Evolution of posaconazole tolerance in *Candida albicans*

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Abstract

While *Candida albicans* is a commensal member of the normal human microbiome, overgrowth can lead to an infection known as candidiasis. Among the repertoire of drugs available to treat candidiasis are the azoles. Resistance to azoles has been well-documented – particularly for fluconazole. However, for the newer azole posaconazole, genetic mechanisms underlying resistance are less well-characterized. This project enacted a selective pressure on strains of *C. albicans* through growth in posaconazole-containing media, with the goal of altering drug susceptibility. Evolved strains were characterized both phenotypically and genotypically. It was found that few replicates evolved true drug resistance, but drug tolerance – the ability to grow at drug levels exceeding the minimum inhibitory concentration – was frequently observed. Using whole genome sequencing, a list of candidate genes of interest involved in posaconazole tolerance were identified. These findings indicate that drug tolerance is an evolvable phenotype in *C. albicans*, and perhaps one that certain strains acquire faster and more readily than resistance under some conditions.

Key words

Candida, posaconazole, drug tolerance, drug resistance

Introduction

Candida albicans is a commensal member of the human microbiome, but overgrowth can lead to candidiasis – a fungal infection that can occur in the mouth, vagina, or bloodstream (CDC, 2019). Candidiasis ranks as the fourth most common type of nosocomial bloodstream infection in the United States (Wisplinghoff et al., 2004). While nosocomial candidiasis can be caused by many *Candida* species, *Candida albicans* is the most common causative organism, accounting for up to 50% of all cases (Wisplinghoff et al., 2014). In the case of vulvovaginal candidiasis (VVC) this proportion is even higher, with *C. albicans* responsible for over 90% of infections (Sobel et al., 1998). By age twenty-five, up to half of all women have experienced at least one clinically confirmed episode of VVC (Hurley and Delouvois, 1979), making treatment of VVC a significant venue for the use of antifungal agents.

Three antifungal drug classes are currently approved for the treatment of *Candida* infections: polyenes, echinocandins, and azoles. Azoles are the broadest and most widely used class of antifungal (Pappas et al., 2016). They disrupt normal cell membrane formation by binding and inhibiting ERG11, which is responsible for the production of ergosterol, a critical component of the fungal cell membrane (Odds et al., 2013). Available azole drugs include fluconazole, itraconazole, and the more recently developed drugs voriconazole, isavuconazole, and posaconazole (Perlin, 2017; Kale and Johnson, 2005). Fluconazole was among the first azole drugs introduced in 1990, followed shortly by itraconazole in 1992 (Nett and Andes, 2015; Pfizer, 2020). Posaconazole, the drug studied in this project, received FDA approval for the treatment of candidiasis in 2006 (PR Newswire, 2006).

Intrinsic resistance to azoles is rare in *C. albicans*, with recorded rates of only 1-2% (Cleveland et al., 2012). However, acquired resistance is observed in patients with recurrent infections following prolonged azole treatments (Kelly et al., 1996). Known contributors to fluconazole resistance include: multiple amino acid substitutions in ERG11 that result in decreased interaction between drug and target (Lamb et al., 2000; Flowers et al., 2015); mutations in and overexpression of *RTA2* (Jia et al., 2008); and up-regulation of drug efflux pumps such as *CDR1, CDR2, MDR1,* and *FLU1* (Sanglard et al., 1995). However, for posaconazole, the underlying genetic mechanisms for resistance are less well-characterized. Studies have shown that *C. albicans* strains with ERG11 mutations that confer resistance to fluconazole do not display the same degree of reduced susceptibility to posaconazole (MacCallum et al., 2010; Sanglard and Coste, 2015; Warrilow et al., 2019). This suggests that posaconazole resistance is acquired via another mechanism, one that has yet to be identified.

In addition to resistance, which refers to fungal growth in the presence of drug concentrations that inhibit susceptible isolates (Brauner et al., 2016), another important parameter in the characterization of drug response is the concept of *tolerance*. Even at concentrations exceeding the minimum inhibitory concentration (MIC), subpopulations of cells may still be capable of growth. Tolerance can be defined as the proportion of a population capable of slow growth at concentrations above the MIC (Rosenberg et al., 2018).

This project enacted a selective pressure on strains of *C. albicans* by prolonged exposure to media containing posaconazole, with the goal of altering and characterizing drug susceptibility. Multiple strains were used to determine the effect of strain background on the development of posaconazole resistance and tolerance. Finally, whole genome sequencing was performed to identify candidate genes involved in altered posaconazole drug response.

Materials and Methods

Strains

Eight strains of *C. albicans* with different genetic backgrounds were selected (P87, CG75, P78048, P75016, P76055, T101, SC5314, and FH1). These strains represent part of a panel of clinical isolates used by our lab group that were selected to capture the phylogenetic diversity of *C. albicans*. The strains selected exhibit a range of intrinsic resistance levels to posaconazole. Here we use 'ancestral strain' to refer to freezer stocks of each strain prepared prior to our experimental exposure to posaconazole and 'evolved strains' to refer strains that resulted from the posaconazole evolution experiment outlined below. All stocks were maintained at -80 °C.

Posaconazole Evolution

The eight strains were streaked onto plates containing YPD agar (2% w/v peptone, 2% w/v yeast extract, 1.8% w/v agar, 1% w/v glucose, 0.00016% w/v adenine sulfate, 0.00008% w/v uridine, 0.1% v/v of each chloramphenicol and ampicillin) from -80°C freezer stocks and grown for 72 hours at room temperature. For each strain, twelve colonies were picked at random, transferred to a 96-well deep well plate in 1mL of YPD media (2% w/v peptone, 2% w/v yeast extract, 1% w/v glucose, 0.00016% w/v adenine sulfate, 0.00008% w/v uridine, 0.1% v/v of each chloramphenicol and ampicillin), sealed with Breathe-Easier sealing membrane (Sigma-Aldrich, St. Louis, MO, USA), and incubated for 24 hours at 30 °C. Ancestral strains were prepared in

triplicate from 100 µL of culture and 100 µL of 30% glycerol and stored at -80 °C. Using 8 different strains and 12 replicates per strain, a total of 96 replicates were prepared.

Each replicate was standardized to an optical density at 600nm (OD_{600}) of 0.01. From the standardized culture, 20 µL was transferred to 180 µL of YPD media (YPD). An additional 20 µL of culture was added to 180 µL of YPD media containing 0.56 µg/mL posaconazole (YPD+POS). Plates were sealed with Breathe-Easier sealing membrane and incubated at 30 °C.

After 72 hours, YPD cultures and YPD + POS cultures were diluted 1:1000 and transferred to fresh media. At the time of each transfer biomass production was determined spectrophotometrically as OD_{600}. A total of five transfers were performed. After the fifth transfer, glycerol stocks were prepared in triplicate for both the YPD and YPD + POS evolved samples in the same manner as outlined above. In this way, replicates were evolved for ~ 50 generations (generations calculated as: $2^x = 1000$; $x = \ln(1000)/\ln(2)$).

An additional evolution experiment was performed using the same ancestral strains. 5 µL of each ancestral freezer strain was added to 195 µL YPD, sealed with a Breathe-Easier sealing membrane, and incubated at 30 °C for 48 hours. Each replicate was standardized to an OD600 of 0.01 before being transferred to YPD and YPD + POS in the manner described above. All other experimental steps remained the same, with the exception of the time between transfers being decreased from 72 hours to 24 hours.

Liquid MIC Assay

5 µL ancestral and evolved freezer strains were added to 195 µL YPD, sealed with Breathe-Easier sealing membrane, and incubated for 72 hours at room temperature. Each

replicate was standardized to an OD600 of 0.01. For the ancestral strains, an MIC assay was prepared as follows: 100 μL of YPD was added to columns 2 through 12 of a 96-well microplate. 100 μL of YPD with 16 μg/mL posaconazole was added to column one, and a 2-fold serial dilution was performed across each row up to and including column 10. 100 μL of each ancestral strain was added to columns 1 through 11, for a total of ten final posaconazole concentrations ranging from 8 μg/mL to 0.015625 μg/mL, and a control column containing no drug. Column 12 contained only YPD. In this fashion, microplates were prepared in duplicate.

For evolved strains, a modified MIC assay was prepared. Four 96-well microplates were prepared each containing 100 μL of YPD with three different concentrations of posaconazole, and a control plate containing 100 μL of YPD with no drug. 100 μL of each evolved replicate was added to each well of the microplates. Final posaconazole concentrations for each plate were 8 μg/mL, 2 μg/mL, 0.05 μg/mL, and no drug. In this fashion, microplates were prepared in duplicate.

All plates (for both ancestral and evolved replicates) were sealed with Breathe-Easier sealing membranes and incubated at 30 °C. Biomass production was measured via spectrophotometer as OD600 at 24, 48, and 72 hours.

Disk Diffusion Assays

5 μL of ancestral and evolved freezer strains were added to 195 μL of YPD, sealed with Breathe-Easier sealing membranes, and incubated at 30 °C for 48 hours. Each replicate was standardized to an OD_{600} of 0.01. 100 μL of standardized culture was spread-plated in duplicate onto plates prepared with 15mL of YPD agar. Posaconazole disks were prepared by adding 4 μL

of 0.625 mg/mL posaconazole to blank antimicrobial susceptibility disks. One disk was applied to the center of each plate. All plates were inverted and incubated at 30 °C. After 24 and 48 hours, each plate was placed on a light box and photographed from above. This process was repeated for a total of two biological replicates.

Photographs were processed using ImageJ (Schneider et al., 2014) by cropping the background, inverting image colours, and adjusting brightness and contrast to obtain bright colonies against a dark background (as in Figure 3C). The adjusted images were analyzed using the *diskImageR* package following recommendations specified in the diskImageR vignette V2 (Gerstein, 2016 https://www.microstatslab.ca/diskimager.html). *diskImageR* analyzes disk diffusion assay images by plotting radial lines from the center of each disk to the edge of the plate. Colony growth corresponds to pixel intensity along each line. Two key metrics were obtained from the *diskImageR* pipeline: RAD and FoG values. RAD refers to the radius of the zone of inhibition, while FoG is a measure of the proportion of growth occurring within this radius. In this project, RAD_{20} and FoG_{20} values were used, referring to the radius at which a 20% reduction in growth occurred, and the fraction of growth occurring within this radius.

RAD and FoG values obtained from the *diskImageR* pipeline were averaged across biological and technical replicates for all strains. For strains P76055, T101, SC5314, and FH1, only one biological replicate was included, as low-quality images prevented the diskImageR maximum-likelihood analysis from running successfully.

A second disk diffusion assay was performed in the same manner for strains P87, CG75, P78048, and P75016 using disks containing 25mg of fluconazole to investigate whether evolution in the presence of posaconazole results in cross-resistance to other azoles. Two

technical replicates and one biological replicate were produced for the fluconazole disk diffusion assay.

Genomic DNA Extraction and Sequencing

Based on preliminary results from the *diskImageR* analysis, strain SC5314 was selected for whole genome sequencing. In addition to these replicates displaying large increases in tolerance, SC5314 is also the *C. albicans* type strain, meaning that a reference genome was readily available for downstream read alignment steps. Genomic DNA was extracted from two ancestral strain replicates and nineteen evolved replicates (twelve replicates from the 72 hour evolution experiment described here, as well as seven replicates from a similar experiment previously completed by Quinn Wonitowy).

30 µL of frozen stock culture was transferred to 2.97 mL of YPD and incubated for 48 hours at 30 °C with constant shaking. Cells were collected by centrifugation at 2 500 rpm for three minutes, and the supernatant discarded. Each sample was vortexed for twenty minutes at 4 °C with 500 µL of TENTS buffer (100 mM NaCl, 10 mM Tris pH 8.0, 1 mM EDTA, 2% Triton X 100, and 1% SDS), ~100 µL of glass beads, and 200 µL phenol:chloroform:IAA, followed by centrifugation for ten minutes at 13 500 rpm. 350 µL of supernatant was transferred to a fresh tube, and DNA precipitated overnight at -20 °C by adding 1 mL of cold 100% ethanol. Each sample was centrifuged again at 13 500 rpm for ten minutes, and DNA resuspended in 50 µL of molecular biology grade water. Samples were treated with 1 µL of 10 mg/mL RNAse A (incubation at 37 °C for one hour) and 2 µL of 20 mg/mL proteinase K (incubation at 37°C for 1.5 hours). 200 µL of water and 300 µL of phenol:chloroform:IAA was added, and samples were

centrifuged at 13 500 rpm for ten minutes. 170 µL of supernatant was transferred to a fresh tube, and DNA was precipitated once more with 4 µL of 5 M NaCl and 400 µL of cold 100% ethanol. After precipitating at least ten minutes at -20 °C, the pellet was left overnight to allow the ethanol to completely evaporate before the DNA was resuspended in 50 µL of water.

DNA quality was assessed using a nanospectrophotometer, and DNA concentration measured using a Qubit 2.0 fluorometer. All samples met the minimum concentration guidelines (between 10 and 100 ng/µL and at least 30 µL total volume) specified by MiGS Sequencing Center (Pittsburgh, USA). Whole genome sequencing was performed by the MiGS Sequencing Center using the Illumina NextSeq 550 platform to a calculated depth of ~40.

Genome Analysis

All reads were quality-assessed using FASTQC and MultiQC before and after trimming (Andrews, 2010; Ewels, 2016). Adaptor sequences and low-quality reads were trimmed using Trimmomatic (v0.36 LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:36 TOPHRED33) (Bolger et al., 2014). Read alignment was performed using the Burrows-Wheeler Aligner MEM algorithm using default parameters (BWA v0.7.17) (Li, H., 2013).

The *Candida albica*ns reference genome (A21-s02-m09-r10) and known polymorphisms were obtained from the Candida Genome Database (http://www.candidagenome.org) on March 12th and March 15th 2020, respectively. After alignment, the following steps were performed using Picard command-line tools (https://broadinstitute.github.io/picard/). Read groups were added using AddOrReplaceReadGroups (RGID=Cell1 RGLB=lib1 RGPL=illumina

RGPU=unit1). Reads were deduplicated using MarkDuplicates (CREATE_INDEX=true) and mate-pair information verified using FixMateInformation (CREATE_INDEX=true).

Variants were analyzed using tools available through GATK (v.4.1.2.0) (McKenna et al., 2010). Base quality scores were first recalibrated using BaseRecalibrator and ApplyBQSR, both with default parameters. HaplotypeCaller (-ERC GVCF) was used to pre-call variants, before combining samples with CombineGVCFs and running GenotypeGVCFs with default paramters.

Variants were divided into two groups using SelectVariants (-select-type SNP, select-type INDELS select-type MIXED). SNPs were quality-filtered using VariantFiltration (-filter "QD < 2.0" -filter "QUAL < 30.0" -filter "SOR > 30" -filter "FS > 60.0" -filter "MQ < 40.0" -filter "MQRankSum < -12.5" -filter "ReadPosRankSum < -8.0"). Indels and other variants were quality-filtered separately from SNPs using different filter parameters (-filter "QD < 2.0" -filter "QUAL < 30.0" -filter "FS >200.0" -filter "ReadPosRankSum < -20.0").

Results were visualized in Integrated Genomics Viewer (Robinson et al., 2011). All replicates were searched manually to identify variants. Candidate genes of interest were identified based on the following criteria. First, candidate variants had to be present only in the evolved replicates, with both ancestral replicates that were sequenced having the wildtype allele. Second, to simplify search efforts, only candidate genes in which the same variant was present in at least five of the evolved strains were considered. Finally, genes meeting the first two criteria were included in the list of putative genes of interest if they had an annotation stating up- or down-regulation by azole drugs. Annotations were obtained from the Candida Genome Database (http://www.candidagenome.org). Genes known to be involved in fluconazole resistance (ERG11, CDR1, CDR2, MDR1, FLU1, RTA2) were also examined.

Results

Posaconazole Evolution

Strain background was correlated with the response to sustained exposure to posaconazole. Over the first, second, and third transfers of the 72 hour evolution experiment, replicates from each strain background displayed a sharp decrease in growth ability (Figure 1). After the third transfer, two different outcomes were observed. In some strains (P87, CG75, P78048, and P75016) the growth ability of each replicate continued to decrease. By the end of the fifth transfer, all or nearly all replicates had gone extinct, as determined by an OD_{600} similar to that of YPD and an inability to revive cells from freezer stocks in subsequent experiments. In P87, all replicates went extinct. In CG75, one replicate survived. In P78048 and P75016, OD_{600} measurements indicated that three replicates survived to the fifth transfer, but only two replicates from each strain could be consistently revived from the evolved freezer stock.

In other strains (T101, SC5314, and FH1) a different outcome was observed. Some replicates were able to recover growth ability over the fourth and fifth transfers. The only strain background in which extinction was observed was strain FH1, in which two replicates went extinct by the fifth transfer. In SC5314, OD600 readings suggested that one replicate went extinct, but because cells could be successfully revived from the evolved freezer stock, it is likely that the cell concentration at the time of the fifth transfer was just very low. Strain P76055 fell between these two outcomes. While no replicates went extinct by the fifth transfer, only some displayed improved growth after the third transfer, while most continued to worsen.

In the 24 hour experiment, replicates from every strain background exhibited very low growth at each transfer (Figure 2). By transfer five, extinction was observed for every replicate.

Resistance and Tolerance

For replicates from every strain background, few improvements in resistance (as measured by the radius of the zone of inhibition through *diskImageR* analysis) between ancestral and evolved replicates were observed (Figure 3A). In strain P76055, only two replicates displayed a smaller zone of inhibition (i.e., increased resistance) than the mean radius observed across the ancestral replicates from the same strain. In T101, six replicates displayed a slight improvement above the mean radius of the ancestral replicates, while for the rest the size of the zone of inhibition remained the same. In SC5314 and FH1, most replicates displayed an increase in the size of the zone of inhibition as compared to the mean of the ancestral replicates, indicating a *decrease* in the level of drug resistance. In CG75, P78048, and P75016, all evolved replicates were less resistant to posaconazole than any of the ancestral replicates.

Different patterns were observed when measuring tolerance (Figure 3B). Though only one or two replicates from strains CG75, P78048, and P75016 survived the 72 hour evolution experiment and could be analyzed, each of these surviving replicates displayed at least 25% more growth within the zone of inhibition than the mean of the ancestral replicates for each strain. Evolved replicates from strain SC5314 also displayed increased drug tolerance, with all evolved replicates exceeding the mean tolerance level of the ancestral replicates. For strain P75066, slight improvements were observed, with seven replicates displaying tolerance levels

greater than the ancestral mean. Evolved replicates from strains FH1 and T101 maintained similar tolerance levels to the ancestral replicates.

Ancestral replicates from the liquid MIC assay displayed abnormally high OD600 readings and were not included in analysis. However, examining the OD600 readings from the plates prepared with evolved replicates in 0.5 μg/mL of posaconazole provides insight into the within-strain variation in biomass production. Strain SC5314 displayed the greatest amount of within-strain variation. This was consistent with the variation seen in resistance and tolerance measurements as determined through *diskImageR* analysis. Despite having a high level of ancestral drug resistance, evolved replicates from strain T101 displayed even lower biomass production in the presence of 0.5 μg/mL posaconazole than the mean growth of an ancestral sample grown in a lower drug concentration (0.12 μg/mL) (Figure 5).

Half of the available strains were used in a disk diffusion assay examining fluconazole tolerance (in which fluconazole disks were used instead of posaconazole), and of these, only evolved replicates from strains P78048 and P75016 grew successfully. Resistance, as measured by the size of the zone of inhibition, was lower in evolved replicates than it was in ancestral replicates (Figure 4A). Tolerance improved in all evolved replicates beyond that of the ancestral replicates (Figure 4B). Despite never having been previously exposed to fluconazole, the response of the evolved replicates to this drug was similar to that seen to posaconazole.

Genome Analysis

In total, five genes of interest were identified based on the criteria previously outlined. IFD6 (NCBI Gene ID: 3644703) contained two single nucleotide variants (SNVs), present in

seven and five of the evolved replicates, respectively. It is provisionally annotated as a member of the aldo-keto reductase family. PNG2 (NCBI Gene ID: 3645568) contained one SNV present in thirteen of the evolved replicates (as a heterozygous SNV in three replicates and as a homozygous SNV in ten), making this the most frequently observed mutation of interest. PNG2 encodes a putative N-glycanase. SNVs were identified in both ALS2 (four SNVs present in seven replicates each) and ALS3 (seven SNVs present in six to seven replicates) (NCBI Gene IDs 3645724 and 3647965, respectively), both annotated as members of the Agglutinin-Like Sequence family, and likely playing a role in cell wall adhesion. The last gene identified was IFC3 (NCBI Gene ID: 3644918) which contained both a two base pair insertion and a two base pair deletion in ten of the evolved replicates. Evolved replicates from two separate but similar evolution experiments were sequenced, and each mutation identified was present in a mix of replicates from each experiment.

The genomes were also investigated for mutations in genes with known associations to fluconazole resistance: *ERG11*, *RTA2, CDR1, CDR2*, *MDR1,* and *FLU1*. However, in each of these genes, the same genotype was observed in both the evolved and ancestral replicates, which is consistent with the hypothesis that although posaconazole and fluconazole are the same class of drug, different mechanisms underlie drug response to these two drugs.

Discussion

Current research into changes in antifungal drug susceptibility are largely targeted at characterizing resistance and identifying associated genes. Tolerance is a less well-characterized and less frequently used metric. However, tolerance has important clinical implications, as slow

growth at drug concentrations exceeding the MIC allows Candida species to persist even after treatment with an antifungal agent in a clinical setting (Rosenberg et al., 2018). Here I found that tolerance is an evolvable phenotype, with all evolved replicates from strains CG75, P78048, P75016, and SC5314 exceeding the mean drug tolerance levels measured across the corresponding ancestral replicates. Interestingly, these strains displayed the lowest ancestral tolerance levels, but the largest improvements in the evolved replicates. In contrast, increased resistance was seen for only two replicates from strain P76055, six replicates from strain T101 (which only improved by a small margin) and one replicate from FH1. This shows that not only can drug tolerance be evolved, but it is a trait that certain *C. albicans* strains may be able to acquire faster and more readily than resistance under some conditions.

I identified five candidate genes of interest for posaconazole tolerance in *C. albicans* strain SC5314. Future investigations will determine whether the replicates in which candidate mutations were identified correlate with the replicates displaying the highest tolerance levels, and conduct more detailed molecular experiments to confirm the role of each of these genes plays in acquired drug tolerance. Each identified gene is only provisionally annotated in the Candida Genome Database, and information regarding biosynthetic and regulatory pathways they are involved in is largely absent from current literature. The most well-characterized of the identified genes are the ALS family genes, which current research suggests play a role in cell adhesion (Gaur and Klotz, 1997). However, as discussed in a 2008 review article by Hoyer et al., research is still ongoing, and other possible roles of ALS family genes in *C. albicans* are still being investigated, including its role in pathogenicity.

While genes currently understood to be involved in fluconazole resistance were investigated, no differences were observed between sequences from the ancestral replicates and

the evolved replicates. This is not unexpected, as increased resistance was not observed among replicates from strain SC5314. The genes identified in this research are therefore not ones associated with drug resistance but are candidate genes involved in posaconazole tolerance.

Due to time constraints, only mutations present in at least five replicates of the SC5314 strain were examined here. However, mutations are a chance event, occurring sporadically and independently in each replicate. Variants occurring in only one or a few evolved replicates are still worth considering, as these could provide different routes towards the evolution of drug tolerance. Future work will develop a more efficient pipeline to identify putative mutations that confer posaconozole resistance or tolerance.

Future experiments will also more fully probe how the number of transfers (i.e., the number of generations in the presence of drug) and the period of time between transfers influences the ability of replicates from different strain backgrounds to acquire resistance and tolerance. Growth ability in the presence of drug typically began to recover between the fourth and fifth transfers in the evolution experiment described here. If additional transfers are performed, will the trend of increasing growth ability continue after the fourth and fifth transfers? If it is the case that drug tolerance evolves faster than drug resistance, perhaps resistance will ultimately be observed if the number of transfers is increased beyond five. In the 72 hour evolution experiment performed here, replicates were able to recover some growth ability in the presence of drug. However, when replicates were transferred to fresh drug daily in the 24 hour evolution experiment, all replicates ultimately went extinct. This potential for slow growth after exposure to drug has potential implications in a clinical setting, particularly for cases where antifungal therapy was abandoned before completion, as it shows that tolerant isolates can arise after drug exposure in strains with no previous intrinsic resistance.

Another future research direction is the study of the potential to acquire cross-resistance to multiple azole drugs. Previous research (Warrilow et al., 2019) has shown that *C. albicans* strains that acquire fluconazole resistance through *ERG11* mutations do not display the same degree of resistance to posaconazole. Does this relationship go both ways, and does it hold true in the case of tolerance as well as resistance? Testing how samples that have evolved posaconazole tolerance respond to fluconazole could provide insight into these questions. Based on preliminary results from the fluconazole disk diffusion assay performed here, it seems that replicates exhibiting posaconazole tolerance also display similar tolerance to fluconazole. However, this relationship needs to be studied in greater detail using additional evolved replicates.

In conclusion, it was found that drug tolerance is a phenotype that can be evolved in a relatively brief time period. Increases in drug tolerance correlated with strain background, with strains having the lowest ancestral resistance levels (such as P78048, P75016, and SC5314) displaying the greatest improvements. The fact that drug tolerance is a readily acquired trait has important clinical implications, as this could provide insight into how and why recurrent *C. albicans* infections occur following antifungal therapy. Finally, a list of candidate genes of interest related to posaconazole tolerance were identified and plans for future research efforts discussed.

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Tables

Table 1 Putative genes of interest relating to posaconazole tolerance.

Gene name	Gene Annotation Notes	Position	Mutation	Number of strains observed in
IFD6	Aldo-keto reductase family member; increased protein correlates with MDR1 overexpression in fluconazole-resistant clinical isolates	chr1:867820 chr1:867829	G/G → G/A G/G → G/A	7 5
PNG2	Putative peptide:n-glycanase; transcription up-regulated by treatment with caspofungin, ciclopirox olamine, ketoconazole, or hypoxia	chr2:1158412	A/A → A/G A/A → G/G	3 10
ALS2	ALS family protein; role in adhesion, biofilm formation induced by ketoconazole, low iron and at cell wall regeneration	chr6:977291 chr6:977293 chr6:977294 chr6:977306	C/C → C/T C/C → C/A A/A → A/T A/A → A/C	7 7 7 7
ALS3	ALS family cell wall adhesin; role in epithelial adhesion; fluconazole-repressed	chrR:1533684 chrR:1533687 chrR:1533705 chrR:1533708 chrR:1533732 chrR:1533750 chrR:1533751	T/T → T/A A/A → A/T G/G → G/A A/A → A/G T/T → T/C T/T → T/G G/G → G/T	7 7 7 7 7 6 6
IFC3	Oligopeptide transporter; fluconazole-induced	chrR:499325 – 494327 chrR:494320	2bp del 2bp ins	10 10

Figure Captions

Figure 1. Growth ability of various strains of *C. albicans* over five subsequent transfers into YPD media supplemented with 0.5µL posaconazole. Biomass was measured via spectrophotometer as optical density as 600nm at the time of transfer, and transfers were performed every 72 hours.

Figure 2. Growth ability of various strains of *C. albicans* over five subsequent transfers into YPD media supplemented with 0.5µL posaconazole. Biomass was measured via spectrophotometer as optical density as 600nm at the time of transfer, and transfers were performed every 24

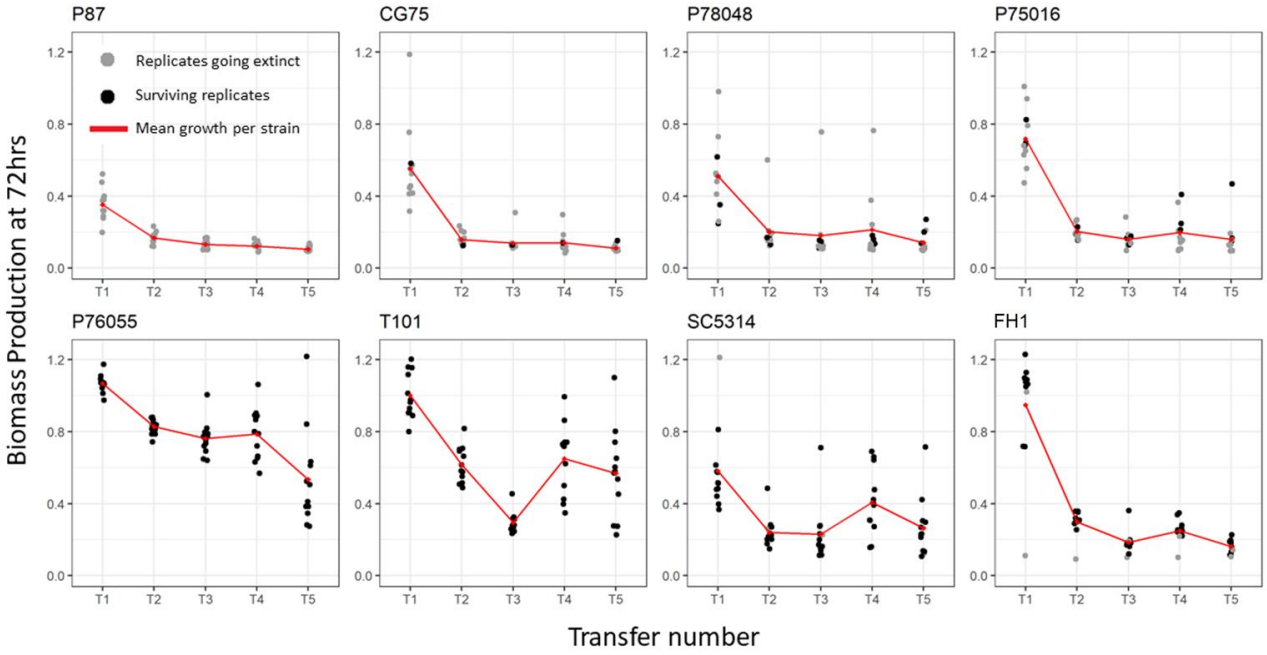
Figure 3. Comparison between ancestral and posaconazole-evolved replicates of *C. albicans*. A) Resistance was determined as the radius from the disk at which there was a 20% reduction in growth ability. B) Tolerance was determined as the fraction of the population growing within the zone of inhibition determined from A). C) Ancestral (top) and evolved (bottom) replicates of *C. albicans* strain SC5314 growing on YPD media in the presence of a posaconazole-containing disk, after processing in ImageJ. The evolved replicate has increased in tolerance, with a large fraction of growth occurring within the zone of inhibition.

Figure 4. Comparison between ancestral and posaconazole-evolved replicates of *C. albicans* in the presence of a fluconazole-containing disk. A) Resistance was determined as the radius from the disk at which there was a 20% reduction in growth ability. B) Tolerance was determined as the fraction of the population growing within the zone of inhibition determined from A).

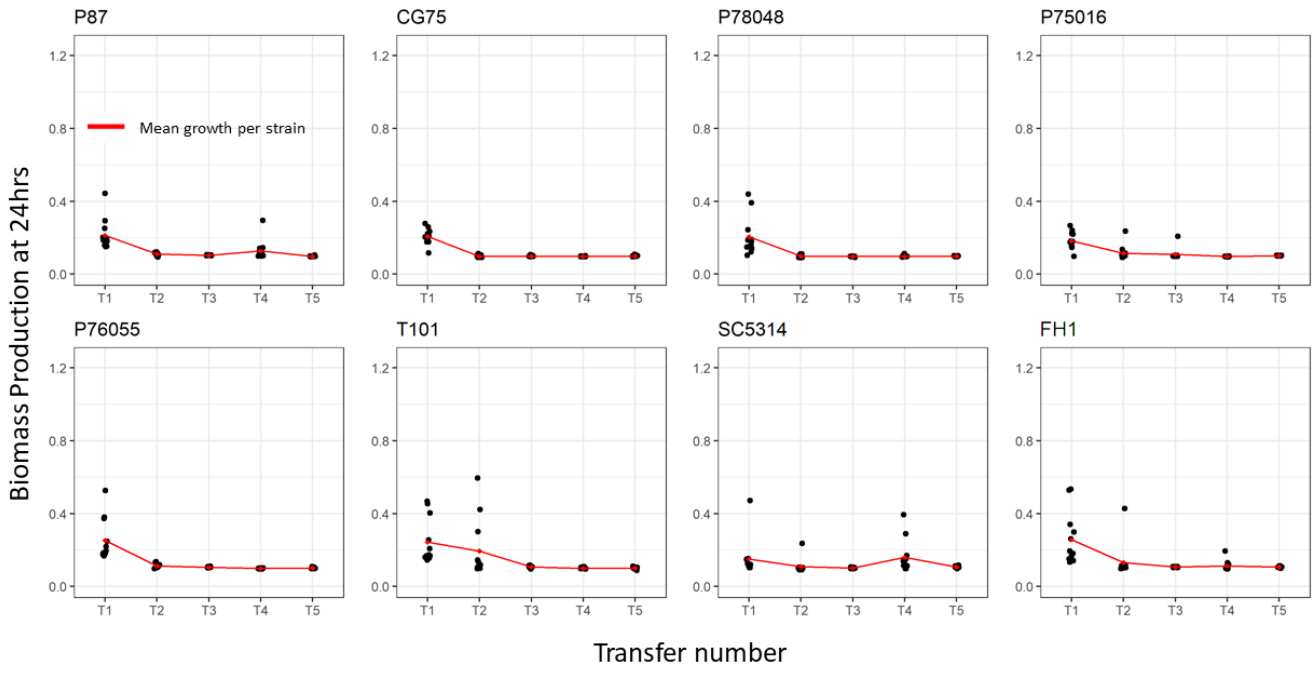
Figure 5. Biomass production for evolved replicates of *C. albicans* after 72 hours of growth in YPD with 0.5 μg/mL of posaconazole. No liquid MIC results were available for ancestral replicates; red squares depict growth at 72 hours in YPD with 0.12 μg/mL posaconazole for the different ancestral replicates from the same strain backgrounds, as measured in a separate but similar experiment.

Figures

Figure 1

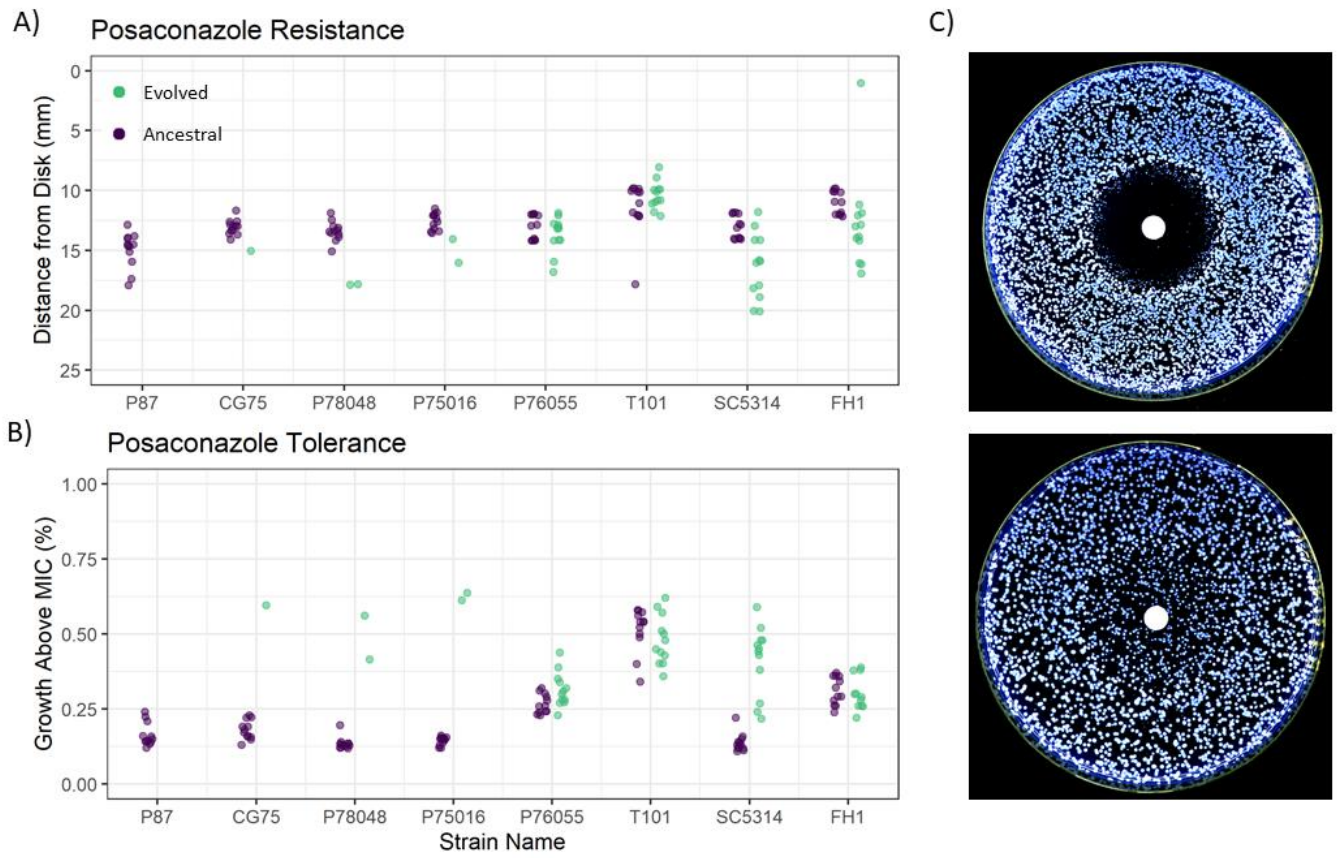


508 Figure 2

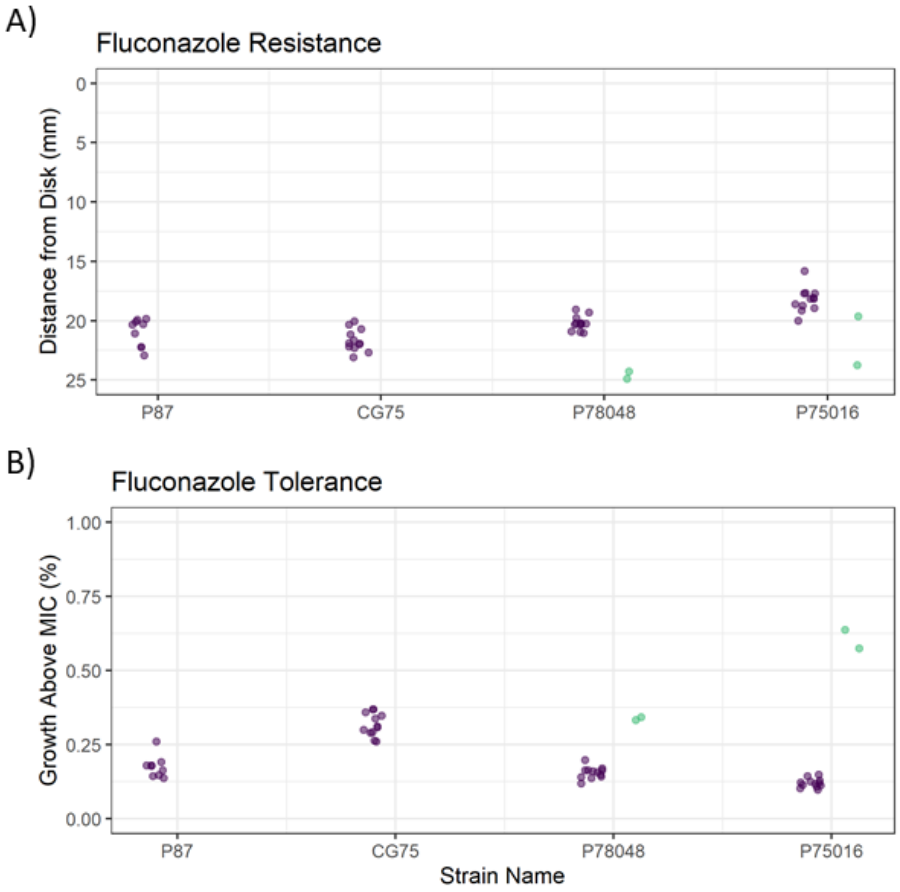


509

Figure 3

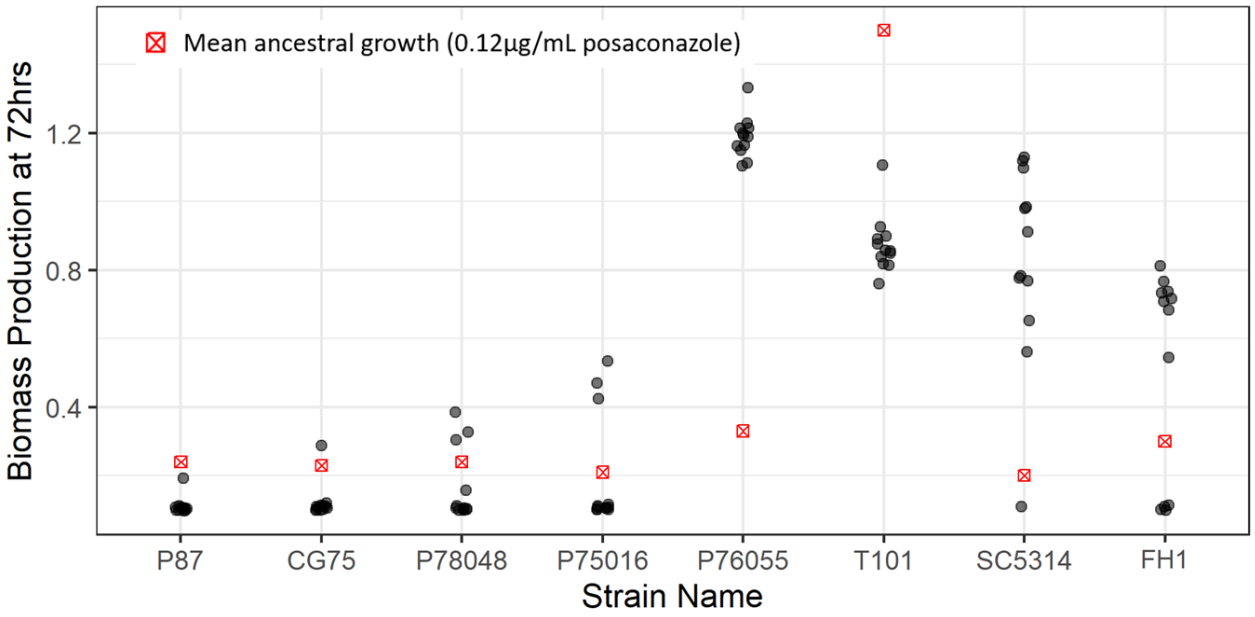

A)
Posaconazole Resistance
Evolved
Ancestral
Distance from Disk (mm)
0
5
10
15
20
25
P87
CG75
P78048
P75016
P76055
T101
SC5314
FH1
B)
Posaconazole Tolerance
Growth Above MIC (%)
1.00
0.75
0.50
0.25
0.00
Strain Name
C)

512 Figure 4



513

514 Figure 5



515