CRISPR-Cas9 Mediated Elimination of Fermentative *S. cerevisiae*

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Abstract

Auto-Brewery Syndrome (ABS) is a rare disorder in which yeast colonies in the gut ferment ingested carbohydrates, leading to the production of ethanol. This can contribute to disorders such as non-alcoholic fatty liver disease. Current methods of ABS treatment, including carbohydrate-restricted diet, probiotics, and antifungals, have been met with varying success. However, modern studies in the use of CRISPR genome editing, which allows researchers to target and cut out relevant genes, indicate that it may be an effective remedy. This research investigated the efficacy of CRISPR gene-editing as a treatment by designing an efficient guide RNA and Cas9 system to target and cut out a vital gene of the ethanol-producing *Saccharomyces cerevisiae*, which would effectively terminate endogenous ethanol production. Plasmids targeting two genes were used. One housekeeping adenine-requiring gene (ADE2) and one essential gene, tRNAHis Guanylyltransferase (THG-1), were each targeted by complementary guide RNA. Each plasmid was then incorporated into *S. cerevisiae* cells using electroporation, followed by analysis of the CRISPR model's efficiency. It was shown that the plasmids caused significant fatal damage to the *S. cerevisiae* cells, demonstrating the potential for CRISPR's use in treating ABS patients.

Introduction

Auto-Brewery Syndrome, a disorder involving fermentation of yeast after eating, causes intoxication without consumption of alcohol. This fermentation often occurs following high-carbohydrate meals or antibiotic therapy (Painter et al., 2020). The syndrome is particularly of interest because it can be detrimental to one's personal life as well as contribute negatively to disorders such as non-alcoholic fatty liver disease (Campo, Eiseler, Apfel, & Pyrsopoulos, 2019).

Previous methods of ABS treatment have failed to be universally effective; however, modern studies in the use of CRISPR genome editing offer a possible solution. The CRISPR-Cas9 method allows researchers to target specific sequences in an organism's DNA in to make intricate edits or, in this case, cut out essential genes. In effect, this technology could be a useful tool for causing death to harmful microflora (Gomaa *et al.*, 2014).

To construct the plasmids, a protospacer adjacent motif (PAM) sequence in the DNA is used to locate the target sequence to be edited. The Cas9 protein and guide RNA (gRNA) then complex to find and cleave the gene of interest for each plasmid.

The main species of yeast implicated in ABS is *Saccharomyces cerevisiae.* This research project aimed to induce targeted fatal damage to the DNA of *S. cerevisiae* using CRISPR-Cas9 gene editing technology. CRISPR-Cas9 plasmids were designed to target two specific genes in *S. cerevisiae*: housekeeping adenine-requiring gene (ADE2) and essential gene tRNAHis Guanylyltransferase (THG-1), that should result in fatal DNA damage. A control plasmid targeting only Leu2 was also created. Each plasmid was incorporated into competent *S. cerevisiae* cells using electroporation, followed by growth on selective media and analysis of the CRISPR model's efficiency.

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Results

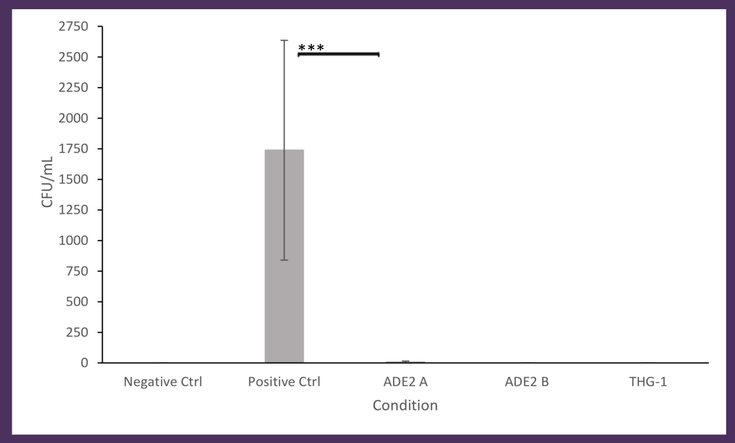


Figure 1. Mean CFU (colony forming units) per mL for *S. cerevisiae* following electroporation. This graph shows the mean CFU growth per mL of cells for each condition. Error bars indicate standard error. *** indicates a p value of 0.000018.

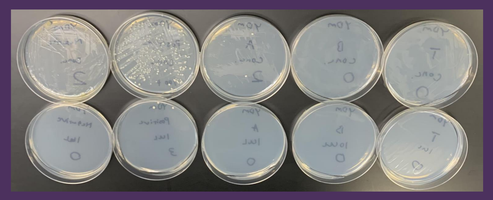
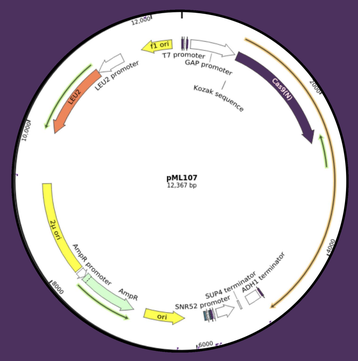


Figure 2. Comparison of growth resulting from concentrated cells and 1 µl loops on drop-out media for each condition. Electroporated cells were plated with concentrated cells (top) and with 1 µl loops on drop-out media (bottom). From left to right: negative control, positive control, ADE2 A, ADE2 B, THG-1.

For each condition, extensive growth on YPD media confirmed cell viability following electroporation. For the positive control which contained a plasmid that only contributed the necessary marker to grow without leucine, there was average growth of 1.74 x 10^3 CFU/mL . The only other condition that produced growth on drop-out media was ADE2 A which had an average growth of 7 CFU/mL. Using a one-sample two-way T-test, we found a significant difference (P=0.000018) between the growth of the positive control and ADE2 A.

Plasmids

Figure 3. Diagram of positive control pML107 plasmid vector with Leu2 marker. This was our base plasmid (empty vector) from which the others were built. The orange arrow indicates the Leu2 marker.



CTAGAACAGTTGGTATATTAGGAGGGGGACAATTGGGACGTATGATTGTTGAGG

CGATGTTATTTTGCCTCAGTGTTATATTGTGGTAAGAATAGACGGCAAGAAGTTT

Figure 4. Potential target sequences for pML107 plasmids. The target sequence for ADE2 (top) and THG-1 (bottom) are shown. Green letters are cut spots and blue letters indicate PAM sequences. Other colors indicate target sequences to build the gRNA to. The sequence with the highest efficacy score (in red) was chosen for this research.

Methods

pML107 plasmids specific to *S. cerevisiae* were created by the University of Utah CORE lab. Each was designed with a different target: the housekeeping Phosphoribosylaminoimidazole carboxylase (ADE2) gene (two identical plasmids from different bacterial colonies), essential tRNA guanylyltransferase (THG-1) gene, and each plasmid with an off-target Leu2 gene. To prove the effectiveness of the electroporation, each set of cells was grown on media lacking leucine to prove they had taken up the Leu2 gene. The negative control was sterile water while the positive control was a plasmid containing only the Leu2 gene, which should allow the cells to grow on selective media.

Electrotransformation

To achieve electrocompetency in the yeast, *S. cerevisiae* cells (ATCC 200901) were grown in yeast peptone dextrose (YPD) broth until reaching an optical density (OD) of 0.5-1.2. The cells were then spun down at 1100 g at 4°C for 5 minutes (Fig. 2). This was followed by decantation of the cells and resuspension in sterilized H2O (ice-cold), followed by another centrifugation and decantation step. Then, the cells were treated with 20 mL of 0.1 M LiTE (16 mL 1M sorbitol plus 2 mL 10xTE buffer plus 2 mL 1 M LiAc). They were then incubated at 30°C for 30 min. Following this, 0.2 mL of 1 M DTT was added, then incubated again at 30°C for 15 min, then decanted.
The cells were washed in sorbitol twice more, with a 5-minute centrifugation step between them. Following the final centrifugation step, aliquots were transferred to sterile microcentrifuge tubes and resuspended in sorbitol.

Electroporation

The following procedure was repeated for each condition: negative control (water), positive control (plasmid with just Leu2 marker), ADE2 A, ADE2 B, and THG-1. Once the *S. cerevisiae* cells were competent, 5 uL of plasmid was added to 100 uL of competent cells. Cells were added to a 0.1 cm chilled cuvette. In which they were electroporated at a pulse charge of 1.85 kV, 200 ohms, 25 uF (pulse time of 3.0-3.4 ms). A sorbitol/YPD (1:1) mixture was added immediately after the pulse then incubated for one hour at 30°C. Cells were then spread on selection media; for each condition, cells were plated on drop-out media with 10 µL loops, YPD media with 10 µL loops, drop-out media at 1 µL, and concentrated (estimated 100 µL) on another drop-out plate (15 dropout, 5 YPD).

Discussion

- Positive control grew and negative did not
  - Plasmid was successfully taken up by cells
  - Indicates successful electroporation
    - Conditions ADE2 B and THG-1 did not grow because their vital genes were successfully cut out by the CRISPR-Cas9 system.
    - Significant difference in growth ($p<0.0001$) between positive control and ADE2 A also indicates electroporation was successful
- Due to limitations in plasmid volume, replicates of this study would be useful to reproduce our findings
- For future research, CRISPR-Cas9 could be used to kill other suspected causative agents of ABS, especially other yeasts.
  - Could eventually be tested in vivo to cure patients that suffer from ABS.
  - It can be possible to one day treat the illness by incorporating a plasmid that targets the THG-1 or ADE2 gene of *S. cerevisiae* or genes of another causative species