DNASCRIPT

APPLICATION NOTE

Gene Knockout Mutagenesis

Mutagenesis studies depend on custom primers to construct cassettes to knockout target genes. These studies can be delayed days, if not weeks, when primers ordered from external suppliers are delayed. In this Application Note we demonstrate the use of primers printed at the bench using Enzymatic DNA Synthesis (EDS) in a gene knockout mutagenesis strategy to identify Candida genes with a putative role in antifungal tolerance. In-house production of PCR primers for next-day construction and screening of gene deletion mutants with DNA Script's novel EDS technology enabled steady progress amidst a global bottleneck in synthetic DNA supply. It also offered full control over primer sequences in a highly competitive area of research.

INTRODUCTION

In response to the growing threat of antifungal resistance to public health, research programs continue to emerge and scale to better understand the mechanisms of resistance in order to guide development of new therapies. These programs can leverage new technologies, such as benchtop Enzymatic DNA Synthesis (EDS), to overcome critical supply bottlenecks and achieve results faster. Candidemia occurs when members of the fungal genus *Candida* infect a patient's bloodstream. Treatment of candidemia is particularly difficult in immunocompromised patients for several reasons, including biofilm formation and resistance/tolerance to antifungal compounds. Three major classes of antifungals that are currently used for the treatment of invasive fungal infection (IFI) include echinocandins, polyenes, and azoles. *Candida glabrata* (Figure 1), the second most prevalent species responsible for IFI,[1] is able to rapidly acquire resistance to echinocandins and is naturally tolerant to azoles.[2,3] Novel antifungal compounds are therefore urgently needed.

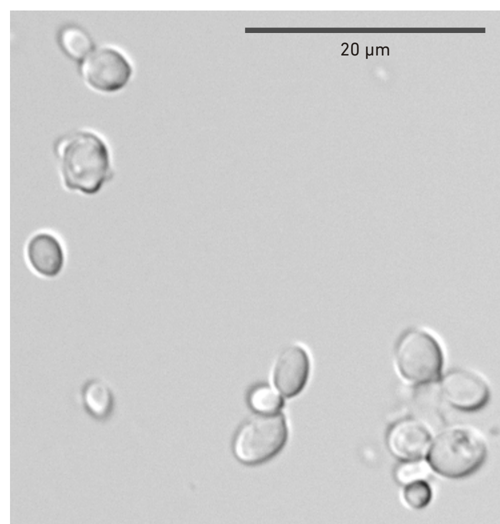

FIGURE 1. *CANDIDA GLABRATA* CELLS, as viewed by light microscopy.

This Application Note demonstrates the use of a reverse genetics approach, similar to that described in Schwarzmüller et al. (2014),[4] to identify and characterize genes involved in antifungal tolerance. Target genes were systematically deleted from *C. glabrata*, and the sensitivity of the resulting mutants to existing anti-fungal compounds was determined. Like most molecular techniques, the gene knockout mutagenesis strategy required access to custom-designed, synthetic DNA oligos.

At the start of this study, primers for the generation and screening of gene knockouts were procured from a commercial supplier. Commercial vendors use centralized manufacturing that relies on supply chain with complex logistics. The COVID-19 pandemic subsequently disrupted the supply chain and impacted access to synthetic oligos with delays of up to three months for standard PCR primers. DNA Script's SYNTAX System[5] was installed at the Institut Pasteur in February 2021, providing the opportunity to produce PCR primers in house for next-day use with the company's novel Enzymatic DNA Synthesis (EDS) technology.[6] This eliminated a major bottleneck and delivered results in time to

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meet project deadlines.

EXPERIMENTAL DESIGN

A gene knockout mutagenesis strategy (Figure 2) was designed for replacing individual, full-length target genes in the *Candida glabrata* (Anderson) Meyer et Yarrow (ATCC 2001)[7] genome with the *C. glabrata TRP1* gene—which complements tryptophan (Trp auxotrophy)[8]—by means of homologous recombination.

Knockout cassettes were constructed by PCR, as shown in Figure 3. Primer cassettes were initially ordered from an external supplier. When shipments from the supplier were delayed, primer cassettes were produced using the SYNTAX System. Each cassette consisted of the *TRP1* gene, flanked by 5'- and 3'-fragments of a targeted gene. *C. glabrata* ATCC 2001 trp- cells were transformed with knockout cassettes and resulting prototrophic mutants were verified by PCR.

The strategy was used to generate a total of 65 gene knockout mutants. Mutants and wild-type (WT) *C. glabrata* were subsequently grown on Trp-deficient SC medium in the presence or absence of one of four antifungal compounds (Table 1). Growth ratios were calculated to identify deleted genes with a putative role in tolerance to existing antifungals.

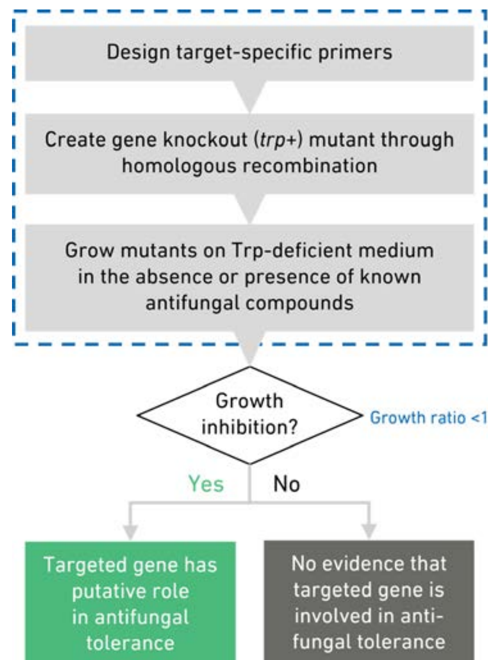

FIGURE 2. KNOCKOUT MUTAGENESIS STRATEGY DEVELOPED FOR THE GENERATION OF *C. GLABRATA* KNOCKOUTS. Boxed steps are outlined in more detail in Figure 3 on the next page. A mutant/WT growth ratio <1 in the antifungal susceptibility assay suggests growth inhibition in the presence of the antifungal compound included in the growth medium—and a putative role of the deleted target gene in the corresponding tolerance pathway.

TABLE 1. ANTIFUNGAL COMPOUNDS USED IN THIS STUDY.

Compound	Class	Mode of action
Amphotericin B	Polyene antibiotics	Forms complexes with ergosterol resulting in the leakage of cytoplasmic content[9]
Caspofungin	Echinocandins	Disrupts fungal cell wall production by inhibiting (1,3)-beta-D-glucan synthase[10]
Fluconazole **Voriconazole**	Triazoles	Disrupts fungal cytoplasmic membrane production by blocking the cytochrome P450-dependent synthesis of ergosterol[11]

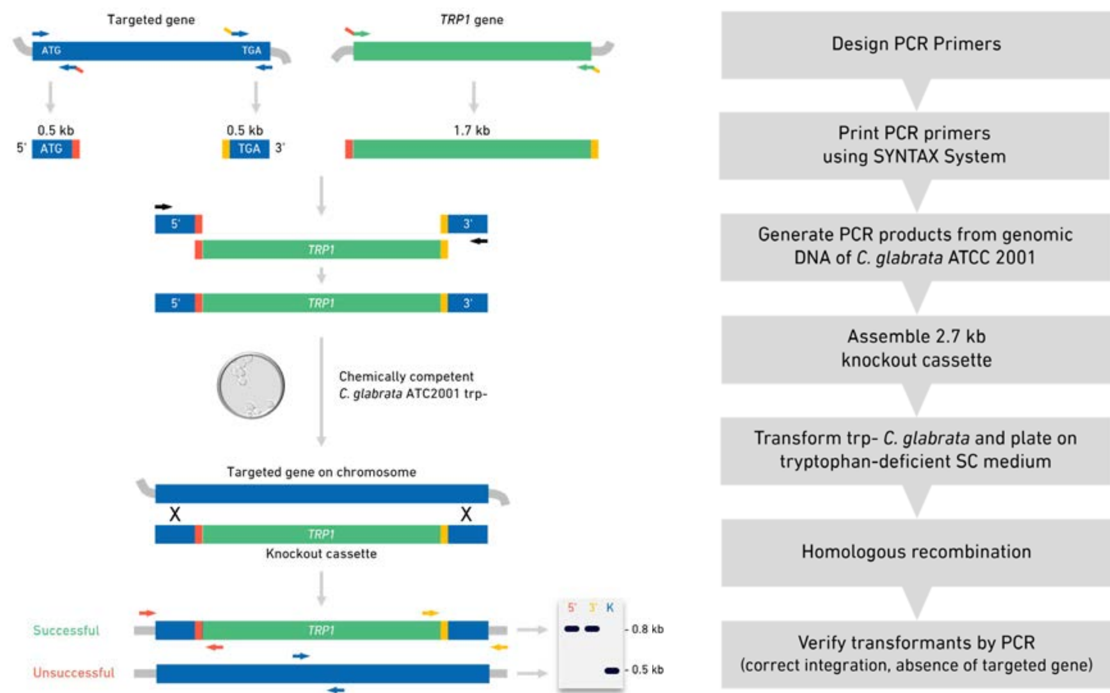

FIGURE 3. CONSTRUCTION OF *C. GLABRATA* KNOCKOUTS. Primers for amplification of the 5'- and 3'-portions of specific *C. glabrata* genes and the *TRP1* gene were designed using Primer BLAST.[12] The *TRP1* gene encodes a 217-amino acid phosphoribosylanthranilate isomerase involved in tryptophan biosynthesis.[8] Amplicons were generated using genomic DNA from the *C. glabrata* reference strain ATCC 2001 as template. Primers were initially ordered from a commercial supplier, but were subsequently produced in-house with the SYNTAX System when orders were delayed for multiple weeks. Chemically competent *C. glabrata* ATCC 2001 trp- cells were prepared, transformed with the knockout cassette (without post-PCR purification), and grown in SC medium as described in Materials and Methods. Colonies were screened by PCR for correct integration and the absence of the targeted gene. Primers, amplicons and gene fragments are not drawn to scale.

RESULTS AND DISCUSSION

Construction of knockout cassettes and *C. glabrata* deletion mutants

Twenty-six (26) gene knockouts were produced before our ability to source primers from our usual commercial supplier was impacted by the COVID-19 pandemic.

Ready-to-use oligos produced in-house using DNA Script's EDS-powered SYNTAX System were subsequently seamlessly integrated into the existing project, and were successfully used to produce an additional 39 gene knockouts. Specifically:

- No changes were required to the tools or strategy used for the design of gene-specific or screening primers.
- All types of amplification reactions (to generate or assemble PCR products, or screen transformants) were performed with existing protocols, without the need for modification. Representative examples of 5'- and 3'- target gene fragments and the *TRP1* locus amplified from *C. glabrata* genomic DNA are given in Figure 4. Overall, no apparent differences in PCR specificity, efficiency, and/or success rates were observed (as compared to results previously obtained with commercial primers; comparisons not shown).

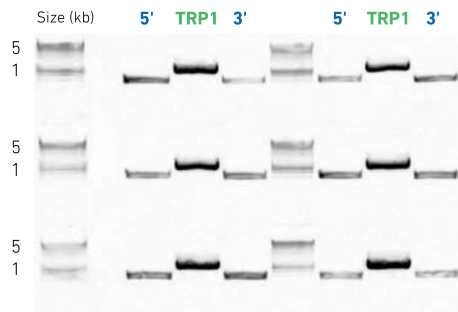

FIGURE 4. REPRESENTATIVE EXAMPLES OF PCR PRODUCTS PRODUCED WITH EDS PRIMERS. Each set of three amplicons represents the 5'- and 3'-portions of a different targeted gene (~0.5 kb each), and the amplified *TRP1* locus (1.7 kb) of *C. glabrata* ATCC 2001.

Results from antifungal susceptibility assays are shown in Figures 5 and 6.

Figure 5 contains representative examples of transformants grown on SC-agar in the presence of each of the four antifungal compounds listed in Table 1 using sub-MIC (Minimum Inhibitoty Concentration) concentrations.

Figure 6 shows the growth ratios for the 65 knockouts generated in this study, as well as 34 additional mutants generated with a similar experimental approach (but different selectable marker) in a different study. A significant number of genes with a putative role in antifungal tolerance were identified. Functional studies are ongoing to determine the precise cutoff for significant growth inhibition to render the assay more predictive of antifungal drug tolerance.

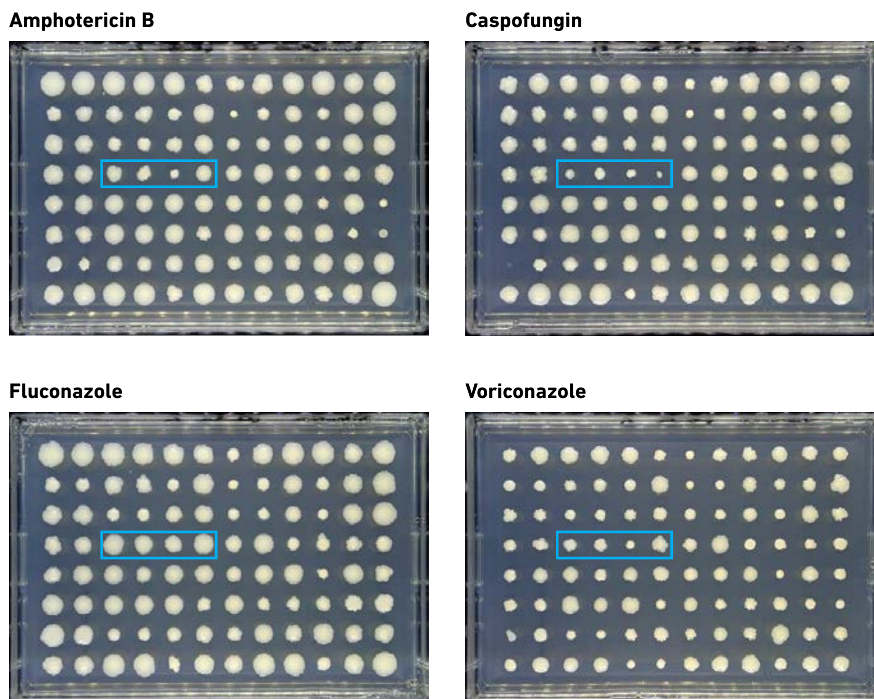

FIGURE 5. REPRESENTATIVE EXAMPLES OF ANTIFUNGAL SUSCEPTIBILITY ASSAYS. Colonies were generated on tryptophan-deficient SC medium, in the presence or absence (not shown) of an antifungal compound at sub-MIC concentration. Plates are oriented with well A1 in the top left corner. The first six wells in the top row contain wild-type *C. glabrata* (reference strain ATCC 2001). Verified deletion mutants (in which a targeted gene was successfully replaced with a selectable marker) were plated in quadruplicate, filling the rest of the plate from left to right and top to bottom. The mutant highlighted in the blue frames was generated with EDS primers. Based on colony diameters of the mutant strain grown in the absence (not shown) and presence of each antifungal compound, the gene deleted from this transformant may play a role in tolerance to all four antifungals, particularly caspofungin and voriconazole.

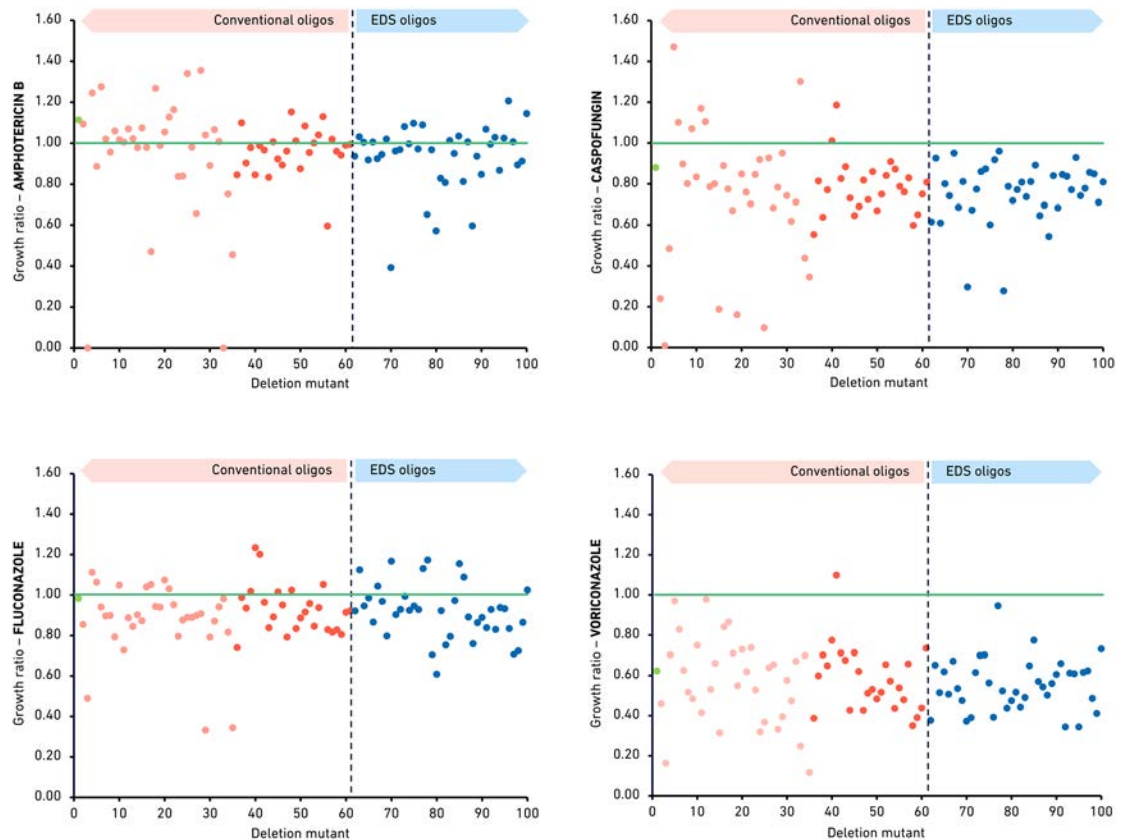

FIGURE 6. RESULTS OF ANTIFUNGAL SUSCEPTIBILITY ASSAYS PERFORMED WITH 99 GENE DELETION MUTANTS GENERATED WITH COMMERCIAL (CHEMICALLY SYNTHESIZED, RED) OR DNA SCRIPT (ENZYMATICALLY SYNTHESIZED IN-HOUSE, BLUE) PRIMERS. Lighter red dots represent mutants constructed in a previous study. Growth ratios were calculated by dividing the average colony diameter of each mutant grown on SC medium in the presence vs. absence of a known antifungal compound. Wild-type *C. glabrata* (green dot) was included as a control. Dots below the green line in each graph correspond to mutants with a growth ratio <1. Targeted genes with a putative role in tolerance to that specific antifungal compound (or class of compounds) are those below the green line (AMPHOTERICINE B and FLUCONAZOLE), or those below the WT green dot (CASPOFUNGIN and VORICONAZOLE).

CONCLUSION

A gene knockout mutagenesis strategy approach was successfully employed to identify fungal target genes with a potential role in drug tolerance to existing antifungal compounds.

Oligos produced with EDS technology were integrated into the project without the need to modify primer design or PCR parameters, and were used to construct more than one-third of a gene deletion library consisting of 99 mutants.

In-house, plate-based synthesis of ready-to-use PCR primers for next-day use eliminated a major experimental bottleneck during the COVID-19 pandemic. It also provides future opportunities to automate aspects of the experimental strategy, thereby accelerating the production and screening of additional knockouts.

In addition to faster iteration and predictable project schedules, in-house oligo production with the SYNTAX System also offered full control over primer sequences in a highly competitive area of research.

MATERIALS AND METHODS

Primer design and production

Oligos were designed using the NCBI primer design tool, Primer Blast.[12] Three types of primers (20-30 nt long) were designed:

- *Two gene-specific primer sets for each gene.* Primers were designed to amplify the 5'- and 3'-regions of individual target genes from the *C. glabrata* reference strain ATCC 2001.[7]
- *TRP1-specific primer sets.* Primers were designed to amplify the 654-bp *TRP1* open reading frame and flanking regions from *C. glabrata* ATCC 2001.
- *Primer sets for screening of transformants.* To confirm successful replacement of a full-length targeted gene with the *TRP1* gene, three sets of target gene-specific primers were employed.

Desalted primers were initially ordered from a commercial supplier, and were used in the construction and screening of 26 gene knockouts. From March 2021, ready-to-use primers for the construction and screening of an additional 39 mutants were produced in-house using a SYNTAX System (DNA Script part number 100296 or 100094) and SYNTAX 60-Cycle Kit, 96 oligos (DNA Script part number 100134) with standard synthesis plates. The system delivers desalted, quantified and normalized DNA oligos with a default 5'-phosphate, ready for use in molecular biology applications.

Construction of knockout cassettes

Genomic DNA, isolated from *C. glabrata* ATCC 2001 with the MasterPure™ Yeast DNA Purification Kit (Lucigen/LGC Biosearch Technologies), was used as the template for amplification of 5'- and 3'-regions of targeted genes, as well as the *TRP 1* gene.

PCR experiments were performed with Expand™ Long Range dNTPack (Roche) using the manufacturer's recommended procedure.

Expand Long Range dNTPack and the specific gene 5F/3R primers were subsequently used in an extension overlap (or fusion) PCR to generate a 2.7-kb knockout cassette for each targeted gene. A modified cycling profile, including a 15-min elongation step (68°C) after the initial denaturation, was used.

Generation and screening of gene knockouts

C. glabrata ATCC 2001 trp- cells were transformed with knockout cassettes in order to replace targeted genes with the tryptophan auxotrophy gene (one knockout cassette per transformation). Preparation and transformation of chemically competent *C. glabrata* ATC2001 trp- cells will be described elsewhere.

Prototrophic transformants were subjected to a PCR-based screen to confirm replacement of the targeted gene with the *TRP1* gene, using DreamTaq PCR Master Mix 2X (Thermo Scientific) according to the manufacturer's instructions. Amplification products were analyzed by agarose gel electrophoresis. .

Antifungal susceptibility assays

Verified mutants (65), as well as wild-type (WT) *C. glabrata,* and 34 gene knockout mutants produced as part of a different study (using the same experimental strategy, but with the *NAT1* gene as a selectable marker for nourseothricin resistance),[8] were plated in 96-well plates with the antifungal compounds and plates were incubated at 37°C for 4 – 5 days, and analyzed using a Phenobooth Colony Imager (Singer Instruments). Imaging software was used to measure the diameter of each colony (WT or gene deletion mutant), grown in the presence or absence of the antifungal compound. Growth ratios (shown in Figure 6) were calculated using the following formula:

$$\frac{\text{Avg. colony diameter on SC + antifungal}}{\text{Avg. colony diameter on SC only}}$$

ACKNOWLEDGMENTS

This study was performed in collaboration with Prof. Christophe d'Enfert, Head of the Fungal Biology and Pathogenicity Unit at the Institut Pasteur, Paris, France. Dr. Colin Clairet from the d'Enfert lab designed all of the primers used in this study, constructed knockout cassettes, generated and screened *TRP1*-complemented knockouts, and performed all antifungal susceptibility assays. The laboratory of Prof. Karl Kuchler, Medical University Vienna, Max F. Perutz Laboratories, Department of Medical Biochemistry, Vienna, Austria is thanked for nourseothricin-resistant mutants included in antifungal susceptibility assays. We also wish to thank Dr. Marc Monot, Head of the Biomics Unit, Department of Genomes and Genetics, Center for Technological Resources and Research, Institut Pasteur and his staff members for hosting the SYNTAX System.

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