure yeast RNA purification kit [Epicentre]) was DNase treated with the Turbo DNA-free kit (Invitrogen). cDNA was synthesized from 300 to 500 ng of DNase-treated RNA from each strain using the high first-strand cDNA synthesis kit (Thermo Scientific), according to the manufacturer’s instructions for random hexamer primer (IDT) and a GC-rich template. Quantitative RT-PCR was performed on a CFX96 real-time system (Bio-Rad), using SsoFast Evergreen supermix (Bio-Rad) with the primers listed in Table S4. Thermocycler conditions were as follows: 95°C for 10 min; 40 cycles of 95°C for 15 s, 60°C for 1 min. Results are summarized in Table S2, including the interaction between strains with low or no Mrr1 activity (U04 mrr1\textsuperscript{Δ}+MRR1\textsuperscript{Y813C}) and high or constitutive Mrr1 activity (U04 mrr1\textsuperscript{Δ}+MRR1\textsuperscript{ ancestral}).
constitutively active Mrr1 and H2O2 (Table S2A), the effect of H2O2 treatment (Table S2B), and all normalized CPM/gene used for this linear model (Table S2C). One U04 mrr1Δ and one U04 mrr1Δ ΔMRR1 ancestral replicate from the 0.5 mM H2O2 treatment condition were excluded from heat maps but not statistical analyses, because they displayed signatures not congruent with the rest of the data and did not cluster with other replicates from those strains.

**Biolog phenotype MicroArray analysis.** The chemicals in Biolog plates PM22D and PM24C were resuspended in 100 µl YPD liquid and transferred to a sterile 96-well polystyrene plate (Fisher). One hundred microliters of cells adjusted to an OD of 0.01 in YPD was added to each well. Plates were incubated at 37°C for 24 h. A control plate containing no drug was grown simultaneously for comparison.

**Plate-based chemical sensitivity assays.** (i) **Serial dilution assays.** Following growth in YPD medium overnight with aeration at 30°C, cells were washed and diluted in water to an OD600 of 1. Serial dilutions of 10-fold were carried out in a microtiter plate to yield six concentrations ranging from approximately 10⁶ cells/ml (for OD600 of 1) to approximately 10⁵ cells/ml. Five microliters of each dilution was applied to YPD plates containing the designated concentration of H2O2, FLZ, or diamide. Images were captured after incubation at 37°C for 24 or 48 h.

(ii) **C. lusitaniae population screen.** Individual isolates were collected from BAL fluid or sputum from the same subject over the course of multiple years; isolates from each sample were saved in a 96-well array format. Isolates were grown in YPD overnight and then transferred to a 384-well plate, with four wells representing each individual isolate. Cultures were spotted onto YPD or YPD supplemented with 8 µg/ml FLZ or 4 mM H2O2 using a 384-pin replicator; the screen performed in singlicate. Plates were incubated for 48 h at 37°C. Growth was scored by eye as completely inhibited, partially inhibited, or uninhibited relative to growth on the YPD-only control.

**Luminex analysis.** Cytokines in BAL fluid samples were measured (pg/ml) in singlicate by Luminex using a Millipore human cytokine multiplex kit (EMD Millipore Corporation, Billerica, MA) according to the manufacturer’s instructions. Assays were performed by the DartLab–Immune Monitoring and Flow Cytometry Resource core at Dartmouth.

**Statistical analyses.** Statistical analyses were performed using GraphPad Prism 9.0.0 (GraphPad Software). Unpaired Student’s t-tests (two-tailed) with Welch’s correction were used to evaluate the difference in FLZ MICs between isolates containing one of two mutations in MRR1. One and two-way analysis of variance (ANOVA) tests were performed across multiple samples with either Tukey’s multiple-comparison test for unpaired analyses or Sidak’s multiple-comparison test for paired analyses. P values of <0.05 were considered to be significant for all analyses performed and are indicated with asterisks or letters in the text as follows: *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001. In figures where the statistics are indicated by lowercase letters, samples marked by the same lowercase letter are significantly different from each other, and samples marked with different lowercase letters are significantly different, as detailed in the figure legends.

**Data availability.** The data supporting the findings in this study are available within the paper and its supplemental material and are also available from the corresponding author upon request. Whole-genome sequences for strains in Fig. 1A were previously published by Demers et al. (10) and can be found in NCBI under BioProject PRJNA433226. The sequence for MRR1 ancestral Sanger sequenced from isolate B_L06, is available in GenBank (MW553730). The raw sequence reads from the RNA-Seq analysis have been deposited into NCBI sequence read archive under BioProject PRJNA680763. Raw and processed RNA-Seq count data are available in Gene Expression Omnibus (GSE162151) and include minor updates to the genome annotation and assembly for C. lusitaniae.

**SUPPLEMENTAL MATERIAL**

Supplemental material is available online only.

**FIG S1** TIF file, 0.3 MB.

**FIG S2** TIF file, 0.4 MB.

**FIG S3** TIF file, 1.0 MB.

**FIG S4** PDF file, 2.4 MB.

**FIG S5** TIF file, 0.9 MB.

**FIG S6** TIF file, 1.4 MB.

**TABLE S1** XLSX file, 2.4 MB.

**TABLE S2** XLSX file, 6.2 MB.

**TABLE S3** DOCX file, 0.1 MB.

**TABLE S4** DOCX file, 0.1 MB.

**ACKNOWLEDGMENTS**

We thank Joachim Morschhäuser and Lawrence Myers for sharing strains, Katja Koeppen for assistance with linear modeling in edgeR, and Amy Biermann for primers.

Research reported in this publication was supported by National Institutes of Health (NIH) grant R01 AI127548 to D.A.H. from the National Institute of Allergy and Infectious Diseases, R01 HL122372 to A.A. from the National Heart, Lung and Blood Institute, and National Institute of General Medical Sciences (NIGMS) of the NIH under award number...
REFERENCES

1. Morley VJ, Woods RJ, Read AF. 2019. Bystander selection for antimicrobial resistance: implications for patient health. Trends Microbiol 27:864–877. https://doi.org/10.1016/j.tim.2019.06.004.

2. Holmes AH, Moore LS, Sundsfjord A, Steinbakk M, Regmi S, Karkey A, Guerin PJ, Piddock LJ. 2016. Understanding the mechanisms and drivers of antimicrobial resistance. Lancet 387:176–187. https://doi.org/10.1016/S0140-6736(15)00473-0.

3. Iwu CD, Korstj L, Quinn AI. 2020. The incidence of antibiotic resistance within and beyond the agricultural ecosystem: a concern for public health. Microbiologyopen 9:e1035. https://doi.org/10.1002/mbo3.1035.

4. Pisoschi AM, Pop A, Georgescu C, Turcuç V, Olah NK, Mathe E. 2018. An overview of natural antimicrobials role in food. Eur J Med Chem 143:922–933. https://doi.org/10.1016/j.ejmech.2017.11.095.

5. Challinor VL, Bode HB. 2015. Bioactive natural products from novel microbial sources. Ann N Y Acad Sci 1354:82–97. https://doi.org/10.1111/nyas.12954.

6. Winstanley C, O’Brien S, Brockhurst MA. 2016. Pseudomonas aeruginosa evolutionary adaptation and diversification in cystic fibrosis chronic lung infections. Trends Microbiol 24:327–337. https://doi.org/10.1016/j.tim.2016.01.008.

7. Lieberman TD, Michel JB, Aingaran M, Potter-Bynoe G, Roux D, Davis MR, McAdam AJ, Priebe GP, Kishony R. 2014. Genetic variation of a bacterial pathogen within individuals with cystic fibrosis and within multiple patient isolates identifies candidate pathogenicity genes. Nat Genet 43:1275–1280. https://doi.org/10.1038/ng.997.

8. Lieberman TD, Flett KB, Yelin I, Martin TR, McAdam AJ, Priebe GP, Kishony R. 2014. Genetic variation of a bacterial pathogen within individuals with cystic fibrosis provides a record of selective pressures. Nat Genet 46:82–87. https://doi.org/10.1038/ng.2846.

9. Ciofu O, Lee B, Johannesson M, Hermansen NO, Meyer P, Hoiby N. 2008. Investigation of the algT operon sequence in mucoid and non-mucoid Pseudomonas aeruginosa isolates from 115 Scandinavian patients with cystic fibrosis and in 88 in vitro non-mucoid revertants. Microbiology (Reading) 154:103–113. https://doi.org/10.1099/mic.0.2007.010421-0.

10. Demers EG, Biermann AR, Masonjones S, Crocker AW, Ashare A, Stajich JE, Hogan DA. 2018. Evolution of drug resistance in an antifungal-naïve chronic Candida lusitaniae infection. Proc Natl Acad Sci U S A 115:12040–12045. https://doi.org/10.1073/pnas.1807098115.

11. Chen SC, Marriott D, Playford EG, Nguyen Q, Ellis D, Meyer W, Sorrell TC, Slavin M. Australian Candidaemia Study. 2009. Candidaemia with uncommon Candida species: predisposing factors, outcome, antifungal susceptibility, and implications for management. Clin Microbiol Infect 15:662–669. https://doi.org/10.1111/j.1469-0691.2009.02821.x.

12. Favel A, Michel-Nguyen A, Peyron F, Martin C, Thomachat L, Datry A, Bouchara JP, Challer S, Noel T, Chastin C, Regli P. 2003. Colony morphology switching of Candida lusitaniae and acquisition of multidrug resist-

13. Asner SA, Giuliani S, Diezi M, Marchetti O, Sanglard D. 2015. Acquired multidrug antifungal resistance in Candida lusitaniae during therapy. Antimicrob Agents Chemother 59:7715–7722. https://doi.org/10.1128/AAC.02204-15.

14. Michel RG, Kinasewitz GT, Drevets DA, Levin JH, Warden DW. 2009. Prosthetic valve endocarditis caused by Candida lusitaniae, an uncommon pathogen: a case report. J Med Case Rep 3:7611. https://doi.org/10.1186/1752-1947-3-7611.

15. Pietrucca-Dilanchian P, Lewis RE, Ahmad H, Lechin AE. 2001. Candida lusitaniae catheter-related sepsis. Ann Pharmacother 35:1570–1574. https://doi.org/10.1348/aph.1.A0177.

16. Desnos-Ollivier M, Moquet O, Chouaki T, Guérin AM, Dromer F. 2011. Development of echinocandin resistance in Clavispora lusitaniae during caspofungin treatment. J Clin Microbiol 49:2304–2306. https://doi.org/10.1128/JCM.00325-11.

17. Hawkins JL, Baddour LM. 2003. Candida lusitaniae infections in the era of fluconazole availability. Clin Infect Dis 36:e14–e18. https://doi.org/10.1086/344651.

18. Atkinson BJ, Lewis RE, Kontoyiannis DP. 2008. Candida lusitaniae fungemia in cancer patients: risk factors for amphotericin B failure and outcome. Med Mycol 46:341–346. https://doi.org/10.1080/13693780801968571.

19. Reboutier D, Piconel N, Boisnard S, Conti A, Chevalier V, Florent M, Girot-Leclerc S, Da Silva B, Chastin C, Fallague K, Favel A, Noël T, Ruprich-Robert G, Chapeland-Leclerc F, Fapon N. 2009. Combination of different molecular mechanisms leading to fluconazole resistance in a Candida lusitaniae clinical isolate. Diagn Microbiol Infect Dis 63:188–193. https://doi.org/10.1016/j.diagmicrobio.2008.10.019.

20. Shen XX, Zhou X, Kominke J, Kurtzman CP, Hittinger CT, Rokas A. 2016. Reconstructing the backbone of the Saccharomycota yeast phylogeny using genome-scale data. G3 (Bethesda) 6:3927–3939. https://doi.org/10.1534/g3.116.034744.

21. Chow NA, Munoz JF, Gade L, Berkow EL, Li X, Welsh RM, Forsberg K, Lockhart SR, Adam R, Alainio A, Alastruey-Izquierdo A, Althawadi S, Arauz AB, Ben-Amri R, Bharat A, Calvo B, Desnos-Ollivier M, Escandon P, Gardam D, Gunturu R, Heath CH, Kurzai O, Martin R, Litvintseva AP, Cuomo CA. 2017. Tracking the evolutionary history and global expansion of Candida auris using population genomic analyses. mBio 11:e03364-19. https://doi.org/10.1128/mBio.03364-19.

22. Du H, Bing J, Hu T, Ennis CL, Nobile CJ, Huang G. 2020. Candida auris: epidemiology, biology, antifungal resistance, and virulence. PLoS Pathog 16: e1008921. https://doi.org/10.1371/journal.ppat.1008921.

23. Franz R, Kelly SL, Lamb DC, Kelly DE, Ruhne M, Morschhäuser J. 1998. Multiple molecular mechanisms contribute to a stepwise development of fluconazole resistance in clinical Candida albicans strains. Antimicrob Agents Chemother 42:3065–3072. https://doi.org/10.1128/AAC.42.12.3065.

24. Morschhäuser J, Barker KS, Liu TT, Bla BWJ, Homayouni R, Rodgers PD. 2007. The transcription factor Mmr1p controls expression of the MDR1 efflux pump and mediates multidrug resistance in Candida albicans. PLoS Pathog 3:e164. https://doi.org/10.1371/journal.ppat.0030164.
25. Schubert S, Rogers PD, Morshchauser J. 2008. Gain-of-function mutations in the transcription factor MRR1 are responsible for overexpression of the MDRI efflux pump in fluconazole-resistant Candida dubliniensis strains. Antimicrob Agents Chemother 52:4274–4280. https://doi.org/10.1128/AAC.00740-08.

26. Dunkel N, Blass J, Rogers PD, Morshchauser J. 2008. Mutations in the multi-drug resistance regulator MRR1, followed by loss of heterozygosity, are the main cause of MDRI overexpression in fluconazole-resistant Candida albicans strains. Mol Microbiol 69:827–840. https://doi.org/10.1111/j.1365-2958.2008.06008.x.

27. Zhang L, Xiao M, Watts MR, Wang H, Fan X, Kong F, Xu YC. 2015. Development of fluconazole resistance in a series of Candida parapsilosis isolates from a persistent candidemia patient with prolonged antifungal therapy. BMC Infect Dis 15:340. https://doi.org/10.1186/s12879-015-0866-6.

28. Branco J, Silva AP, Silva RM, Silva-Dias A, Pina-Vaz C, Butler G, Rodrigues AM, Miranda IM. 2015. Fluconazole and voriconazole resistance in Candida parapsilosis is conferred by gain-of-function mutations in MRR1 transcription factor gene. Antimicrob Agents Chemother 59:6629–6633. https://doi.org/10.1128/AAC.00842-15.

29. Kannan A, Asner SA, Trachsel E, Kelly S, Parker J, Sanglard D. 2019. Comparative genomics for the elucidation of multidrug resistance in Candida lusitaniae. mbio 10:e02512-19. https://doi.org/10.1128/mbio.02512-19.

30. Iyer KR, Camara K, Daniel-Ivad M, Trilles R, Daniel-Ivad M, Trilles R, Fossen JL, Krugel H, Fiedler G, Haupt I, Sarfert E, Simon H. 1988. Analysis of the nourseothricin resistance gene (nat) of Streptomyces noursei. Gene 62:209–217. https://doi.org/10.1016/0378-1119(88)90559-8.

31. Schubert S, Barker RC, Naidzi S, Schneider S, Schneider S, Dierolf F, Dunkel N, Aid M, Boucher G, Rogers PD, Raymond M, Morschhauser J. 2011. Regulation of efflux pump expression and drug resistance by the transcription factors Mrr1, Upc2, and Cap1 in Candida albicans. Antimicrob Agents Chemother 55:2212–2223. https://doi.org/10.1128/AAC.01343-10.

32. Rognon B, Kozovska Z, Coste AT, Pardini G, Sanglard D. 2006. Identification of promoter elements responsible for the regulation of MDR1 from Candida albicans, a major facilitator transporter involved inazole resistance. Mol Microbiol (Reading) 52:3701–3722. https://doi.org/10.1099/mic.0.29277-0.

33. Schubert S, Popp C, Rogers PD, Morshchauser J. 2011. Functional dissection of a Candida albicans zinc cluster transcription factor, the multidrug resistance regulator Mrr1. Eukaryot Cell 10:1110–1121. https://doi.org/10.1128/EC.05100-11.

34. Rognon B, Kozovska Z, Coste AT, Pardini G, Sanglard D. 2006. Comparison of genome expression profiles of Candida albicans azole-resistant clinical isolates and laboratory strains exposed to drugs inducing multidrug transporters. Antimicrob Agents Chemother 48:3064–3079. https://doi.org/10.1128/AAC.48.8.3064-3079.2004.

35. Kwak MK, Ku M, Kang SO. 2018. Inducible NAD(H)-linked methylglyoxal oxidoreductase regulates cellular methylglyoxal and pyruvate through enhanced activities of alcohol dehydrogenase and methylglyoxal-oxidizing enzymes in glutathione-depleted Candida albicans. Biochim Biophys Acta Gen Subj 1862:18–39. https://doi.org/10.1016/j.bbagen.2017.10.003.

36. Biermann AR, Demers EG, Hogan DA. 2020. Mrr1 regulation of methylglyoxal oxidoreductase regulates cellular methylglyoxal and pyruvate through enhanced activities of alcohol dehydrogenase and methylglyoxal-oxidizing enzymes in glutathione-depleted Candida albicans. Biochim Biophys Acta Gen Subj 1862:18–39. https://doi.org/10.1016/j.bbagen.2017.10.003.

37. Biemann AR, Demers EG, Hogan DA. 2020. Mrr1 regulation of methylglyoxal oxidoreductase and methylglyoxal-induced fluconazole resistance in Candida lusitaniae. Mol Microbiol 115:115–130. https://doi.org/10.1111/mmi.14604.

38. Calabrese D, Bille J, Sanglard D. 2000. A novel multidrug efflux transporter gene of the major facilitator superfamily from Schizophyllum commune. Antimicrob Agents Chemother 44:213–219. https://doi.org/10.1128/AAC.44.1.213-219.2000.

39. Hampe IAI, Edgerton J, Edgerton M, Morshchauser J. 2017. An acquired mechanism of antifungal drug resistance simultaneously enables Candida albicans to escape from intrinsic host defenses. PLoS Pathog 13:e1006655. https://doi.org/10.1371/journal.ppat.1006655.

40. Alonso A, Martinez JL. 2001. Expression of multidrug efflux pump SmoDEF by clinical isolates of Stenotrophomonas maltophilia. Antimicrob Agents Chemother 45:1879–1881. https://doi.org/10.1128/AAC.45.6.1879-1881.2001.

41. Prasinski Albrigt L, Hussey DL, Brandis G, Hughes D. 2017. Fitness cost constrains the spectrum of mdr mutations in ciprofloxacin-resistant Escherichia coli. J Antimicrob Chemother 72:3016–3024. https://doi.org/10.1093/jac/dkx270.
74. Brown RK, Kelly FJ. 1994. Evidence for increased oxidative damage in *Candida albicans*: absence of a general stress response. Mol Biol Cell 1:4140–4147. https://doi.org/10.1091/mbc.06-08-0546.

69. Gilchrist C, Stelkens R. 2019. Aneuploidy in yeast: segregation error or adaptation mechanism? Yeast 36:525 and/or 132F to K143R in Turkey. Antimicrob Agents Chemother 64:e010001-20. https://doi.org/10.1128/AAC.010001-20.

66. Arastehfar A, Daneshnia F, Hilmio D, Rigolli P, Coen Y, Irikura M, Pan W, Liao W, Hagen F, Kostrzewa M, Perlin DS, Lass-Flörl C, Boekhout T. 2020. First report of candidemia clonal outbreak caused by emerging fluconazole-resistant *Candida parapsilosis* isolates harboring Y132F and/or Y132F production of a constitutive Mrr1 activity. *nobio.asm.org*