High Throughput Screening of Random Aneuploidy Strain Library to Reveal Mul<-Drug Resistance and Tolerance in *Candida albicans*

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Abstract

Candida albicans is a common skin and gut microbe; however, hospitalized, and immunocompromised individuals are at elevated risk of developing candidemia, a serious bloodstream infecKon. Candidemia paKents experience high rates of treatment failure and mortality, which can be aNributed to drug resistance or tolerance. Drug tolerance is a disKnct to drug resistance and allows cells to grow slowly in the presence of drug; however, the mechanisms that govern drug tolerance remain poorly understood. We discovered that rapid genomic alteraKons, such as changes in chromosome number referred to as aneuploidy, prior to drug exposure promoted tolerance to two drugs belonging to a class of drugs called azoles. This project aims to invesKgate the comprehensive spectrum of aneuploidies that contribute to drug tolerance and determine the similariKes and differences in drug responses to various classes of anKfungal drugs. The experimental approach will include screening the diverse collecKon of aneuploid strains with representaKve drugs from different drug classes, then applying machine learning clustering algorithms to the screening dataset. Understanding the geneKc mechanisms underlying the physiological response to anKfungal drugs will yield novel insights into their roles in drug tolerance.

Background

Candida albicans account for most systemic bloodstream *Candida* infections with a high mortality and treatment failure rate between 40-50%.[1,2] Patients with candidemia systemic bloodstream *Candida* infection have a high rate of treatment failure and mortality attributed partly to the development of antifungal drug tolerance.[3,4] Drug tolerance, distinct from resistance and often overlooked in clinical assays, is a phenomenon in which strains with a drug-susceptible genotype exhibit slow growth in inhibitory drug concentrations.[4,5] The mechanisms governing drug tolerance remain poorly understood, as the contributions of genetic and physiological responses to antifungal drugs are uncertain. Antifungal drug tolerance relies on lowering intracellular drug concentration and activating stress response pathways that regulate cell wall and membrane integrity, protein translation, or other pathways to alleviate drug stress.[3,4,5] Whereas the evolution of drug resistance coincides with the accumulation of point mutations that directly affect the regulation of the drug target and drug efflux pumps.[3]

C. albicans has a repertoire of mechanisms that generate rapid and extensive genomic diversity and plasticity to adapt to environmental and drug stress. *C. albicans* undergo large scale genomic changes, such as aneuploidy and loss of heterozygosity events (LOH), at a much higher rate than single nucleotide mutations.[6] Typically, aneuploidy is unfavorable as the imbalance of gene copy number alters the global gene expression, resulting in cellular stress17 However, under various physiological stressors, certain aneuploidies confer with improved

growth and fitness, as *C. albicans* can tolerate trisomy of each chromosome.[7] Large-scale genomic changes can arise with and without stress, showcasing the level of genomic plasticity and adaptability under various conditions.

In parasexual reproduction diploid, having two copies of each of the eight chromosomes*, C. albicans* of opposite mating types fuse together and promotes mitotic crossing-over.[8] The newly formed tetraploid cell, having four copies of each chromosome, then undergoes concerted chromosome loss (CCL), which generates extensive and variable levels of genomic recombination.[9] CLL reduces the level of ploidy to near diploid levels and drives random aneuploidy and LOH events.[8] Parasexual reproduction results in phenotypic diversity as progeny displays both ranges in phenotypes and, in many cases, increases in virulence fluconazole resistance compared to parental strains.[10]

In contrast, when fungal cells are exposed to myriad stressors within their host, that can induce aneuploidy and LOH events to respond to drug stress. During an infection within a host, temperature, oxidative, or antifungal drug stress increases the rates of aneuploidy and LOH events.[11,12,13] Thus, widespread genomic changes happen frequently during adaptation to host related stresses. Early observations of aneuploidy driving drug resistance were found in clinical isolates containing an isochromosome 5L (i5L) segmental aneuploidy, which increases the copies of the drug target and drug efflux pumps.[14] In the absence of fluconazole, clinical isolates rapidly lose the characteristic i5L aneuploidy due to the gains in fitness.[14] The current understanding of the relationship and intersection between drug tolerance and the acquisition of aneuploidy has been through evolution experiments. In a study involving clinical isolates, most strains demonstrated temperature-enhanced tolerance and exhibited rapid emergence of tolerant colonies.[15] All the evolved progenitors with elevated tolerance had recurrent aneuploidy that included chromosome R, and the loss of the aneuploidy was associated with the loss of the tolerant phenotype.[15] The underlying activation of stress response genes is essential to drug tolerance, whereas the accumulation of aneuploidy accelerates the trajectory and dynamics of adaptation to drug stress. The regulation of tolerance may be partly due to overlapping stress response pathways. Experimental evolution in the presence of tunicamycin, inducer of endoplasmic reticulum stress, resulted in the acquisition of trisomy of Chr2, which in turn facilitated the development of cross-tolerance to the echinocandin, caspofungin, and the chemotherapy hydroxyurea.[16]

It is now understood that the evolution of drug tolerance and cross-tolerance relies on the activation of stress response pathways via exposure to drug stress, accompanied by the development of aneuploidy. Nonetheless, it remains unexplored whether pre-existing aneuploidy alone is sufficient to promote drug tolerance to antifungals, and we do not know the entire spectrum of aneuploidies and LOH events that contribute to the tolerance phenotype.

Preliminary Data

To begin to investigate these questions we constructed a diverse putative aneuploid strain library of 707 isolates by plating tetraploid strains on stress conditions known to induce chromosome loss. The generation and screening of the random aneuploid library at a low and

high concentration of fluconazole revealed a wide range of tolerant phenotypes ploidy among the two strain backgrounds and four stress conditions (Figure 1). Twenty strains exhibiting elevated minimum inhibitory concentration (MIC) or supra-MIC growth (SMG) compared to the diploid reference and parental tetraploid strain were then selected for flow cytometry ploidy analysis. Progenitors ranged in ploidy from near-diploid to near-tetraploid that were associated with fluconazole tolerance (Figure 1). Nine strains with a high degree of fluconazole tolerance were selected for whole genome sequencing to determine the exact aneuploidies and regions of LOH (Figure 2). Nine unique karyotypes were observed with aneuploidies of Chr2, Chr4, Chr5, Chr6, Ch7, and ChrR in various combinations with partial or whole chromosome LOH events (Figure 3). Lastly, to determine whether the highly fluconazole-tolerant strains were cross tolerant to other azoles, an itraconazole MIC assay was conducted. Tolerance to fluconazole generally correlated with higher tolerance to itraconazole, but LB46_sorbose_F7 displayed higher resistance and/or tolerance to certain azoles, when baring a trisomy of Chr4 and Chr6 and tetrasomy of Chr7 (Figure 2). Through parasex recombination and concerted chromosome loss, we determined that pre-existing aneuploidy is sufficient to promote fluconazole tolerance. This finding suggests that the tolerant-aneuploid phenotype might be sufficient to promote multi-drug resistance and tolerance to other antifungals. Furthermore, I propose to investigate the comprehensive spectrum of aneuploidies and LOH events that contribute to drug tolerance, and determining how similar and different it is to other antifungal drugs.

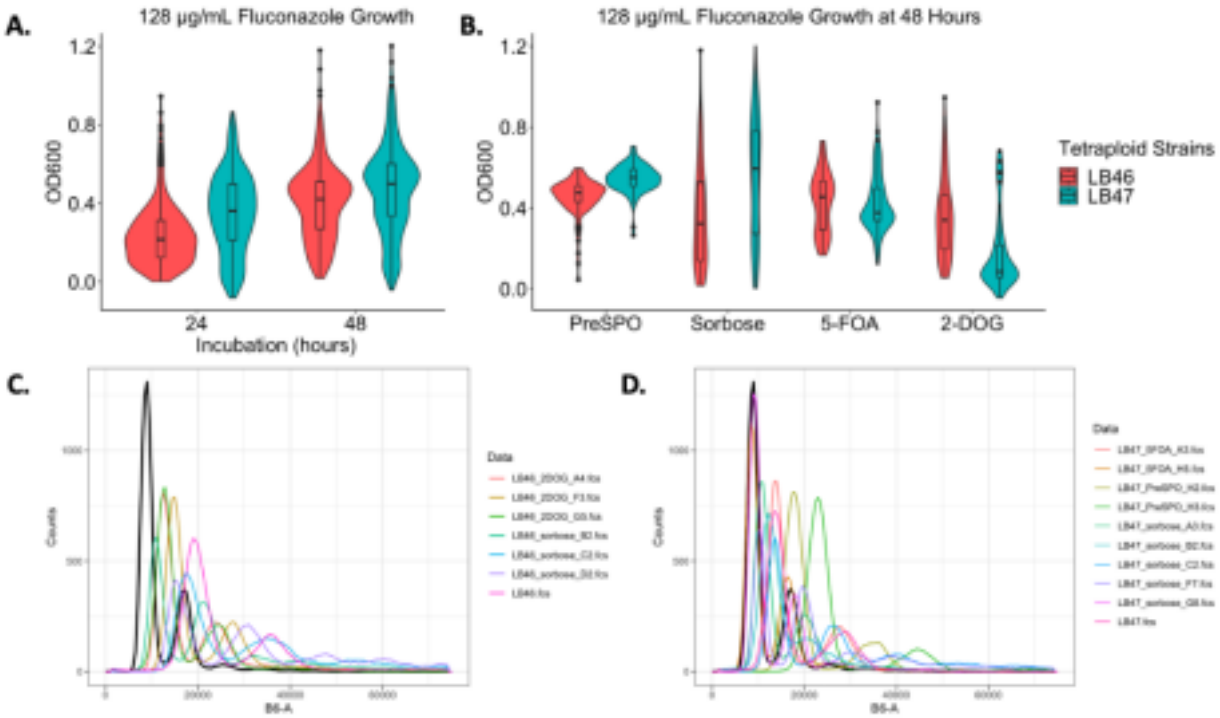


Figure 1: Screening of random aneuploid library revealed spectrum of tolerant phenotypes and levels of ploidy. The OD_{600nm} served as the proxy of growth. A) Growth of tetraploid progenitors at 128 µg/mL of fluconazole at 24 and 48 hours. B) Growth at at 128 µg/mL of fluconazole at 48 hours across stress condiKons. Flow cytometry was employed propidium iodine was conducted to measure the amount of DNA. Ploidy analysis of C) LB46 and D) LB47

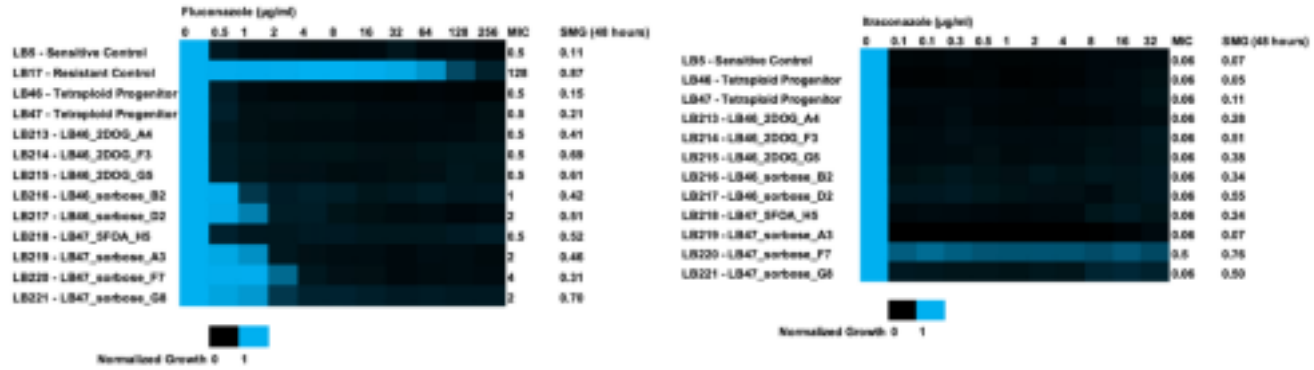


Figure 2: DeterminaKon of fluconazole minimum inhibitory concentraKon (MIC) and supra-MIC growth (SMG) revealed near-diploid progenitors displaying significant increase in fluconazole and itraconazole tolerance that were selected for whole genome sequencing. One strain exhibiKng significant level of resistance and tolerance to itraconazole. The MIC at 24 hours serves as the measure of resistance and the SMG at 48 serves as the measure of tolerance.

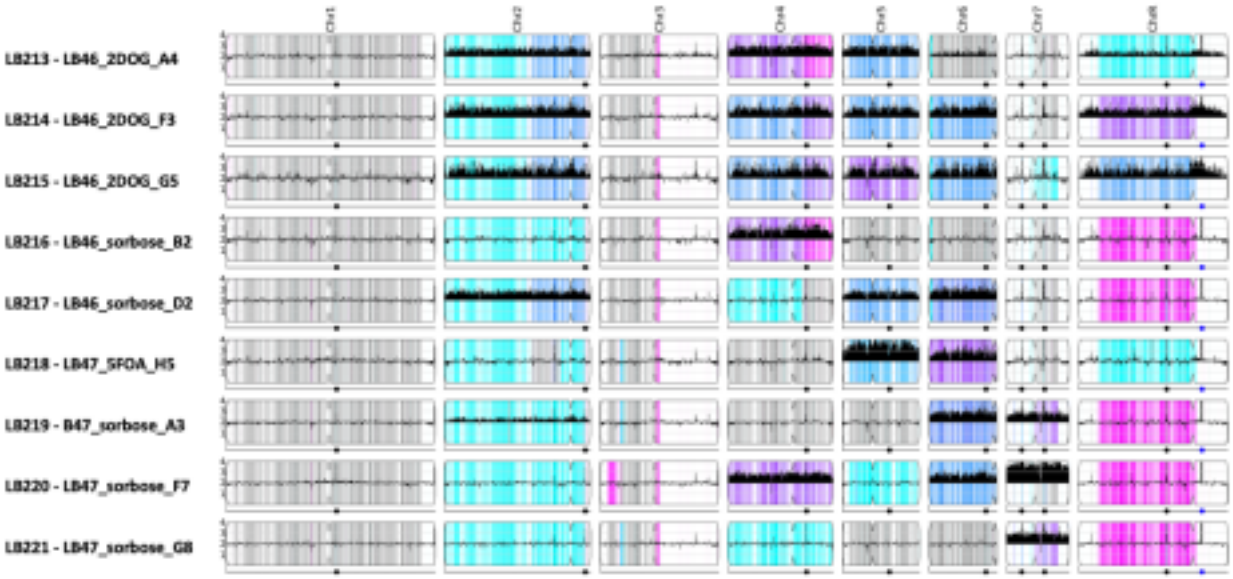


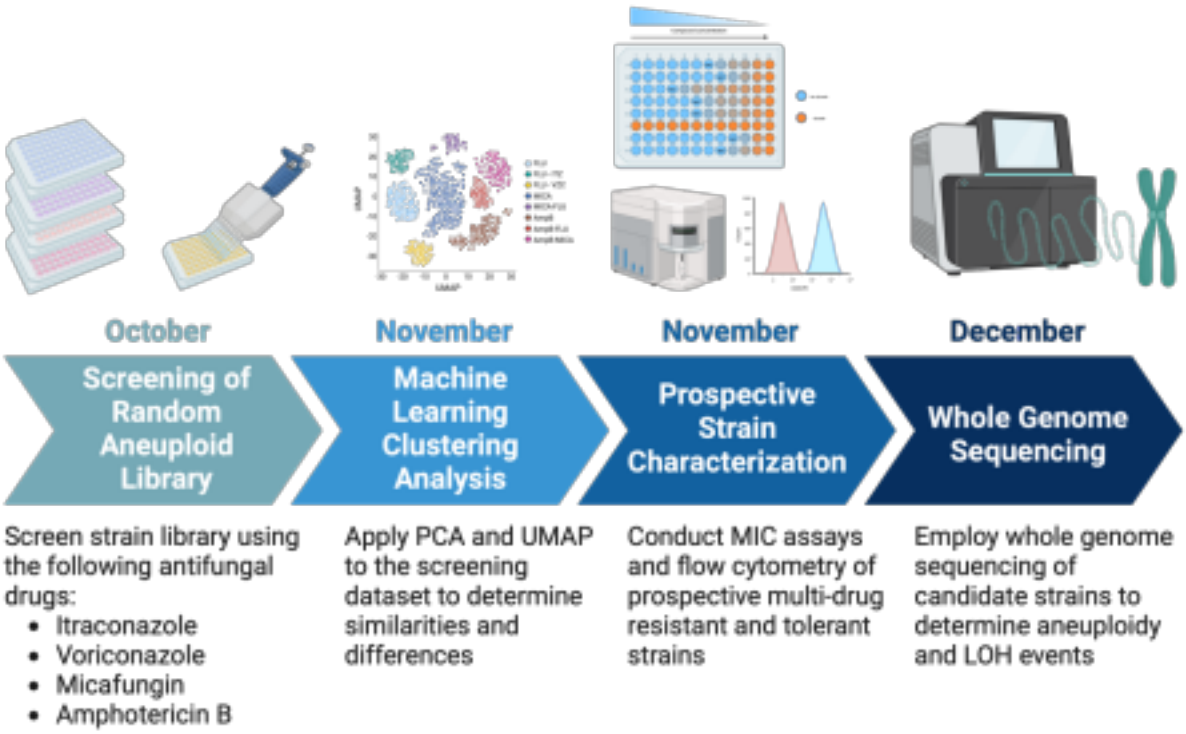
Figure 3: Nine disKnct karyotypes were observed involving several aneuploid combinaKons with parKal or whole chromosome LOH events. Illumina whole genome sequencing data ploNed using Yeast Mapping Analysis Pipeline.[17] Sequence read depth was converted to copy number and indicated on each chromosome. Chromosomal color indicates allelic raKo. Gray denotes heterozygous, cyan and pink denotes homozygous, dark blue and purple denotes heterozygous with 2 or more alleles.

Experimental Approach

Screening the random aneuploidy library will aid in establishing the range of aneuploidies associated with drug tolerance. In addiKon, it will help determine how similar or different aneuploid combinaKons effect drug responses. The random aneuploid strain library will be screened using representaKve drugs from all anKfungal drug classes: voriconazole, itraconazole, severing as representaKve azoles, the echinocandin micafungin, and the polyene amphotericin B. Strains will be diluted 1:000 into YPAD media containing the drugs being tested. OD_{600nm} readings will be taken at 24- and 48-hour post-inoculaKon with a microplate reader. Any

isolates growing at 24 hours will be classified as resistant and any strains that grows at 48 hours and not 24 hours will be classified as drug tolerant. ProspecKve strains will be further classified by MIC assay. Subsequently, using the data generated, clustering algorithms such as principal component analysis (PCA) and uniform manifold approximaKon and projecKon (UMAP) will be applied. These machine learning techniques will hopefully idenKfy how similar or different aneuploid combinaKons affect anKfungal drug responses. Analysis will either be coded in R or python. ProspecKve strains will be further characterized using the MIC assay, flow cytometry, and eventually whole genome sequencing.

Workflow Timeline



Statement of Presenta<on

If awarded with the SHUR grant, I will present my research findings to the Gustavus Adolphus College academic community at the CelebraKon of CreaKve Inquiry (Spring Research Symposium) in May of 2024.

Budget

Items	Purpose	Source	Quantities	Cost
Micafungin (5mg)	Representative echinocandin	MedChemExpress	1	$70 $15 shipping
Amphotericin B (100mg)	Representative polyene	MedChemExpress	1	$55 $15 shipping

Illumina Whole Genome Sequencing	Used to identify gene mutations and chromosome alterations	SeqCenter	2	$105 $30 shipping
96 Well Plates	To conduct screening experiments and MIC assays	VWR	1	$115 $10 shipping

Total = $520

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