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Evolution of drug resistance in an antifungal-naive chronic Candida lusitaniae infection

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The limited number of available antimicrobials necessitates strategies to better enable their judicious use in appropriate cases to prevent further development of drug resistance (1). The ability to determine drug susceptibility in single-species infections can be complicated by heterogeneity within the infecting population. Heterogeneity in drug resistance can result from coinfections by phylogenetically distinct strains with allelic differences that affect drug sensitivities (2, 3). However, the diversification of microbes within chronic infections may be an even more important driver of allelic heterogeneity and the development of drug resistance differences. Analysis of Helicobacter pylori isolates from ulcers or Mycobacterium tuberculosis isolates from the lung have shown that isolates derived from the same strain can have different levels of antimicrobial resistance (4, 5). Diversification of bacteria within chronic lung infections associated with the genetic disease cystic fibrosis (CF) has also been shown to lead to heterogeneous drug resistances within the population (6–8).

The discovery of a CF patient with a high burden chronic fungal infection containing phenotypically heterogeneous isolates provided the opportunity to analyze fungal population structure. These analyses led us to discover a striking, and possibly unprecedented, level of heterogeneity in the sequence of a single drug resistance-related gene among the haploid Clavispora (Candida) lusitaniae isolates that are otherwise genetically similar. Unlike some of the diploid Candida species, such as Candida albicans, which are common members of the human microbiome, C. lusitaniae is most often isolated from environmental samples. Like other Candida species, C. lusitaniae can cause both acute and long-term infections (9). C. lusitaniae is particularly notorious for its rapid development of resistance to multiple antifungals during therapy (10–12) and is phylogenetically closely related to Candida auris (13), multidrug-resistant strains of which have caused outbreaks in recent years (14).

Our work shows the presence of a complex, dynamic, and structured population of C. lusitaniae within a single infection. Through the analysis of over 300 C. lusitaniae isolates from a single patient we observed heterogeneity in fluconazole (FLZ) resistance and found that this heterogeneity was largely caused by the presence of at least 12 different alleles of MRR1, which encodes a drug-resistance regulator. The enrichment of non-synonymous mutations in MRR1 greatly exceeded the heterogeneity at any other locus. Here we have explored factors that may have contributed to the selection for drug-resistant populations in heterogeneous single-species infections.

Candida | drug resistance | evolution | fungi | heterogeneity

Significance

Drug-resistant subpopulations of microbes or tumor cells are difficult to detect but can confound disease treatment. In this deep characterization of a chronic fungal infection, we report unprecedented heterogeneity in the drug-resistance-related gene MRR1 among Clavispora (Candida) lusitaniae isolates from a single individual. Cells expressing Mrr1 variants that led to drug resistance, by elevated expression of the MDR1-encoded efflux protein, were present at low levels in each sample and thus were undetected in standard assays. We provide evidence that these drug-resistant fungi may arise indirectly in response to other factors present in the infection. Our work suggests that alternative methods may be able to identify drug-resistant subpopulations and thus positively impact patient care.


The authors declare no conflict of interest.

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Data deposition: Reads for genome sequences and RNA sequencing data have been deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (accession no. SRP133092). Data are also available through GitHub (available at https://github.com/stajichlab/C_lusitaniae_popseq). All sequence files related to this work can be found in NCBI (BioProject PRJNA433236). Whole Genome Shotgun project de novo assemblies are deposited in NCBI (accession nos. OORB00000000–OORB00000000).

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subpopulations and highlight the importance of assessing and treating fungal populations during the management of disease.

**Results**

*C. lusitaniae* Coisolates Are Phenotypically and Genotypically Heterogeneous. Analysis of bronchoalveolar lavage (BAL) fluid from a subject with CF detected >10^7 cfu/mL of *C. lusitaniae* in both the right upper lobe (UL) and right lower lobe (LL) of the lung with very few coinfecting bacteria (subject 6 in ref. 15, subject A here). *C. lusitaniae*, while rarely encountered in CF, has been detected in CF respiratory sputum previously (16). The patient history revealed non-albicans Candida species were detected in sputum cultures collected 6 mo before the BAL, suggesting that *C. lusitaniae* may have been present for a prolonged period. *C. lusitaniae* isolates recovered from UL and LL BAL fluid (n = 74 and 68 isolates, respectively; Fig. 1A) varied in colony color when grown on the chromogenic medium CHROMagar Candida (17, 18), indicating isolates differed in enzymatic activities (Fig. 1B). Additional *C. lusitaniae* isolates were obtained from an archived sputum sample (Sp1, n = 82 isolates) collected 1 mo before the BAL, confirming the persistence of *C. lusitaniae* in the lung for at least 1 mo. The Sp1 coisolates were also phenotypically heterogeneous on CHROMagar Candida medium (Fig. 1B).

We performed whole-genome sequencing (WGS) for 20 isolates from the UL, LL, and Sp1 samples (n = 7, 9, and 4 isolates, respectively) chosen to represent different CHROMagar phenotypes. Genomic analysis indicated that the isolates were more closely related to each other than to other sequenced strains, ATCC 42720 and CBS 6936, and thus shared a recent common ancestor (Fig. 1C). Pairwise analyses of the 20 clinical isolate genomes found 24–131 SNPs between any two isolates, with 404 high-confidence interisolate SNPs (45% nonsynonymous) in total (SI Appendix, Fig. S1 and Dataset S1). Similarly, 76–179 insertions or deletions (INDELs) differed between any two isolates, with 536 INDELs in total. The INDELs were primarily short, with >60% being less than 3 nt in length and 80% being intergenic (SI Appendix, Fig. S1 and Dataset S2). Phylogenetic analysis using either SNPs or INDELs found similar relationships between isolates (Fig. 1C and SI Appendix, Fig. S2). Isolates did not cluster by the lavage or sputum sample of origin, indicating that genomic heterogeneity was not solely explained by spatial separation (UL vs. LL) or population changes over time (Sp1 vs. BAL). Additionally, phylogenetic analysis of SNPs and INDELs did not cluster isolates by colony color on CHROMagar Candida medium, suggesting this is likely a complex trait which we will not explore further here.

Although it is possible that some genomic heterogeneity was present within the original infecting inoculum, analysis of copy number variation has suggested that the genomes of these isolates were changing within the context of this infection (SI Appendix, Fig. S3A). We identified a duplication of chromosome 6R in the four Sp1 isolates obtained at the earlier time point, which suggests it may have been ancestral, that was repeatedly lost in UL and LL isolates from separate clusters within the cladogram (SI Appendix, Fig. S3B). Overall, however, the number of differences between the isolates is much smaller than that which is reported for differences between fungal strains within a species (19, 20) (Fig. 1C). Thus, these data indicate that these coisolates are recently diverged and are evolving within the infection in ways that lead to phenotypic heterogeneity.

**Allelic Heterogeneity in MRRI Confers Differences in FLZ Resistance.** Evaluation of the number of mutations per gene found that the most heterogeneous locus among the 20 sequenced isolates was CLUG_00542, an ortholog of *C. albicans* MRR1 (SI Appendix, Fig. S4). CLUG_00542 is referred to hereafter as MRR1. Among the 20 *C. lusitaniae* clinical isolate genomes we found that MRR1 contained 13 nonsynonymous SNPs and two INDELs, but no synonymous SNPs, within 12 distinct MRR1 alleles (Figs. 1C and 2 A and B). No other gene contained more than two non-synonymous SNPs and only a few genes contained more than two INDELs (Fig. 24 and Datasets S1 and S2). Phylogenetic analysis suggested that eight of the MRR1 alleles arose independently and the remaining four alleles had two mutations (two SNPs or one SNP and one INDEL) that arose sequentially (Fig. 1C). Alignment of *C. lusitaniae* MRR1 with *C. albicans* orthologs in other Candida species revealed that the SNPs and INDELs generally fell within regions of moderate to high protein sequence conservation (Fig. 2B). The absence of mutations in MRR1-adjacent genes indicated that this locus was not in a hypervariable region of the genome, and the absence of synonymous SNPs in MRR1 indicated that the sequence heterogeneity was not solely due to high rates of nucleotide substitution (Datasets S1 and S2). Together, these data suggest that MRR1 was under strong selection. Heterogeneity in MRR1 is of interest because prior studies in other Candida species have shown that constitutively active variants of the Mrr1 transcription factor can be selected for during azole therapy and are capable of conferring FLZ resistance (21–23). In this case, there was no history of antifungal

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is a known target of FLZ and increased expression of ERG11 has been shown to increase FLZ resistance in other Candida species (27). Thus, this duplication may explain the slightly elevated FLZ MIC of U02 [MRR1]\^{Δ}L10] relative to other isolates with the same MRR1 allele (U07 and L15) (SI Appendix, Fig. S6A).

To assess the impact of differences in Mrr1 on the transcriptome, we performed an RNA sequencing (RNA-Seq) analysis and identified 19 differentially expressed genes between representative FLZ\(^2\) isolates, with Mrr1 variants Y813C (U04), Y813N (L10), or H467L (L17), and FLZ\(^2\) isolates, with Mrr1 variants L1191H\(^{+}\)Q1197\(^{+}\) (U05) or Y1126N\(^{+}\)P1174P\(^{tr}\) (U07) (SI Appendix, Table S1). One of the most differentially expressed genes was MDR1 (CLUG\(_{1938}\)Q1939 [SI Appendix, Fig. S8]) or MFS7 (28), which encodes an ortholog of the C. albicans Mdr1 multidrug efflux transporter. In C. albicans, Mdr1 contributes to the elevated FLZ MIC in strains with constitutively active Mrr1 variants (22, 29, 30). Quantitative RT-PCR analysis found that MDR1 expression was similar among isolates, with the same MRR1 allele and again significantly higher in FLZ\(^{2}\) isolates than in FLZ\(^2\) isolates (Fig. 2D; \(P \leq 0.01\)). FLZ MIC correlated with MDR1 levels across all isolates (SI Appendix, Fig. S6C). MDR1 was deleted from isolates with either high Mrr1 activity (U04, MRR1\(^{Δ}L10\)) or low Mrr1 activity (U05, MRR1\(^{Δ}L10\)Q1197\(^{+}\)). Deletion of MDR1 in U04 caused a sixfold decrease in FLZ MIC and a 26-fold decrease in the MIC for cerulenin, another substrate of C. albicans Mdr1 (31). In contrast, deletion of MDR1 in U05 did not alter the MIC for these compounds (Fig. 2E). These data indicate that Mdr1 activity differs between isolates and can contribute to FLZ\(^2\) in C. lusitaniae.

In addition to MDR1, the C. lusitaniae Mrr1 regulon contained CLUG\(_{02968}\) (Cao19r.7306), CLUG\(_{01281}\), and CLUG\(_{04991}\) (putative methylgluxol reductases with sequence similarity to CaGRP2) and multiple putative oxidoreductases (SI Appendix, Table S1). Some of the C. albicans orthologs and genes with similar predicted functions are also regulated by C. albicans Mrr1 (30). MRR1 itself was not differentially expressed between the resistant and susceptible isolates.

High Mrr1 Activity Confers Resistance to Host and Microbial Factors. Although MRR1 appeared to be under positive selection within the C. lusitaniae population in the lung, even in the absence of antifungal treatment, this gene did not appear to be under strong selection in the laboratory (SI Appendix, Fig. S9). Passage strains with either high (L17, MRR1\(^{Δ}L10\)) or low (U05, MRR1\(^{Δ}L10\)Q1197\(^{+}\)) FLZ resistance in a defined medium did not yield populations with significantly different FLZ resistance profiles. Thus, we proposed that Mrr1 variants that confer high Mdr1 activity could have been selected for in response to immune system components or by factors produced by confecting microbes. Prior data have indicated that high Mdr1 activity in C. albicans contributes to resistance to histatin 5 (Hst 5), a peptide secreted by the salivary glands as part of the innate immune system (32, 33). We found that deletion of either MRR1 or MDR1 from the FLZ\(^{2}\) U04 (MRR1\(^{Δ}L10\)) isolate reduced survival in the presence Hst 5 by twofold (Fig. 3A; \(P < 0.001\)), confirming the necessity of Mrr1 and Mdr1 for Hst 5 resistance. In addition to host defenses, the lungs of patients with CF are typically polymicrobial environments filled with molecules produced by a variety of bacteria (15, 34). Phenazines, produced by the common CF pathogen P. aeruginosa, are known to inhibit the growth and metabolism of some Candida species (35) and can be found at high concentrations in CF sputum (36). We found a role for high Mrr1 activity and concomitant high MDR1 expression in protection against these phenazine toxins. Deletion of either MRR1 or MDR1 from the FLZ\(^{2}\) isolate U04 (MRR1\(^{Δ}L10\)) increased the zone of clearance around phenazine-producing P. aeruginosa colonies (Fig. 3B; \(P < 0.001\)). The U04 clinical isolate and its mrr1Δ and mdr1Δ derivatives grew equally well in the presence of P. aeruginosa colonies that could not produce phenazines (Fig. 3B). Analysis of all 20 clinical isolates revealed an inverse correlation between zone of

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**Fig. 2.** Multiple nonsynonymous SNPs in MRR1 increase FLZ resistance via up-regulation of MDR1 expression. (A) Number of nonsynonymous (blue) and synonymous (red) SNPs within each gene. (B) Schematic of Mrr1 depicting the locations of the amino acid changes caused by the 13 nonsynonymous SNPs and two INDELs. Mrr1 is represented by a heat map of sequence conservation, described in SI Appendix, Fig. S4, with increased conservation represented by a gradient from white (dark gray) to white (red) colors. The color of the line marking the location of each mutation corresponds to the sequence conservation score of the affected amino acid. (C) Log-transformed FLZ MICs (micrograms per milliliter) of mating progeny, measured at 48 h, obtained by crossing the FLZ\(^{2}\) 2383 (MRR1\(^{Δ}L10\)) strain to FLZ\(^{2}\) clinical isolate L17 (Rx\(^{+}\), MRR1\(^{Δ}L10\), progeny \(n = 30\)) or U04 (Rx\(^{+}\), MRR1\(^{Δ}L10\), progeny \(n = 28\)); isolates are grouped by MRR1 allele. Red lines indicate the mean FLZ MIC for the parental strain for each MRR1 allele. Mean ± SD of three independent measurements are shown, \(*\*\*\cdot P < 0.0001\). (D) MDR1 expression (exp) for FLZ\(^{2}\) [MRR1\(^{Δ}L10\)H467L] and [MRR1\(^{Δ}L10\)Q1197\(^{+}\)] and FLZ\(^{2}\) (MRR1\(^{Δ}L10\) and MRR1\(^{Δ}L10\)) isolates with the same MRR1 allele (\(n = 3\), colored to match phylogenetic tree in Fig. 1C). MDR1 expression was normalized to ACT1 levels. Data represent the average of three independent replicates, a-b and c-d \(P < 0.01\). (E) FLZ and cerulenin (CER) MICs for WT (solid) and the isogenic mdr1A derivatives (striped) of the FLZ\(^{2}\) U04 (MRR1\(^{Δ}L10\)) and FLZ\(^{2}\) U05 (MRR1\(^{Δ}L10\)Q1197\(^{+}\)) isolates measured at 48 h. Mean ± SD for four independent replicates shown, \(*\*\*\cdot P < 0.0001\); ns, not significant.

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treatment, yet a fraction of the 224 BAL and SpI isolates exhibited high resistance to FLZ, but not to other classes of antifungals including polyenes and echinocandins (amphotericin B and caspofungin, respectively; SI Appendix, Fig. S5). Further analysis revealed that isolates with the MRR1\(^{Δ}L10\)H467L (S18, S20, and L17) and MRR1\(^{Δ}L10\)S (S19, L09, and L10) alleles had FLZ minimum inhibitory concentrations (MICs) of 16 μg/mL, which is clinically resistant and notably higher than the reported MIC\(_{\text{SI}}\) for C. lusitaniae (0.5 μg/mL) (24). In contrast, isolates with the MRR1(L1191H\(^{+}\)Q1197\(^{+}\) (S21, L14, and U05) and MRR1(L1191H\(^{+}\)P1174P\(^{tr}\) (L15, U02, and U07) alleles had FLZ MICs of 0.5–2 μg/mL (SI Appendix, Fig. S6A). Mating FLZ-resistant (FLZ\(^{2}\)) clinical isolates with an FLZ-sensitive (FLZ\(^{2}\)) strain of a distinct genetic background confirmed that Mrr1\(^{Δ}L10\)H467L and Mrr1\(^{Δ}L10\)S variants conferred a high FLZ MIC (Fig. 2C; \(P < 0.0001\)). No FLZ\(^{2}\) progeny were detected in a similar mating assay using an FLZ\(^{2}\) clinical isolate (SI Appendix, Fig. S7). Thus, the heterogeneity in MRR1 alleles created a C. lusitaniae population with heterogeneous levels of resistance to FLZ.

Other genes known to impact FLZ resistance in Candida species like UPC2 (CLUG\(_{39001}\)) and TAC1 (CLUG\(_{02369}\)) (26) did not contain SNPs or INDELs (Datasets S1 and S2). There was also no correlation between the chromosome 6R duplication, found in nine isolates, and FLZ MIC (SI Appendix, Fig. S3C). One isolate, U02 [MRR1\(^{Δ}L10\)P1174P\(^{tr}\)], had a duplication of the left arm of chromosome 6 where ERG11 (CLUG\(_{04932}\)) is located (SI Appendix, Fig. S3). Erg11, a lanosterol 14α-demethylase,
inhibition due to phenazines and either FLZ MIC or MDRI expression (SI Appendix, Fig. S6 D and E), suggesting Mrr1 activity correlates with phenazine resistance. Deletion of either MRR1 or MDRI does not alter other phenotypes including growth rate or colony color on CHROMagar medium (Fig. 3C). While we cannot know if either Hst 5 or phenazines selected for high Mrr1 activity in these C. lusitaniae isolates, these examples demonstrate how strains with high Mrr1 activity might be more fit in vivo even in the absence of azole drugs.

Isolates Containing Constitutively Active Mrr1 Variants Are Minor Members of the Population. We performed an unbiased pooled sequencing analysis of ~70 isolates from the UL, LL, and Sp1 samples (Fig. 1A). Single-isolate WGS data were used to establish thresholds for the identification of biallelic positions within the pooled WGS data (7% of reads per position for novel SNPs and 5% for previously confirmed SNPs) (Fig. 4A). In the PoolSeq data, there was striking heterogeneity in nucleotide frequency within the MRR1 locus relative to single-isolate WGS data (Fig. 4B). The MRR1-adjacent gene CLUG_00541 and other loci analyzed were not similarly heterogeneous (Fig. 4B).

Using the 5% threshold for previously confirmed SNPs, we found 11 of the 13 SNPs identified by single-isolate WGS within the pools. Of these, the MRR1 SNPs found in FLZ-resistant isolates (encoding H467L, E722K, Y813N, and Y813C Mrr1 variants) represented 18, 22, and 12% of the reads in the Sp1, UL, and LL pools, respectively (Fig. 4B). Although the respective ratios of each SNP changed between samples, SNPs indicative of FLZ-resistant isolates were present at low levels in all three samples, even in the absence of prior antifungal therapy. Using a more stringent threshold for the detection of novel SNPs, those not found among the 20 sequenced genomes, we identified four new SNPs in MRR1, all of which were nonsynonymous, further underscoring the level of heterogeneity at this locus (Fig. 4B).

Unrecognized FLZ-Resistant Subpopulations May Contribute to Treatment Failure. To complete the longitudinal analysis of this infection, we analyzed an additional sputum sample from this subject (Sp2) collected after a 4-mo course of oral FLZ treatment (started after the BAL sample was collected) and 5 mo with no prescribed antifungal therapy. Abundant C. lusitaniae were found at this time point, indicating either FLZ treatment failed to clear this infection or that recolonization occurred after FLZ therapy was completed. Among the 83 Sp2 isolates analyzed, we found a range of FLZ susceptibilities (2 to >32 μg/mL). The median FLZ susceptibility for isolates was >32 μg/mL (Fig. 4C), which was higher than for the Sp1 population (2 μg/mL; SI Appendix, Fig. S5A). Sequencing of MRR1 from nine isolates revealed that the fixed SNPs present in all Sp1 and BAL isolates relative to other genomes, such as ATCC 42720, were still present in Sp2 isolates (SI Appendix, Fig. S4), indicating a single-strain background was present at all three time points over these 10 mo. MRR1 alleles in Sp2 isolates encode Mrr1 variants different from those found in the Sp1 and BAL populations (Mrr1-K922E, K922E+N459H, F1123V, F1123Y, and E1122D+F1123L; Fig. 4C), suggesting that heterogeneity in the population either arose a second time or that multiple MRR1 alleles persisted despite FLZ treatment. We propose that while factors other than FLZ exposure lead to heterogeneity in MRR1 and thus a range of FLZ susceptibilities, the presence of isolates with high Mrr1 activity enabled the persistence of C. lusitaniae and perhaps the emergence of an even more resistant population upon FLZ treatment. It was interesting to observe three different variants of F1123 and additional alleles with two non-synonymous changes within MRR1.

Because routine clinical microbiological assessment of drug resistance profiles involves the analysis of only one or a few representative isolates from a clinical sample, it was not surprising that the small percentage (10–20%) of FLZ-resistant isolates within the BAL and Sp1 populations escaped detection. To aid in the identification of drug-resistant subpopulations, we propose the use of pools of isolates in MIC assays. The FLZ MIC for U04 (16–32 μg/mL) in a 24-h-endpoint assay was similar to that obtained for a 9:1 mixture of FLZ (U05, MRR1ΔY813KΔI122V) and FLZ (U04, MRR1ΔY813C), isolates, a U04 culture with a starting inoculum comparable to that in the 9:1 mixture (U04 10%), and a complex mixture of all UL isolates (Fig. 4D). Although it is surprising that a small subpopulation of drug-resistant isolates can be assessed by MIC within 24 h, further analysis of growth kinetics in the presence of FLZ showed that small resistant subpopulations within mixed populations, as in the 9(0.05):1 (U04) and mixture of all UL isolates samples, became detectable within 18 h (SI Appendix, Fig. S10 B and C). Thus, pooled analysis of isolates may be a method by which we can detect the presence of drug-resistant subpopulations before or early on in treatment, thereby decreasing the incidence of treatment failure.

Discussion
Understanding fungal population structure within chronic infections, and awareness of the potential for the development of antifungal resistance in the absence of selection by drug, may improve individual treatment strategies. Furthermore, the common practice of analyzing only one or two isolates per infection (37–39) or per timepoint within a patient (12, 40) may not be sufficient to appreciate the phenotypes and genotypes within a large population. The analysis of drug resistance at a population level may also be important for other fungal pathogens as azole resistance is found to be heterogeneous among isolates of Aspergillus fumigatus and C. albicans both in subjects without recent triazole treatment (41) as well as in individuals undergoing active azole treatment (42).

Azole antifungals are widely used for treatment and prophylaxis (43–45) and act by inhibiting Erg11, an enzyme essential for ergosterol biosynthesis (46). In prior reports of FLZ-resistant C. albicans, Candida parapsilosis and C. lusitaniae, the strains were isolated from patients who had received FLZ therapy (12, 40, 47). In cases where increased FLZ resistance is due to activation of Mrr1, only one mutated allele was found per patient, in contrast to the 17 alleles found here. The stable genomic diversity observed here, resulting in the up-regulation of MDRI through hyperactivation of Mrr1, was the main driver of heterogeneous levels of FLZ resistance between isolates. Although we identified one chromosomal duplication that included ERG11, other mechanisms of FLZ resistance including increased activity of the Tac1 transcription factor to up-regulate expression.
of CDRI/2-encoded ABC transporters and mutations that alter FLZ binding to Erg11 were not observed (reviewed in ref. 48).

Determining what stimuli within chronic infections can contribute to the selection for antifungal resistance, either through MRR1 or other factors, will enable strategies to decrease resistance development. For example, coinfection with phenazine-producing P. aeruginosa or chronic oral colonization (where Hst 5 is abundant) may be risk factors for emergent FLZ\textsuperscript{R} isolates. Fungal species frequently cocolonize with bacteria and thus the effects on drug resistance may be widespread. Here, we propose that the diversity of the C. lusitaniae population may have been a consequence of the long duration of the infection, spatial complexity within the lung, and the presence of diverse selective pressures imposed by the immune system or coinfecting microbes. These factors could have contributed to both the repeated selection for and persistence of cells with different Mrr1 variants, which led to heterogeneous drug resistance within a single infection. Other chronic infections, such as those associated with bronchiectasis, oropharyngeal candidiasis, biofilms on indwelling artificial surfaces, and fungus balls may also have sufficient temporal and spatial complexity to result in heterogeneous populations. Chronic colonization of host environments that promote the selection for isolates with azole resistance have the potential to contribute to the initial evolution of epidemic multidrug-resistant strains, such as those that arose in C. auris (14).

In light of the potential benefits of high Mrr1 activity in the CF lung, the presence of Mrr1 variants with C-terminal truncations and low Mrr1 activity in this in vivo population is perplexing. This may indicate that there are differences between Mrr1 activity assessment in vitro and activity in vivo, or that there is a cost for Mrr1 activity in some genetic backgrounds. Although we focused on the heterogeneity in MRR1 and its link to the heterogeneous levels of FLZ resistance within the infecting population, these isolates and data may provide insight into the activities of different naturally occurring Mrr1 variants as well as other genetic changes that occur in this naturally evolved yeast population. For example, the genome data from the single and pooled isolates from the Sp1 and the UL and LL BAL samples revealed SNPs in genes encoding proteins with similarities to bile acid carriers, proteins involved in calcium homeostasis, and proteins with domains associated with signaling or protein stability. Future studies on serial samples from chronic infections may reveal other loci that enable opportunistic pathogens to adapt to the human host or influence drug treatment.

**Materials and Methods**

Methods describing the growth conditions, genome sequencing and analysis, transcript sequencing and analysis, mutant construction, mating experiments, drug susceptibility assays, in vitro evolution, Hst 5 sensitivity assay, zone of inhibition by P. aeruginosa, and statistical analysis are described in detail in SI Appendix, Supplemental Materials and Methods. Data and code availability statements are also included in SI Appendix, Supplemental Materials and Methods.

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