PRIMARY RESEARCH OPEN ACCESS

Sequencing and Detection of Two Missense Variants at Chromosome 7 as Potential Azole Resistance Markers in *Saccharomyces Cerevisiae*

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Abstract
Introduction: Widespread practices of over-prescribing antibiotic, antiviral, and antifungal drugs have sparked concern over the risk of antimicrobial resistance arising in bacterial, viral, and fungal pathogens. This risk threatens to jeopardize the efficacy of many drugs being prescribed today for said infections.
Azoles are a major class of antifungal drugs, presenting the need for research efforts on mechanisms of azole resistance.
My objective was to perform the genomic sequencing and variant profiling of a Baker's Yeast (*Saccharomyces cerevisiae*) strain that displays resistance phenotype when plated with Clotrimazole, a type of azole antifungal.
Methods: Through short-read genomic sequencing and subsequent variant calling, putative antifungal resistance genotypes were elucidated in a clotrimazole-resistant yeast strain.
Results: Variant calls at the *PDR1* and *ERG25* loci reveal two potential Clotrimazole-resistance genotypes.
Discussion: These variants are both missense mutations involving a single-nucleotide change to the reference genomic loci in question.
Although *PDR1* variants are putative markers of azole resistance in yeast, the emergence of a novel *ERG25* variant that may contribute to this phenotype has not yet been.
Conclusion: Follow-up experiments will need to include induced missense mutations at the *ERG25* loci and selection assays to confirm that the described variants described here indeed constitute azole resistance.
Establishing a knowledgebase of resistance marker genes and putative resistance variants for model organisms such as *Saccharomyces cerevisiae* can guide investigations into orthologous proteins in other species that may become responsible for future antifungal-resistant infections.

Keywords: sequencing; genomics; drug resistance; antimicrobial resistance; pandemic; yeast; public health; Illumina

Introduction

Azoles are a major family of antifungal drugs that inhibit fungal growth by inhibiting the synthesis of fungal plasma membranes, ergosterol [1]. Azoles compromise Ergosterol synthesis by the targeting Lanosterol demethylase, an enzyme that is responsible for the 14-alpha demethylation of an ergosterol synthesis of Lanosterol, an intermediate molecule in the ergosterol synthesis pathway.

Prescribing antimicrobial drugs is commonplace in healthcare systems worldwide to combat illness [2]. However, excessive administration of these drugs has contributed towards a growing health crisis: antimicrobial resistance (AMR) [3]. AMR is a phenomenon that arises from pathogens that evolve drug resistance mechanisms to canonical therapeutics. In 2016, the Center for Disease Control and Prevention estimated the prescription of over 47 million antibiotic courses that year, comprising the 30% of antibiotics recommended annually. This trend of increasing risk poses AMR to be a future global health threat [4]. Azole resistance genotypes have been documented in transcription factor (TF)-encoding loci, such as *ERG11*, that encode regulators of efflux pump-encoding genes, such as *CDR1* and *CDR2* [5].

Baker's yeast, also known as *Saccharomyces cerevisiae (S. cerevisiae)*, is a versatile eukaryotic model organism a genome spanning 12,100,000 base pairs over 16 chromosomes and an additional 2-micron plasmid [6] The ability to identify strains of *S. cerevisiae* that are resistant to common antifungals, may help reveal new insights into the functional mechanisms of AMR. Functional assays such as comparing colony growth of azole-resistant versus azole-sensitive strains on a plate with media and an contains azole drug, allows for the clear visualization of these AMR phenotypes *in vitro*. Genomic sequencing serves to verify that observed azole-resistance phenotypes from functional assays are indeed a product of genetic variation at a hypothesized locus.

Here, I document results from paired-end sequencing of *S. cerevisiae* F10, a clotrimazole-resistant strain interest that is derived from a clotrimazole-sensitive parental strain, A10.

Methods

Genomic DNA (gDNA) Preparation

Single colonies of parental A10 and mutant F10 yeast were isolated from source plates that were provided by a third party, and streak plated on Yeast Extract-Peptone-Dextrose (YPD) media. Single colonies on this rich media were then isolated and diluted at $5x10^{0}$ μM, $5x10^{-1}$ μM, $5x10^{-2}$ μM, $5x10^{-3}$ μM, $5x10^{-4}$ μM, 5 $x10^{-5}$ μM with nuclease-free water. From each dilution, 3μL was spotted onto a new plate containing YPD media. Single colonies were then isolated from the YPD media. gDNA was isolated using RNAse and YeaStar Genomic DNA Kit (ZYMO Research).

Quality checks on the gDNA isolation was done using fluorometry (Qubit) and visualized on 1% EX-gel (Figure 1B). Solid single colonies of A10 and F10 were individually plated on media-rich plates deficient of histidine, uridine, or tryptophan, and incubated for 3 days at 30°C. The formation of visible single colonies indicated the emergence of auxotrophic phenotypes. Specifically, the F10 strain displayed histidine and uridine auxotrophy (Figure 1A).

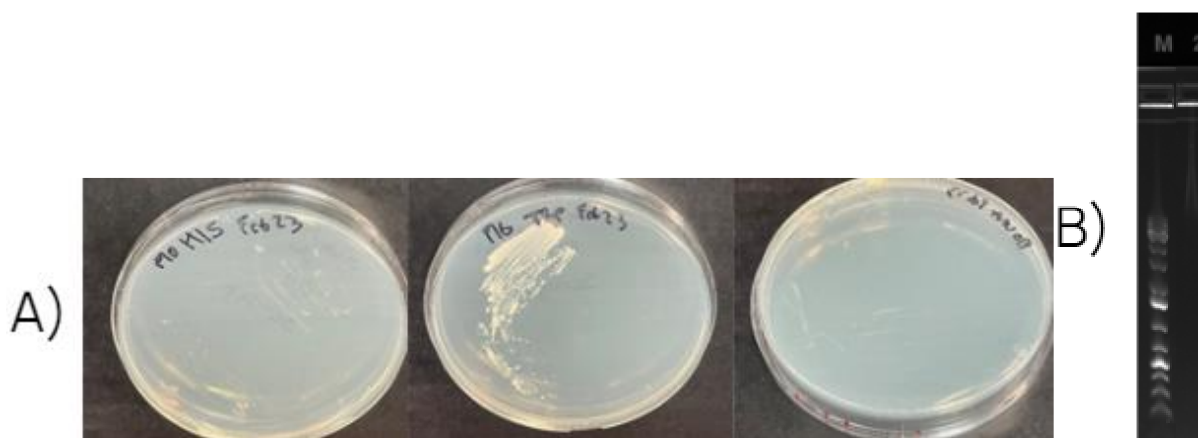

Figure 1. Preliminary quality control experiments on yeast cell line viability and gDNA purity. The copyright of this figure belongs to the author(s).
A) *Functional screening of F10 for auxotrophic phenotypes shows a histidine- and uridine-auxotrophic phenotype. (Left to right: HIS = Histidine-deficiency plate, TRP = Tryptophan-deficiency plate, URA = Uridine-deficiency plate)*
B) *Visualization of isolated genomic DNA on 1% agarose gel (M = Ladder, Well 2 = F10)*

Illumina Sequencing Library Preparation

Libraries of ~300 base pairs (bp) gDNA fragments were generated using 10μL of Tn5 transposase-containing tagmentation buffer, alongside 5μL of sample gDNA and 5μL of attached adapters for downstream Illumina sequencing and Polymerase Chain Reaction (PCR) amplification steps. Illumina sequencing indexes were ligated with a library-specific barcode using low-cycle PCR (Nextera) at 4000 RPM for 1 minute. PCR-amplified libraries were purified using 30μL of AMPure Beads (Beckman Coulter Life Sciences), quantified via quantitative real-time polymerase chain reaction (qPCR), and diluted to 10pM in nuclease-free water for Illumina paired-end sequencing.

Sequencing Analysis

Illumina sequencing reads were processed as FASTQ files on Galaxy [7] and assessed for quality of base calls with a quality score (Q) threshold of Q=30. (Figure 2)

DOI Link: https://doi.org/10.26685/urncst.363

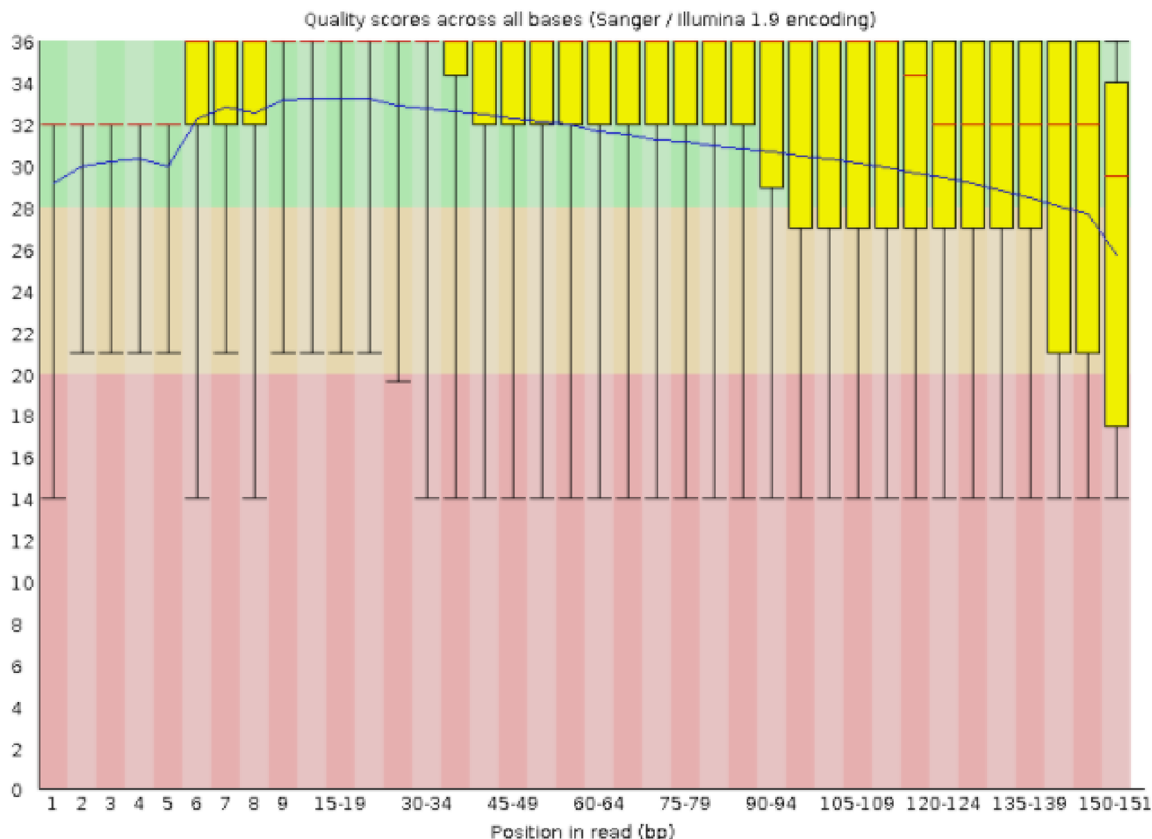

Figure 2. Per-base quality score graph, Q-Scores decrease below acceptable threshold around 124bp into each read (Q score cut-off = 30). Generated from Galaxy.

Multiple sequence alignment was performed using Bowtie [8] to map paired end sequencing reads of F10 and A10 to the *S. cerevisiae* reference genome database, SacCer3 (Genbank Accession: GCA_000146045.2). Variant calling based on the mapped F10 and A10 sequences was done using Bedtools [9]. F10 variant calls were filtered out if they were found both in A10 and F10, had a quality score < 120, or had no genomic orientation.

Remaining variant calls at genes that have been documented to arise azole-resistance were then inputted into Variant Effect Predictor [10] for predictions on their contributions to azole resistance phenotypes.

Results

Here, I report paired-end sequencing variant calls on two reads of a single F10 sample. The two variants can be found at the pleiotropic drug response 1 (*PDR1*) and ergosterol biosynthesis (*ERG25*) loci on chromosome 7 (Table 1).

Table 1. Outline of the two genetic variants discussed in this report. A SIFT score of below 0.5 indicates a variant that is predicted to significantly alter a phenotype

Mutant	Locus	Chromosome	Nucleotide Change	Amino Acid Change	Variant	SIFT Score
T304N	*PDR1*	VII	C → A	T → N	Missense (Conservative)	0.29
W155L	*ERG25*	VII	T → G	W → L	Missense (Non-conservative)	0.00

W155L is a Significant Missense Variant at the *ERG25* Locus in F10 Yeast

Analysis of variant calls from paired-end sequencing data revealed a G→T transversion at the *ERG25* locus, a gene characterized by its role in the ergosterol synthesis pathway [11,15]. This variant alters the codon at position 155 in the *ERG25* open reading frame from “5’-TGG-3” to “5’-TTG-3”, resulting in the translation of a leucine (L) in place of a tryptophan (W).

This variant was assigned a sorting intolerant from tolerant (SIFT) score of 0 on Variant Effect Predictor, indicating a highly confident predicted impact on the F10 phenotype (Table 1).

T304N is a Significant Missense Variant at the *PDR1* Locus in F10 Yeast

A missense variant was detected at the *PDR1* locus, which encodes a transcription factor that regulates pleiotropic drug responses [11]. This variant is characterized by C→A transversion and a codon change of “5’-ACC-3” to “5’-AAC-3”, resulting in the translation of an asparagine (N) instead of a threonine (T). Analysis on Variant Effect Predictor yielded a SIFT score of 0.29, indicating a highly confident predicted impact on the F10 phenotype (Table 1).

Discussion

Variant calls on two reads of a single F10 paired-end sequencing run implicated two potential missense variants of interest for clotrimazole resistance. The two variants can be found at the pleiotropic drug response 1 (*PDR1*) and ergosterol biosynthesis (*ERG25*) loci on chromosome 7 (Table 1), with both representing missense, non-conservative changes to the genomic DNA (gDNA).

The *PDR1* gene on chromosome 7 encodes PDRp1, a zinc-cluster transcription factor that regulates recruitment of zinc-cluster proteins to genomic regions known as pleiotropic drug regulatory elements (PDREs) [12]. Transcriptional activity of PDRp1 is attributable to a prominent trans-activation domain that spans amino acid positions 1054-1062. [11]

The change from a threonine to asparagine in position 301 of PDRp1 would therefore not directly alter the trans-activation domain. Furthermore, T and N are both similarly sized polar amino acids, classifying this variant as a conservative missense. These two datapoints postulate that the functional effect of this missense mutation on the F10 phenotype would not be sufficient to confer azole resistance. Gain of function variants in *PDR1* have previously served as a putative markers of azole resistance in *S. cerevisiae* [13].

A variant of interest of mutant was also found at *ERG25,* a gene on chromosome 7 that encodes a metho steryl oxidase. Metho steryl oxidases catalyze ergosterol demethylation and yield viable substrates for the ergosterol synthesis pathway [14, 15]. The catalytic activity of methol steryl oxidases is attributed to a fatty acid hydroxylase domain spanning amino acids 147-282 of the polypeptide [11]. W155L is a non-conservative missense variant, given that tryptophan (W) is a larger and thereby more steric amino acid than leucine (L). Given that this non-conservative missense variant occurs within the fatty acid hydroxylase domain, it can be hypothesized that W155L serves as a gain-of-function variant of metho steryl oxidase.

Next Step: Plasmid Rescue to Assay of Function Hypotheses of T304N and W155L

Follow-up experiments will be required to further characterize the T304N and W155L variants and establish a definitive link between these mutations and azole resistance phenotypes. This is exacerbated by a key limitation in this experiment, being that only one sample from the F10 and A10 strains was sequenced over a singular paired-end sequencing run. Given that the hypothesis for both these variants are that they are gain of function mutants, plasmid rescue experiments are an elegant method that exploits the diploid and haploid flexibility of *S cerevisiae.*

The T304N mutation is a hypothesized gain-of-function variant at the *PDR1* locus. Given the fact that *PDR1* is a transcription factor and a master regulator of numerous genes, a gene knockout followed by functional screen would not yield a clear link between variant and azole resistance.

Alternative approaches may include introducing a knock-out at the endogenous *PDR1* locus in wild type yeast using a digest of two restriction enzymes that recognizes consensus sequences on the 5’ and 3’ ends of the *PDR1* ORF. followed by transformation of a T304N mutant *PDR1* rescue plasmid. This plasmid would also encode *eGFP* to act as a reporter gene for transformation success.

Plasmid rescue and wild type cells without *PDR1* knockout would both be spotted on clotrimazole + YPD and YPD control plates and observed for growth of viable colonies. If T304N is indeed a *PDR1* gain-of-function variant that confers azole resistance, significant growth of viable green colonies should be observed on both clotrimazole + YPD and YPD control plates (Figure 3A).

This experiment can be replicated to test the hypothesis that W155L is a gain-of-function mutant. This would entail knocking out endogenous *ERG25* using two different restriction enzymes on the 5’ and 3’ ends of the *ERG25* ORF, then introducing a W155L *ERG25* rescue plasmid that contains *eGFP* or another reporter gene (Figure 3B).

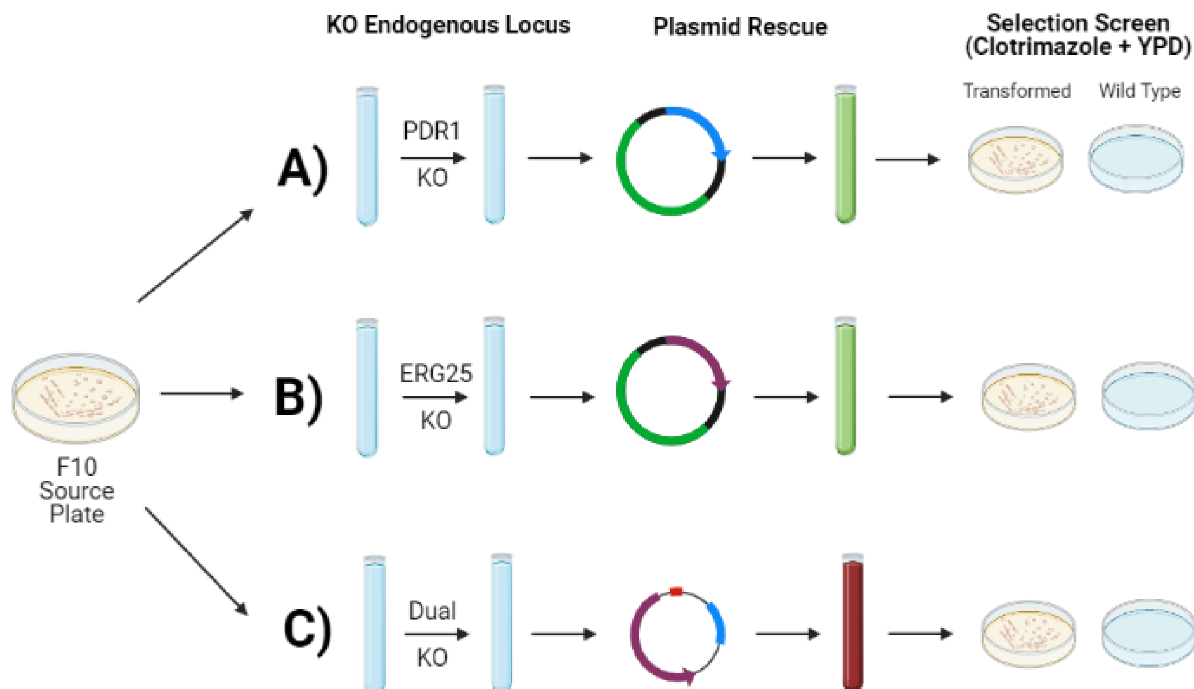

Figure 3. Outline of the proposed plasmid rescue experiment. A) Rescue with eGFP+ plasmid that contains T304N mutant PDR1, B) Rescue with eGFP+ plasmid that contains W155L mutant ERG25, C) Rescue of double knockout yeast with mCherry+ plasmid that contains both mutant ERG25 and PDR1 (Dual KO = Knock out of endogenous ERG25 and PDR1) Created with Biorender.com.

In the case where the plasmid rescue cells do not yield significant viable colony growth in the above assays, there lies an additional possibility that the contributions of W155L at *ERG25* or T304N at *PDR1* alone are not sufficient to induce clotrimazole resistance, however the combined contributions of both variants are sufficient. The previously described experiments can be amended to include dual knockouts of both the *ERG25* and *PDR1* loci. The rescue plasmid would encode W155L mutant *ERG25*, T304N mutant *PDR1*, and an *mCherry* reporter. A selection screen involving plating red transformed cells and wild type cells would be done under the same conditions as outlined above, with the red transformed cells expected to viably grow on azole + YPD and YPD control plates (Figure 3C).

Conclusions

Antimicrobial-resistant infections present a major global health threat that will need to be addressed in years to come. Proactive research into the mechanisms of AMR can serve to inform more robust future public health measures, as well as guide diagnostic and novel drug development efforts that aim to pre-emptively address this incoming health crisis.

Here I describe the sequencing and profiling of a Clotrimazole-resistant strain of *S. cerevisiae*, revealing two relevant genomic features. One feature is a putative resistance variant at the *PDR1* locus, and the other is a potentially novel variant that was detected at the *ERG25* locus.

The prospect of finding novel, defining variants that arise of AMR in model organisms, may provide a viable framework for profiling and anticipating how AMR will arise in pathogenic strains. By anticipating the mechanisms of AMR, experiments to uncover new therapeutics can be gudided with the intention of gaining a head start on this looming crisis.

List of Abbreviations Used

AMR: antimicrobial resistance
PDR1: pleiotropic drug resistance 1
ERG25: ergosterol biosynthesis 25
DNA: deoxyribonucleic acid
A: adenine
T: thymine
C: cytosine
G: guanine
bp: base pairs

nt: nucleotide
gDNA: genomic geoxyribonucleic acid
PCR: polymerase chain reaction
PDREs: pleiotropic drug response elements
PDR1: pleiotropic drug response 1
PDRp1: pleiotropic drug response protein 1
eGFP: enhanced Green Fluorescent Protein
YPD: yeast extract-peptone-dextrose
SIFT: sorting intolerant From Tolerant
W: tryptophan
N: asparagine
T: threonine
L: leucine

Conflicts of Interest

The author declares that they have no conflict of interest pertaining to the project or any of the described experiments.

Ethics Approval and/or Participant Consent

No ethics research board approval or consent was obtained in these experiments, as the project scope did not encompass any *in vivo* studies or involve patient-interfacing research.

Authors' Contributions

MT: Performed experiments described, interpretation and analysis of the data, drafted and finalized the manuscript

Acknowledgements

I would like to thank the undergraduate teaching staff at the Department of Molecular Genetics in the University of Toronto for providing the course environment that made this project possible. A special thank you to Lamisa Mizan and Dr. Atina Cote for their technical mentorship and guidance throughout the development of this project. Additionally, I would like to express my gratitude to Professor Maitreya Dunham (PhD), University of Wahsington, for providing the parental yeast strains used in this study.

Funding

This study was not funded.

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Article Information
Managing Editor: Jeremy Y. Ng
Peer Reviewers: Siobhan O'Brien, Robyn Loves
Article Dates: Received Mar 27 22; Accepted Jul 02 22; Published Jul 28 22

Citation
Please cite this article as follows:
Trinh M. Sequencing and detection of two missense variants at chromosome 7 as potential azole resistance markers in *Saccharomyces cerevisiae*. URNCST Journal. 2022 Jul 28: 6(7). https://urncst.com/index.php/urncst/article/view/363
DOI Link: https://doi.org/10.26685/urncst.363

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