Characterizing the Role of the Lipid Metabolism-Associated Efflux Protein EncT in the Pathogenesis of *Cryptococcus neoformans*

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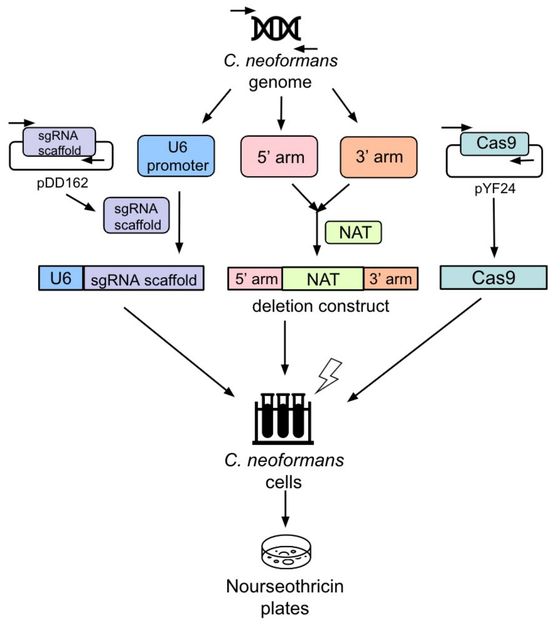
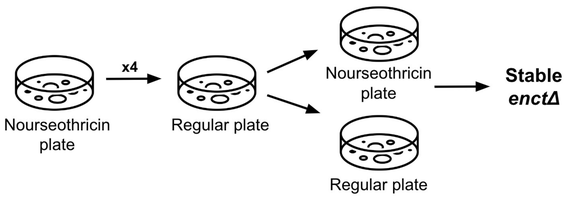
Introduction

Cryptococcus neoformans is an ubiquitous fungal pathogen that is life-threatening for immunocompromised patients. Specific fungal signaling lipids, termed eicosanoids, have been associated with increased virulence in this pathogen. Since *C. neoformans* lacks common enzymes associated with eicosanoid biosynthesis in humans, this pathway presents novel genes which could be used as potential drug targets. Our study focuses on *EncT*, a poorly characterized gene encoding an efflux pump, which our lab has found to be most upregulated during eicosanoid production in *C. neoformans*. To evaluate the potential role of this gene in virulence, we used CRISPR technology to generate an *EncT* knockout (*enctΔ*) strain. We then conducted *in vitro* virulence assays of the *enctΔ* strain and the wild-type strain (H99) to assess changes in temperature sensitivity and virulence factors. These tests help us to characterize the potential role of the *EncT* gene in the virulence of this pathogen.

Experimental Methods

Transient CRISPR-Cas9 Coupled with Electroporation (TRACE)





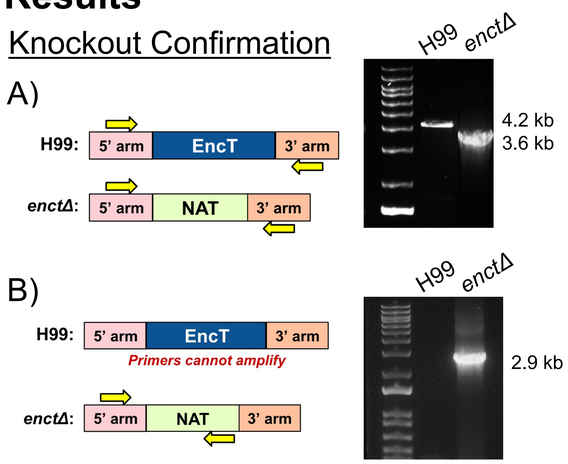
To generate the *enctΔ* strain, the Transient CRISPR-Cas9 Coupled with Electroporation (TRACE) method was used. The U6 promoter and the 5' and 3' arms of the *EncT* gene were amplified from the *C. neoformans* genome. The arms were fused to NAT, the gene that encodes resistance to the antibiotic nourseothricin, to generate a deletion construct. Cas9 and the sgRNA scaffold were amplified from plasmids. The scaffold was fused to the U6 promoter.

The final sgRNA construct, the deletion construct, and the Cas9 were purified and introduced into *C. neoformans* cells via electroporation, and the cells were plated on nourseothricin media.

After transformants grew on the plates, these cells underwent stability testing through multiple passages onto non-selective media followed by return to selective media to ensure stable gene deletion.

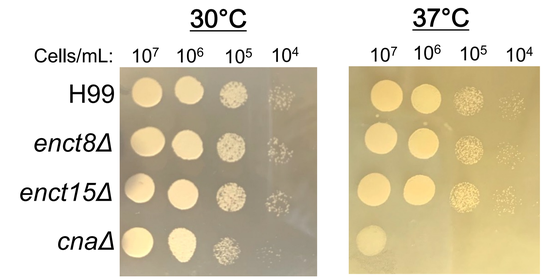
Results

Knockout Confirmation



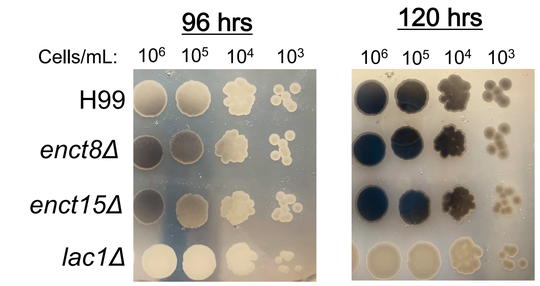
To confirm *EncT* gene deletion, cellular DNA was extracted and amplified with primers that bind in both H99 and *enctΔ* but result in different sizes (A) and with primers that only amplify *enctΔ* (B). Two *enctΔ* strains (*enct8Δ, enct15Δ)* were identified by this method.

Temperature Assays



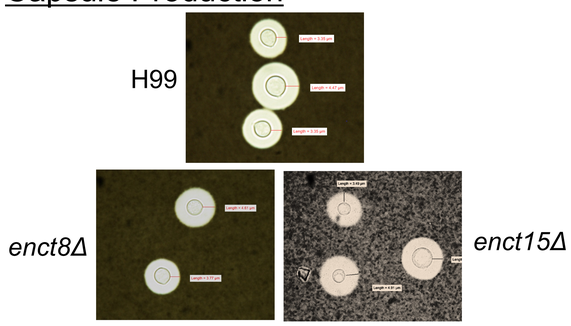
Cells were grown on regular media for 24 hours. The calcineurin knockout (*cnaΔ*) strain is a mutant control with decreased growth at 37°C.

Melanin Production Assay



Cells were grown on L-DOPA (media that enables production of melanin, shown as a black color change). The laccase knockout (*lac1Δ*) strain is a mutant control that doesn't produce melanin.

Capsule Production



Cells were grown in capsule-inducing conditions (PBS+10% FBS at 37°C in 10% CO_2), stained with India ink, and viewed for the presence of capsule via light microscopy at 1000x total magnification.

Conclusions

The TRACE method resulted in the identification of two *enctΔ* strains which successfully passed stability testing and confirmation by PCR and gel electrophoresis.

Based on the temperature assays, the two *enctΔ* strains showed no difference in growth at 30°C nor 37°C compared to the H99 strain, which has a functional *EncT* gene.

In the melanin production assay, both *enctΔ* strains showed earlier melanin production than H99, which could indicate enhanced protection against the host immune response when the *EncT* gene is suppressed. This is further supported by the retention of capsule in both *enctΔ* strains, but the effects of these findings need to be further studied.

Future Directions

Future directions include conducting more *in vitro* assays to analyze changes in virulence factors including the production of urease and sensitivity to both oxidative stress and antifungal treatment. We will also use the TRACE method to generate an *EncT* reconstituted strain to corroborate that any changes in virulence observed with *enctΔ* are due to the lack of the *EncT* gene. Further, H99, *enctΔ,* and the reconstituted strain will be given to mice to evaluate the pathogenicity of the *enctΔ* strain in an *in vivo* model. We will evaluate the presence of the fungi in the lung and the dissemination in other organs, and we will analyze the host immune response.

References & Acknowledgements

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