Characterizing the role of a hypothetical protein in the pathogenesis of *Cryptococcus neoformans*

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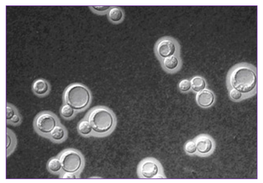
Abstract (shorten)

Cryptococcus *neoformans* is a fungal pathogen that mainly affects immunocompromised patients and is opportunistic as it invades the central nervous system. In the Wormley research lab, we are currently working with multiple genes that have been shown to be involved in lipid metabolism in C. *neoformans* (Cn). Using the TRACE procedure; Transient CRISPR-Cas9 coupled with Electroporation (TRACE) is hypothesized to be a reliable method in order to knock out genes in C. *neoformans.* This specific project we have been working on will lead to a knockout by using CRISPR methodology to create a C. *neoformans* deletion construct for an associated gene.

Introduction

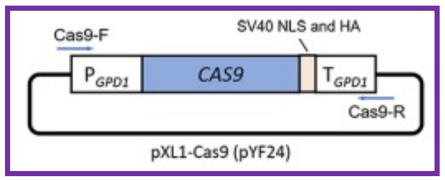
We are analyzing identified genes that have been found to be upregulated in C. *neoformans*, multiple of which have been shown to be involved in lipid metabolism and virulence. By characterizing the role of these genes and certain proteins this project aims to deepen the knowledge of the roles of lipids in pathogenesis and hopefully develop ways to combat infection of people with weakened immune systems. We will generate a knockout (KO) using the TRACE technique to achieve this. The overall problem in this study is the implications these fungal proteins may have and the lack of understanding surrounding their involvement which is essential to research in order to create a pathway leading toward potential drug targets.

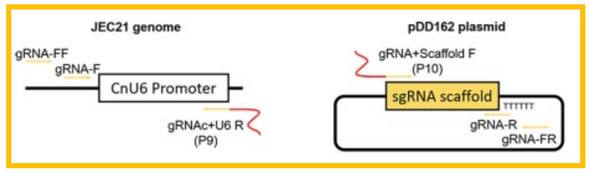


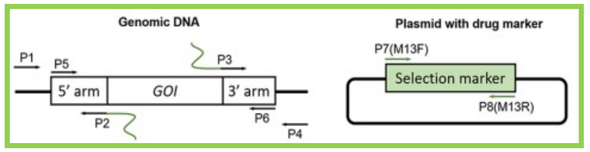
Methodology

The methodology of this project includes the amplifying promoter and end sequences from the unidentified protein to fuse the primer with sgRNA to create a construct ultimately. From here, amplifying the 'arms' of the target gene, and the selection marker from a plasmid (in this case nourseothricin [NAT]) will use PCR to fuse the marker and arms together and create the deletion construct. Once we confirm the gene has been knocked out we will analyze its role in virulence by assessing phenotypic characteristics *in vitro* and *in vivo*.

Experimental methods

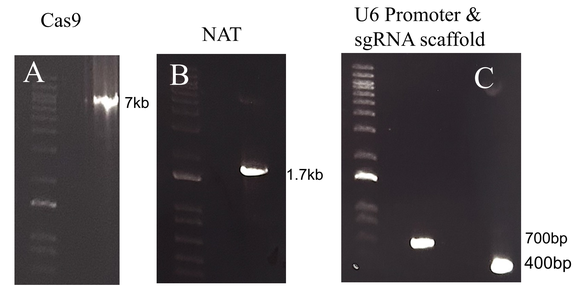


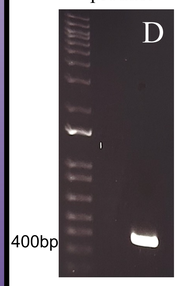




- **Cas9**
- Cas9 construct with the constitutive promoter is amplified from a plasmid (PCR)
- **sgRNA**
- Amplify U6 promoter from Cn genome (PCR) & amplify sgRNA scaffold from pDD162 plasmid (PCR)
- Fuse promoter & sgRNA scaffold (joint PCR, which uses overlapping primers to combine the constructs)

Preliminary Results





The U6 promoter and the 5' and 3' arms of the unknown gene were amplified from the C. neoformans genome, with the U6 promoter amplified from serotype JEC21, and the arms amplified from H99. The arms (5' and 3') were fused to the selection marker NAT, the gene that encodes resistance to an antibiotic. Cas9 was amplified from plasmid pYF24. This construct creates a double-stranded DNA break at the site of the gene. The sgRNA scaffold was amplified from plasmid pDD162 and fused to the U6 promoter. This construct is used to guide the Cas9 to the specific gene.

A. Cas9
B. NAT construct
C. RNA cassette (U6 promoter with JEC21 (p9) and scaffold/terminator with pDD162 (p10))

Future Directions

- After confirmation of PCR by electrophoresis, we will proceed to PCR purification by ethanol precipitation in order to measure the concentration of the sgRNA scaffold.

<u>For Deletion Construct</u>

- Amplify the selection marker (NAT) using pZPZ plasmid as the template with primers M13F and M13R
- Using H99 DNA makes two different reactions for each arm of the gene
- Fusion PCR using a ratio of 1:2:1
- Amplify the full-length deletion construct
- Confirm via electrophoresis

Acknowledgments

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