Mutating the ADE2 Gene in *Saccharomyces cerevisiae* Using the CRISPR-Cas9 System and Homology Directed Repair

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Abstract

CRISPR-Cas9 was discovered in bacteria as an adaptive immunity mechanism (Li et al, 2021). CRISPR-Cas9, known as Clustered Regularly Interspaced Short Palindromic Repeats and CRISPR-associated proteins, utilizes a guide RNA to direct its endonuclease activity (Harrison et al, 2014). We explored this mechanism by mutating the ADE2 gene in *Saccharomyces cerevisiae*, also known as brewer's yeast. ADE2 is utilized for purine synthesis in yeast while already in the presence of adenine (Bharathi et al, 2016). By targeting this gene, we introduced the CRISPR-Cas9 mechanism and homology directed recombination templates and were able to analyze phenotypic and genotypic changes. When ADE2 is mutated, we can observe a red pigment that accumulates within the yeast (Gedvilaite et al,1994). Our experiment shows the types of mutations induced through homology directed repair vs. non homology directed repair and HDR incorporation rate into yeast cells. In our experiment we saw that HDR templates were introduced on average 3 times into a yeast cell with an 91% induction rate of point mutations resulting in premature stop codons. 90% of frameshift mutations were produced by the NHEJ repair mechanism. We also observed that cells not incorporated with HDR had induced deletion mutations rather than point mutations through NHEJ.

Introduction

CRISPR-Cas9 is a powerful genetic tool that is used in the natural world by bacteria against foreign invaders. Clustered Regularly Interspaced Short Palindromic Repeats and CRISPR-associated proteins paired with a guide RNA complex get directed to the site of interest which includes a protospacer adjacent motif sequence (PAM) that is identified by the Cas9 protein and performs endonuclease activity (Wilson et al, 2018). CRISPR's unique gene editing through a guide RNA endonuclease activity ability is increasingly becoming an interest in the field of biology. We chose to explore the CRISPR-Cas9 system with yeast, as it is a relatively simple and widely available organism. The plasmid which was transformed into our yeast was chosen due to its possession of the ampicillin resistance from *E.coli* and Cas9 expression, making it an ideal plasmid for CRISPR (Laughery et al, 2015). Yeast encodes the ADE2 gene, a gene important for purine biosynthesis in *Saccharomyces cerevisiae* (Ugolini et al, 1996). When ADE2 is functioning properly, wild type yeast produces a white phenotype. Through an auxotrophic process, when mutated, a red pigment in the yeast cell accumulates and produces a red phenotype as well as genotypic changes (Pronk, 2002). Our goal in this experiment was to introduce CRISPR-Cas9 and gRNA

and pML-104 plasmid complex into yeast cells to create double standard breaks in the targeted sequence of the ADE2 gene. By this process we could overwhelm the yeast with uniquely mutated HDR templates to allow the yeast to repair itself with the donor DNA, in turn producing a yeast cell that contained a mutated ADE2 gene. In our experiment, we aimed to analyze what type of mutations were introduced through the CRISPR-Cas9 system and HDR mechanism vs what mutations were introduced by non-homologous end joining. Homology directed repair utilizes donor DNA as its repair mechanism (Sansbury et al, 2019). On the other hand, non-homologous end joining joins the broken DNA strands together and often results in errors during repair (Sharma et al, 2010). Utilizing CRISPR's endonuclease activity we created a double stranded break in our target sequence and sequentially designed and created copies of an HDR template with a gRNA and PAM sequence to guide our Cas9 protein. We also examined how many average HDR templates will be included into a cell when paired with the CRISPR-Cas9 system.

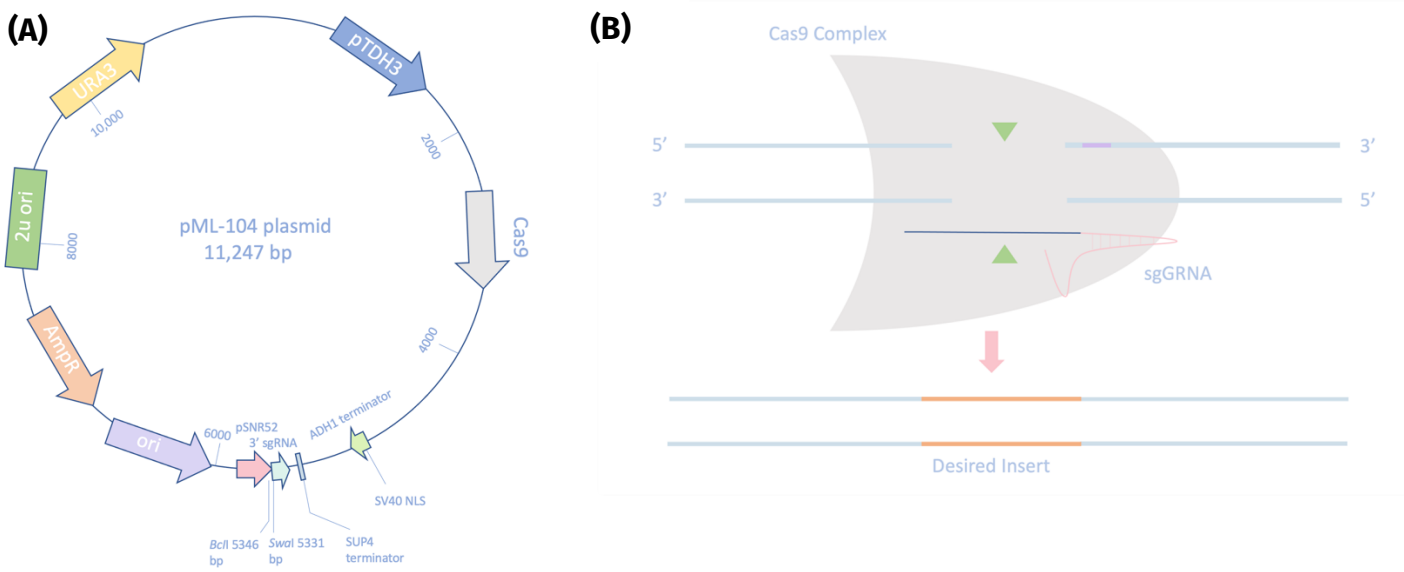


Figure 1:
Overview of pML-104 plasmid and Cas9 complex
(A) Image of the pML-104 plasmid cultured in *dam-/dcm-* strain of *E.coli*. The plasmid is 11,247 bp and contains the URA3 marker for yeast selection, AmpR resistance from *E. Coli*, Cas9 protein from *Streptococcus pyogenes*, the origin of replication for yeast 2u ori, and ori as the origin of replication for *E.coli* (Laughery et al, 2019). *BcII* and *SwaI* are Type II restriction enzyme cut sites between which our gRNA was incorporated and cut close to the recognized DNA sequence (Pingoud et al, 2011). **(B)** Diagram of homology directed repair complex. The gRNA complex will guide Cas9 to desired template through recognition of PAM sites (purple). By inducing double stranded breaks by the CRISPR-Cas9 gRNA complex and introducing HDR templates with

mutations into the yeast, aiming for yeast uptake of the HDR template through homology directed repair rather than non-homologous end joining (NHEJ).

Methods

Analyzing Sequences to Mutate and gRNA Design

We began our experiment by exploring gene sequences through bioinformatics software ApE. We analyzed the sequence of the ADE2 gene through the BLAST database and inputting it into ApE where we explored possible sites to induce mutations. Once our target region was elected our goal was to design a gRNA and HDR template (Figure 4A). Utilizing BLASTP we explored what sequences were conserved within *Saccharomyces cerevisiae* and other species. To design our gRNA, we utilized the CRISPRdirect program. Our 100-base pair HDR templates were designed with 3 premature stop codons and 3 point mutations and ordered from a company. To ensure that Cas9-gRNA did not cut again, the PAM sequence was also mutated. 3 mutations were introduced downstream of the gRNA sequence while an addition stop codon was introduced in the PAM sequence. A single point mutation was made upstream of our gRNA (Figure 4B).

Restriction Enzyme Digest and Bacterial Transformation

We digested our plasmid to allow the gRNA complex to ligate with the pML-104 plasmid. We performed a double digest as well as single and no digest for negative controls. Digestion of oligo plasmid occurred between the *BcII* and *SwaII* restriction enzyme sites. To analyze our results, we analyzed previous classes gel electrophoreses results with a *HindIII* ladder. After digestion, a column clean-up of our plasmid was performed to ensure removal of stuffer and to allow greater efficiency of ligation. During column cleanup, DNA was bound to a silica column while unwanted particles flowed through the column. Once our oligo gRNA was hybridized we transformed *E.coli* with our cut pML-104 plasmid + gRNA (cut plasmid, insert, ligase) as well as control 1 (cut plasmid, insert, no ligase) and control 2 (cut plasmid, ligase, no insert).

Analyze HDR Templates and Miniprep

Our HDR templates were synthesized and amplified commercially. Polymerase chain ration, also known as PCR, was performed to synthesize double stranded HDR templates. PCR cycling conditions of our HDR templates comprised of of 95° C for 2 minutes, 2 cycles of 48° C annealing for 30 seconds and a 72° C extension for 15 seconds (McDonnel et al). Primer sequences included a forward primer of 5'-CGGACAAAACAATCAAGTATG-3' and reverse primer of 5'-GGAGAATTTTCAGCATCTAG-3'. To ensure purity of our HDR template a polymerase chain reaction column clean-up was performed. Warm elution buffer was added to our column cleanup to maximize product yield. To analyze our HDR templates we performed gel electrophoresis. Miniprep cultures were prepped for pML-104 plasmid gRNA with bacterial colony and 10 ul ampicillin in LB media and incubated overnight.

Extracting pML-104 gRNA Plasmid and Yeast Streaking
Before our plasmid extraction, *E.coli* bacterial cultures were grown. Due to this laboratory being online, our bacterial cultures were prepared beforehand. QIAprep Alkaline lysis plasmid purification with a an alkaline SDS solution and high salt buffer was performed to extract pML-104 plasmid from bacteria. To assess purity and concentration of plasmid, sample was loaded on a nanodrop. Data was not derived from nanodrop because this laboratory was performed online. Nanodrop evaluation was followed by gel electrophoresis was used to visualize plasmid. Yeast was spread all around YPD plates and grown at room temperature for 2 days.

Yeast Transformation Analysis and Phenotype Assessment
Yeast was transformed with transformation mix and treatments (pML-104 control, pML-104 gRNA and HDR, pML-104 gRNA, and water) and incubated for 24-48 hours. Following incubation period, yeast was plated onto YC-ura dishes from each transformation tube and incubated again for 2 days. Phenotypic assessment of colonies was carried out post incubation.

Extracting Genomic DNA and ADE2 PCR
Genomic DNA was extracted from yeast and was succeeded by PCR to amplify our desired ADE2 gene region with primer pairs (Figure 2A) prepared ahead of time. PCR cycling conditions included denaturing at 95° C for 30 seconds, annealing at 45-60° C for 30 seconds and 72° C for 30 seconds, and extension at 15° C for 2 minutes and utilized *Taq* polymerase vs DNA polymerase.
Individually we extracted gDNA for two cultures which included red colonies and HDR templates (698 and 699) to send for sequencing.

(A)

Primer	Melting Point
D-FW 5' – TTGTTGCATGGCTACGAACC – 3'	59° C
E-RV 5'- CTTTACAACGAAGTTACCTCTTCCA -3'	59° C

Figure 2:
PCR Primers
(A) Primers D-FW and E-RV were used for our ADE2 gene PCR. These primer pairs gave us an expected PCR length to be around 679 base pairs when analyzed on a gel.

Analyzing PCR of ADE2 and Sending for Sanger Sequencing
Our amplified ADE2 region was assessed by gel electrophoresis. PCR samples were then cleaned through a column cleanup and checked for purity was a nanodrop.

Samples were then prepped and sent for Sanger sequencing, a chain termination method utilizing fluorescent labeling and ddNTP's (Crossley et al, 2020). Chromatograms and sequences were analyzed using ApE individually with each student analyzing 4 sequences, including pML-104 gRNA and HDR with red/pink phenotype (Figure 6C and D), pML-104 grNA with red/pink phenotype, pML-104 gRNA and HDR with white phenotype, and pML-104 gRNA with white phenotype Following individual analysis of sequencing data, multiple sequence alignments were compared to the wildtype ADE2 gene and later pooled into an Excel file with data from other classes.

Results

Bacterial Transformation

E.coli transformations were analyzed using previous classes pooled data (Figure 3A). Bacteria was transformed with uncut pML-104 plasmid, cut pML-104 plasmid + gRNA (cut plasmid, insert, ligase) as well as control 1 (cut plasmid, insert, no ligase) and control 2 (cut plasmid, ligase, no insert). Uncut pML-104 plasmid with intact ampicillin resistance cassette yielded the highest number of colonies and had nearly two-fold more colonies than cut pML-104 plasmid. Controls 1 and 2 yielded similar number of colonies while no colonies grew with water only. When ligase or insert was no included in our transformations, we saw a significant decrease in colony growth compared to the uncut plasmid.

(A)

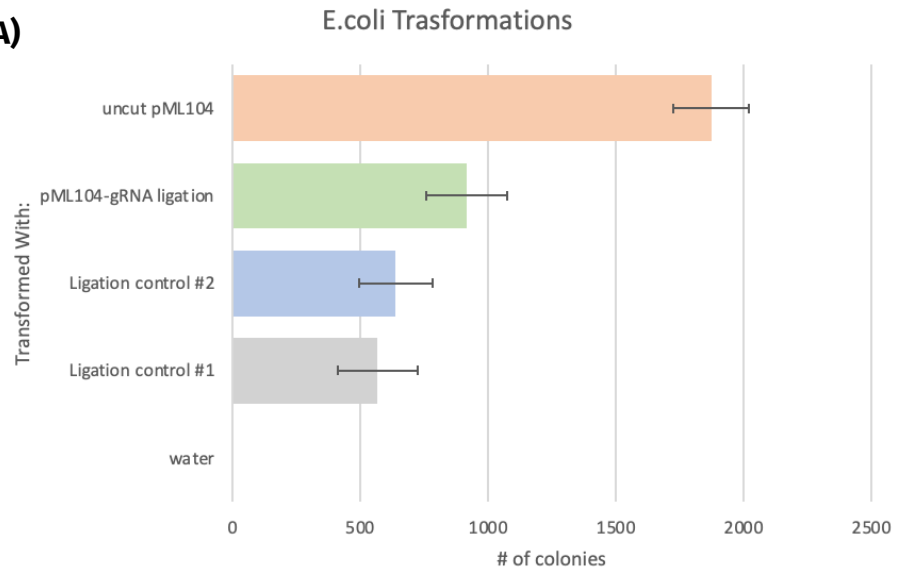


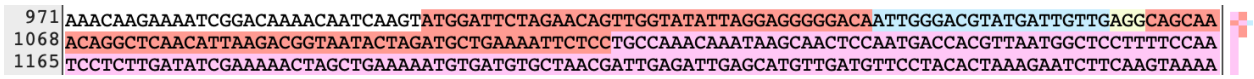
Figure 3:
E. coli Transformations
(A) Transformation data from previous classes. 5 different transformations were analyzed, and colonies were counted for each transformation. Uncut pML-104 plasmid had the highest transformation efficiency due to plasmid and ampicillin resistance marker being intact and grew nearly double the number of colonies as cut

pML-104. Standard deviation between the 5 transformations was calculated and included in error bars.

HDR and gRNA Construction

Mutated HDR contained 3 premature stop codons and 2 point mutations (Figure 4B). Our gRNA, which was included in the HDR template, included a premature stop codon as well as a mutated PAM site (Figure 4B).

(A)



(B)

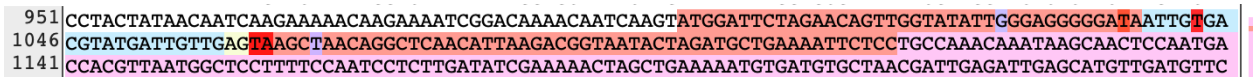


Figure 4:
HDR and gRNA Design

(A) Original unmutated HDR template (salmon), coding region (pink), gRNA (blue) and PAM (yellow). Unhighlighted region is the noncoding region. HDR template is 100 base pairs. **(B)** Mutated HDR template with premature stop codons (red) and point mutations (purple). PAM site was also mutated to prevent recutting by gRNA.

Plasmid Extraction

To analyze purity of our plasmid extraction we utilized a nanodrop. We received a concentration of 444.8 ng/ul with A260/280= 1.90. Our pML-104 plasmid ran at 11.2 kb when gel electrophoresis was performed.

Yeast Streaking and Transformations

Our yeast plate (before transformations) did not achieve any yeast growth therefore another groups yeast was utilized for transformations. We analyzed 4 different yeast transformations including a pML-104 control, pML-104 gRNA and HDR, pML-104 gRNA only, and water. For individual yeast transformation results we found the pML-104 control produces 63 white colonies. Yeast transformations with pML-104 gRNA and HDR produced 50 red colonies. pML-104 gRNA produced 1 colony white colonies, and no colonies and negative control (water) produced 0 colonies (Figure 11). Group transformations were analyzed and pooled into an excel sheet. PML-104 gRNA and HDR was nearly two-fold fraction of red colonies/total colonies if pML-104 gRNA and forty-fold that of pML-104 with no gRNA. Water only resulted in no colonies. Overall, we saw a greater number of red colonies when both gRNA as well as HDR are

included in our treatments compared transformations with no gRNA or HDR templates.

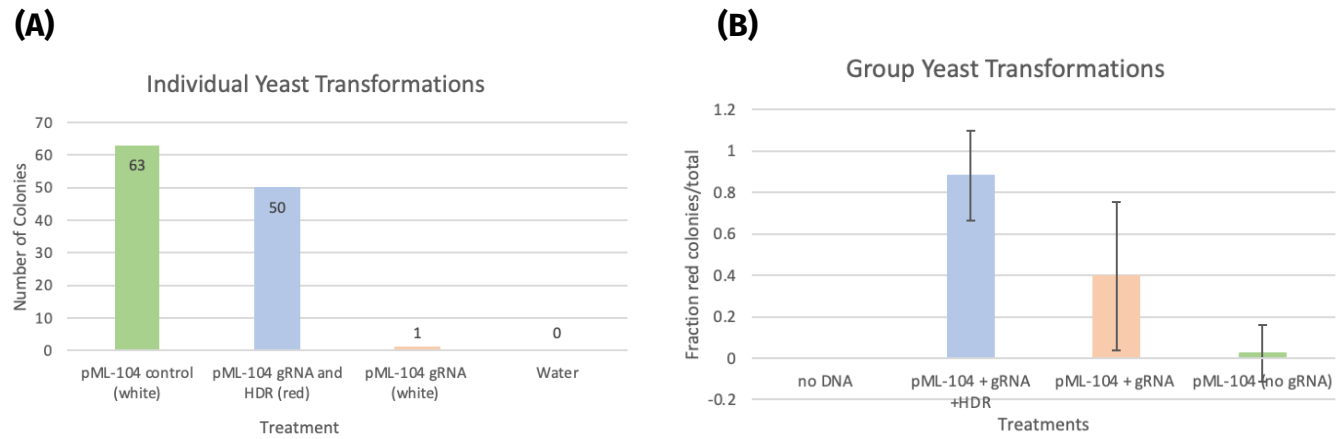


Figure 5:

Individual and Group Yeast Transformations

(A) Data was taken from 4 individual YC-ura containing *Saccharomyces cerevisiae*. Each plate containing a different transformation. Number of colonies in each plate is displayed. Standard deviation was not calculated because data about each treatment was only taken from a single plate. **(B)** Group result transformations from 296 replicates was included. Any transformation that resulted in inconclusive fraction of red colonies/total (resulted in zero) was discarded. Standard deviation was derived from each transformation and included in error bars.

ADE2 PCR and Sanger Sequencing

We analyzed culture 698 and 699 which included red colonies and HDR. Primer pairs were designed outside of our laboratory and resulted in a product that was around 679 base pairs. Following column cleanup of PCR products nanodrop results yielded A260/A280 values for 698 was 2.5 with a concentration of 75.3 ng/ul and 2.61 for 699 with a concentration of 54.0 ng/ul. When analyzing pooled sequencing results for ADE2 chromatograms and sequences, we found that most mutations containing HDR templates included point mutations resulting in premature stop codons (Figure 6A). NHEJ mutations indicated high levels of deletions, resulting in a frameshift in the sequence. As our HDR template was designed with point mutations which resulted in premature stop codons, our chromatogram and sequence analysis reflects that of our HDR templates. A total of 192 sequences were analyzed and any poor-quality chromatograms were not included in the data.

Red phenotype yeast colonies on average had incorporated more HDR templates that colonies producing white phenotypes (Figure 6B)

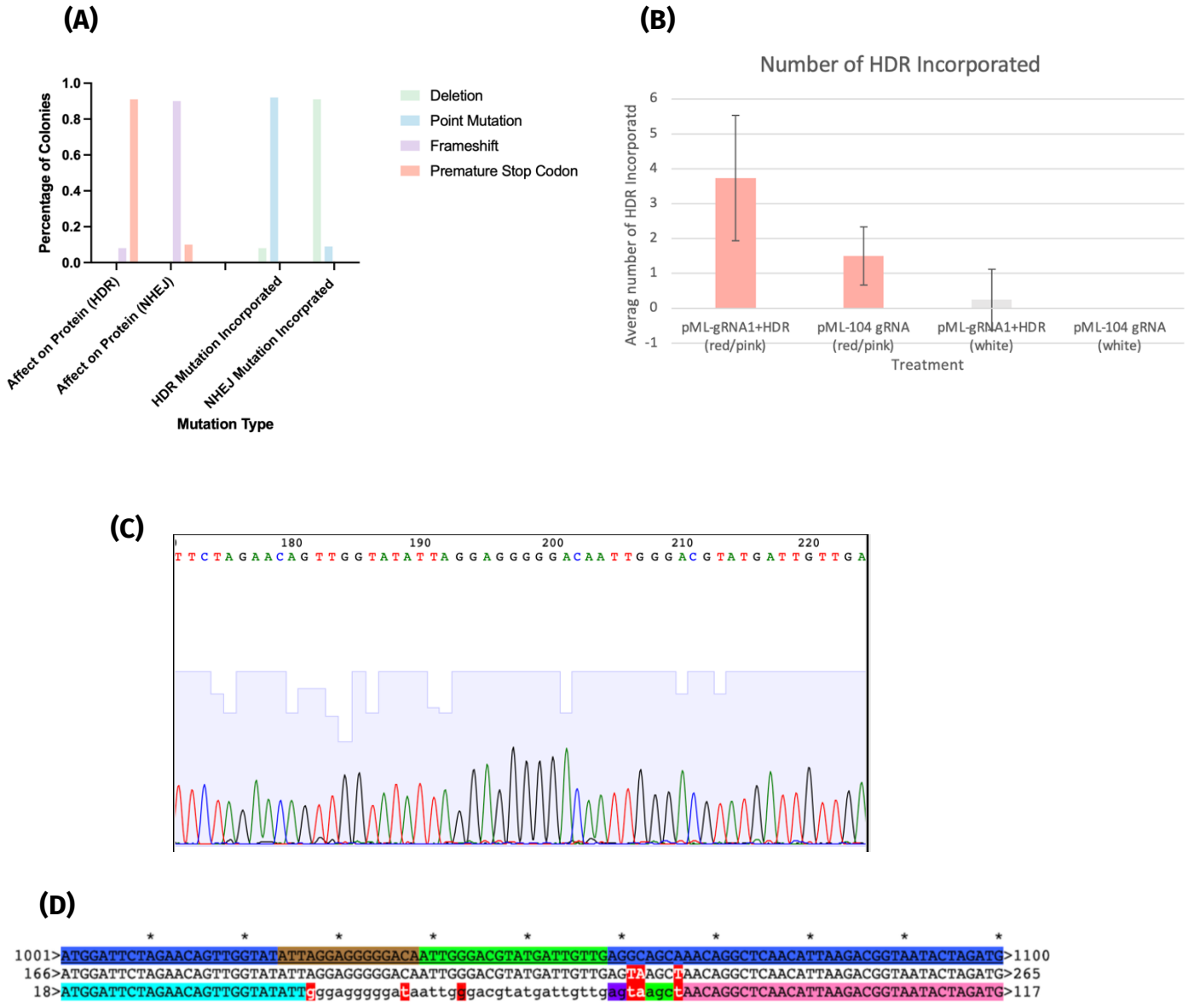


Figure 6

Mutation Types and HDR Templates Incorporated

(A) HDR templates incorporated into our ADE2 gene had the greatest impact on the protein through premature stop codon mutations. Percentage of HDR related premature stop codons amounted to 91% with 9% coming from NHEJ. HDR template resulting in premature stop codons mutations were mainly due to point mutations, whereas NHEJ produced deletions which resulted in a frameshift of the protein. Data consists of 71 replicates and was calculated by a two-way ANOVA test. Standard deviation was not included. Sequences which resulted in poor chromatograms were discarded. **(B)** Colonies containing PML-104 gRNA and HDR had on average 3 HDR

templates implemented that produced a red phenotype, while colonies that produced white phenotype averaged less than one HDR implemented. Standard deviation was included in error bars. **(C)** Chromatogram of Sanger sequence 698 which was transformed pML-104 gRNA and HDR1 and had a red/pink phenotype. Nucleotide A is labeled fluorescently with green, G with black, C with blue and T with red. **(D)** Mutated 698 sequence with 3 incorporated HDR templates between (middle row). Coding sequence is labeled in blue, gRNA in brown, and HDR template is included in light blue, purple, and pink.

Discussion

In our experiment we tested what types of mutations CRISPR-Cas9 can induce when paired with a homology directed repair template. By designed HDR templates with desired mutations and pairing it with the CRISPR-Cas9, we were able to transform yeast colonies and observe phenotypic and genotypic changes.

Due to the nature of the course, many of our beginning laboratories were performed online. Often, we had to use previous course data to analyze results, and our class HDR templates and gRNA's were all designed outside of the laboratory identically. Therefore, our data can only represent for our induced mutations in the HDR. Future experiments may need to be performed with variable amounts of mutations and in different locations of the ADE2 gene to be representative of the scope of CRISPR-Cas9's endonuclease activity. Our HDR templates and mutations were included in the coding region of the ADE2 gene so future experiments may need to be carried out to demonstrate the CRISPR-Cas9 system and HDR mechanism when different regions of the ADE2 are targeted, for example the promotor.

Our yeast originally did not grow. Possible causes may have been contamination or yeast was killed off during sterilization. We utilized another groups yeast experiments for the rest of our transformations. When performing individual yeast transformations (Figure 5A) we observed an unexpectedly low number of colonies for our pML-104 gRNA transformation. A possibility of this result may have been poor sterilization or incorrect spreading technique. When assessing purity of our samples during gRNA extraction and after PCR of our ADE2 genes, our values indicated RNA contamination. Although we saw contamination, samples were used and later discarded if chromatograms were poor quality. When data was pooled we a significant fraction of red colonies when transformed with pML-104 gRNA and HDR (Figure 5). This showcases that HDR was getting incorporated into our ADE2 gene and inducing mutations at a much higher rate than just a plasmid with gRNA, a plasmid with no gRNA, or water. Our experiments also are effective at showing what types of mutations HDR vs NHEJ induces. We see a clear indication that HDR induces point mutations which often results in a premature stop codon. We expected these results because we designed our HDR templates to include 3 premature stop codons. NHEJ, an error prone method of repair, often resulted in expected deletions as its mechanism is simply ligating strands back together. We also saw that when yeast was overwhelmed with HDR templates, we noticed a significantly higher rate of HDR incorporated with an average of 3 HDR templates incorporated into the gene.

Overall, our experiments displayed the incredible activity of the CRISPR-Cas9 and homology directed repair mechanisms in *Saccharomyces cerevisiae.* CRISPR is an ever-growing field of interest in biology and may one day have ground a groundbreaking role in medicine, genetics, and molecular and cell biology.

This report is formatted per Journal of Cell Biology Guidelines.

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