Genetic Engineering of Probiotic Yeast for Improved Secretion of Antimicrobial Molecules

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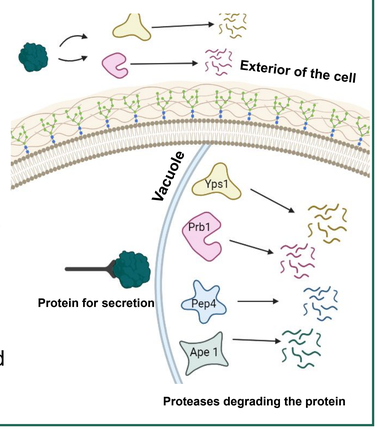
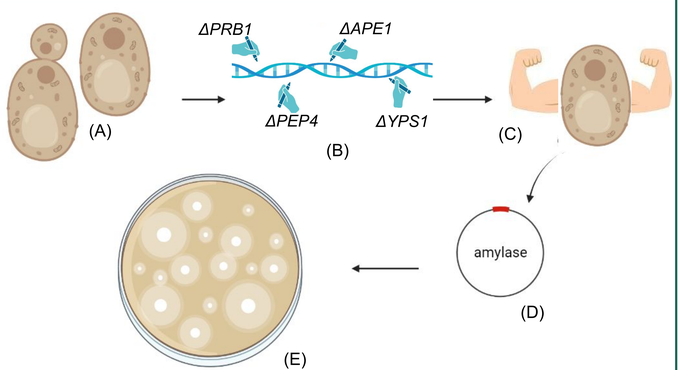
ISEST

Introduction

- *Clostridium perfringens* is a pathogenic bacteria that is found in the gut and is the main cause of necrotizing enteritis (NE), which is characterized by small intestinal inflammation.
- It releases toxins that result in symptoms including diarrhea, bloating, and nausea. Broiler chickens are commonly infected with this pathogen as a result of contamination in their feed. Fatality rates are close to 50%, which costs the chicken industry $6 billion annually.
- The infection has frequently been treated with antibiotics. However, they were prohibited from being included in poultry feeds in 2006 due to increased prevalence of antibiotic-resistant strains.
- Probiotics are live cells that help modulate the digestive environment; they have been found to be an effective alternative to antibiotics. However, their natural defenses against infections are insufficient.
- This study's goal is to precisely kill *C. perfringens* by modifying the probiotic yeast, *Saccharomyces boulardii*, to better secrete antimicrobial peptides. This was done by removing proteases implicated in the degradation of these antimicrobial peptides.



Methods

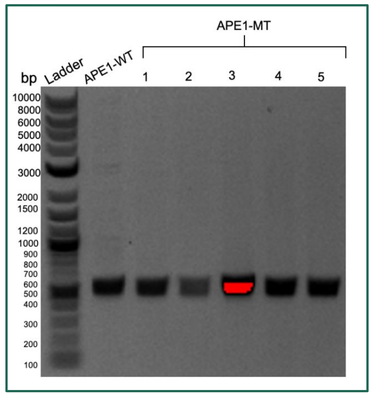


(A) Modified *Saccharomyces boulardii* was used for this project. This probiotic yeast already had the *ΔPRB1* and *ΔPEP4* genes mutated by CRISPR-Cas9.
(B) This yeast strain was further modified through transformation with CRISPR-Cas9 plasmids and donor DNAs that would introduce premature mutations to the *ΔAPE1* and *ΔYPS1* genes
(C) These mutations were hypothesized to produce yeast with better secretory activity. These mutations were validated by sanger sequencing of the strain's genome.
(D) An amylase gene was transformed into this newly modified strain to test for increased secretory activity.
(E) Analysis of this increased activity was done by spotting the yeast cells on starch plates and observing halo formations from starch digestion.

Results

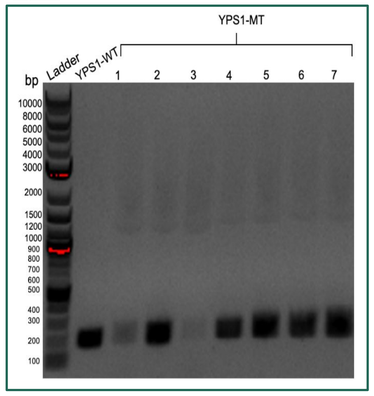
Mutagenesis of *S. Boulardii* by CRISPR-Cas9 was Validated by Polymerase Chain Reaction

Fig 1.0:



Figures 1.0 - 1.1: To increase secretion of the heterologous endolysin gene, key proteases implicated in degradation of proteins in the yeast secretory pathway were mutagenized using CRISPR-Cas9. A PCR (polymerase chain reaction) was performed to confirm the mutation of the APE1 gene (Fig 1.0) and the YPS1 gene (Fig 1.1).

Fig 1.1:



Sequencing Shows Successful Truncation of Secretion-dampening Genes in *S. Boulardii*

Fig 2.0:

WT APE1 Gene:
…GGTTACGGAAGAATTGCTGTTGCTCCCTAT
GGAGGTACACTGAATGAATTGTGGCTAGACA
GAGACCTAGGTATTGGTGGTCGCNNNNN…

Fig 2.2:

WT YPS1 Gene:
…CCAAGTTCGTCAAGTTGCCCTTTCATAAGCTT
TACGGGGACTCGCTAGAAAATGTGGGAAGCGA
CAAAAAACCGGAAGTACGCCTATTGAAGAGG…

Fig 2.1:

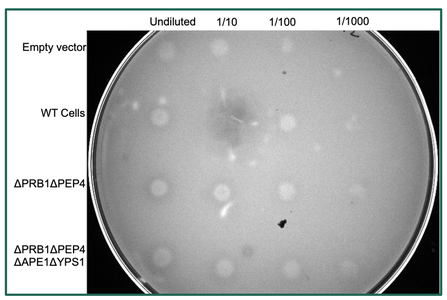
MT APE1 Gene:
…GGTTACGGAAGAATTGCTGTTGCTCCCTAT
GGAGGTACACTGAATGAATTGTGGCTAGACA
GATAATAGCCNANNNNNNNTNANNAGN…

Fig 2.3:

MT YPS1 Gene:
…CCAAGTTCGTCAAGTTGCCCTTTCATAAGCT
TTACGGGGACTCGCTAGAAAATGTGGGAAGCG
ACAAAAAACCGGAAGTATAATAGCCGACTTAT…

Figures 2.0 - 2.3: CRISPR-Cas9 was performed by transforming yeast cells with CRISPR-Cas9 plasmids with gRNAs complementary to proteases' gene regions in the yeast genome. Donor DNAs were then introduced to add premature stop codons to the gene sequences (highlighted in brown in figures 2.1 and 2.3), resulting in a non-functional enzyme. Sanger sequencing of the genome revealed the mutations were successful.

Amylase Activity Shows Improved Secretory Ability of Mutagenized *S. boulardii*



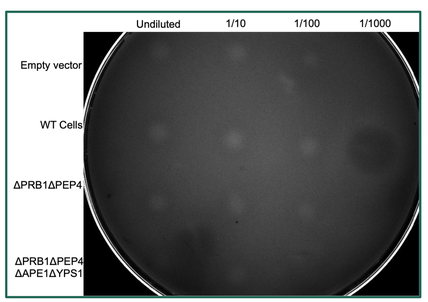


Figure 3.0 : To validate for increased secretion, an amylase gene was transformed into mutagenized yeast cells and quantified by halo formation on starch plates. Cells included wild-type *S. boulardii, ΔPRB1ΔPEP4 S. boulardii* and *ΔPRB1ΔPEP4ΔAPE1ΔYPS1 S. boulardii.* The transformed cells were grown on synthetic media plates and colonies that appeared were patched and grown in synthetic broth. Serial dilutions of the cultures were made and plated on 2% starch (and 0.5% dextrose) plates to evaluate improved amylase activity.

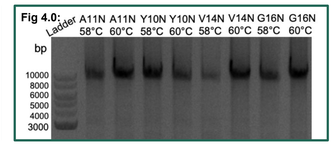
Conclusions

- Utilized CRISPR-Cas9 technology to engineer a distinct variant of *S. boulardii*:
  - Created mutant strains by deleting *ΔAPE1* and *ΔYPS1* genes.
- Verification through plating experiments:
  - Confirmed successful expression of amylase gene in modified S. boulardii.
  - Knockout of proteases shows increased secretion of heterologous genes.
- Strategic deletions of crucial protease genes within the yeast secretory pathway:
  - Purposeful removal of non-essential protease genes.
  - Resulted in a noteworthy improvement in heterologous amylase secretion.
- Significance of the findings:
  - Highlights the potential for refined protein secretion pathways in engineered yeast strains.

Future Directions

- Putting nanobody in yeast capable of blocking *C. perfringens* adhesion to the gut.
- Developing a nanobody library to find potential higher binders to *C. perfringens* to block its adhesion to the intestines.

Figure 4.0: Agarose gel Electrophoresis showing newly constructed plasmids (shown around 10 kb) for the nanobody library.



- A subclinical challenge model to be established using broiler chickens gavaged for *C. perfringens* colonization. Model to see if a diet supplemented with our engineered probiotic can better treat the resulting necrotizing enteritis disease.

Fed positive control: growth-promoting antibiotic

Fed negative control: control diet sans antibiotics

Fed experimental diet: wild-type For engineered *S. boulardii* expressing endolysin

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