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

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Management of the limited number of antimicrobials currently available requires the identification of infections that contain drug-resistant isolates and the discovery of factors that promote the evolution of drug resistance. Here, we report a single fungal infection in which we have identified numerous subpopulations that differ in their alleles of a single gene that impacts drug resistance. The diversity at this locus was markedly greater than the reported heterogeneity of alleles conferring antibiotic resistance in bacterial infections. Analysis of genomes from hundreds of Clavispora (Candida) lusitaniae isolates, through individual and pooled isolate sequencing, from a single individual with cystic fibrosis revealed at least 25 non synonymous mutations in MRR1, which encodes a transcription factor capable of inducing fluconazole (FLZ) resistance in Candida species. Isolates with high-activity Mrr1 variants were resistant to FLZ due to elevated expression of the MDR1-encoded efflux pump. We found that high Mrr1-regulated Mdr1 activity protected against host and bacterial factors, suggesting drug resistance can be selected for indirectly and perhaps explaining the Mrr1 heterogeneity in this individual who had no priorazole exposure. Regional analysis of C. lusitaniae populations from the upper and lower lobes of the right lung suggested intermingling of subpopulations throughout. Our retrospective characterization of sputum and lung populations by pooled sequencing found that alleles that confer FLZ resistance were a minority in each pool, possibly explaining why they were undetected before unsuccessful FLZ therapy. New susceptibility testing regimes may detect problematical drug-resistant subpopulations in heterogeneous single-species infections.

Candida | drug resistance | evolution | fungi | heterogeneity

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 genomically 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

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.
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 earliest 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 MRR1 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 2A and B). No other gene contained more than two nonsynonymous SNPs and only a few genes contained more than two INDELs (Fig. 2A 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 *Mrr1* 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 duringazole therapy and are capable of conferring FLZ resistance (21–23). In this case, there was no history of antifungal

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**Fig. 1.** *C. lusitaniae* coisolates are phenotypically and genomically heterogeneous. (A) Schematic of isolate acquisition from patient samples. Primary BAL fluid or sputum samples were plated. Numerous colonies from each plate were streak-purified and saved to represent the population within each sample. Following species identification by ITS1 sequencing, phenotypic and genomic analyses were performed for the indicated number of isolates per sample. (B) Phylogenetic distribution of *C. lusitaniae* isolate colony color on CHROMagar Candida medium, example of colony colors shown above graph. From left to right, colors include blue/green, brown/green, green, and purple/mauve. The Sp1 (sputum) sample was obtained approximately 1 mo prior (T = −1 mo) to the UL and LL BAL samples (T = 0). (C) Gray inset contains a maximum-likelihood tree for the 20 subject A clinical isolates compared with ATCC 42720 and CBS 6936. The expanded maximum-likelihood tree shows the relationship between subject A isolates based on interisolate SNPs found through WGS, and bootstrap values are shown at every branch point; arms are colored by *MRR1* allele. Isolate identifiers are color-coded by sample of origin: Sp1 (black), UL BAL (red), and LL BAL (blue). *MRR1* alleles are denoted by the amino acid changes caused by non-synonymous SNPs and INDELs; asterisk indicates stop codon. Amino acid numbers are based on the *MRR1* reannotation in SI Appendix, Fig. S4. One nucleotide INDELs in codons 1174 and 912 cause an amino acid change in the latter case and frame shift mutations that resulted in premature stop codons, noted by “tr” for truncation, at N1176 and L927, respectively.
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 non-synonymous 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 cool (dark blue) to warm (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\(^{2388}\) (MRR1\(^{2388}\)) strain to FLZ\(^{2}\) clinical isolate L17 (MRR1\(^{-}\), progeny \(n = 30\)) or U04 (MRR1\(^{+}\)), Mrr1 \(^{R}\), 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 \pm SD of three independent measurements are shown, \(****P < 0.0001\). (D) MDR1 expression (exp) for FLZ\(^{2}\) [MRR1\(^{2388}\)Q1197*] and FLZ\(^{2}\) [MRR1\(^{2388}\)] and MRR1\(^{2388}\) 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 significantly different, \(****P < 0.0001\). (E) FLZ CER for FLZ\(^{2}\) U04 (MRR1\(^{R}\)) and FLZ\(^{2}\) U05 (MRR1\(^{R}\)) isolates measured at 48 h. Mean \pm SD for four independent replicates shown, \(****P < 0.0001\); ns, not significant.

High Mrr1 Activity Confers Resistance to Host and Microbial Factors. Although MRR1 appeared to be under positive selection in 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\(^{2388}\)) or low (U05, MRR1\(^{1126N+P1174P}\)) 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 coinfecting 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\(^{1126N+P1174P}\)) isolate reduced survival in the presence Hst 5 by twofold (Fig. 34; \(P < 0.0001\)), 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 Pseudomonas 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 similar 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\(^{1126N+P1174P}\)) increased the zone of clearance around phenazine-producing P. aeruginosa colonies (Fig. 3B; \(P < 0.0001\)). The U04 clinical isolate and its mrr1\(^{Δ}\) and mdr1\(^{Δ}\) 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...
inhibition due to phenazines and either FLZ MIC or MDR1 expression (SI Appendix, Fig. S6 D and E), suggesting Mrr1 activity correlates with phenazine resistance. Deletion of either MRR1 or MDR1 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 ofazole 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. L4). 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 Pool-Seq 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 strains (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, red symbols). 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 encoded 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-endpoint assay was similar to that obtained for a 9:1 mixture of FLZ (U05, MRR1(Y1019G)Y1019G) and FLZ (U04, MRR1(Y1019G)) isolates, a U04 culture with a starting inoculum comparable to that of 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 U05(1):U04 (10%) and mixture of all UL isolates samples, became detectable within 18 h (SI Appendix, Fig. S10). Additionally, after 18 h of growth in FLZ, the culture starting at 9(U05):1(U04) was predominantly composed of U04 (MRR1(Y1019G)) cells (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 *A. 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 MDR1 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 *ERG11* and perhaps the emergence of an even more resistant population upon FLZ treatment.
of CDR1/2-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 <i>P. aeruginosa</i> or chronic oral colonization (where Hst 5 is abundant) may risk factors for emergent FLZ-resistant isolates. Fungal species frequently coexist with bacteria and thus the effects on drug resistance may be widespread. Here, we proposed that the diversity of the <i>C. lusitaniae</i> 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 coexisting 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 <i>C. auris</i> (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. These findings 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, 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 <i>P. aeruginosa</i>, 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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