Tao3 mediates a phenotypic switch between amoeba-adapted and mammalian-adapted forms of Cryptococcus neoformans
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ABSTRACT
Many microbes are capable of changing phenotypes more frequently than due to background mutation rates alone, and this ability is coupled to pathogenesis. The human pathogenic yeast Cryptococcus neoformans is found in the environment in soil, pigeon guano and tree species, and has long been thought to be a eukaryotic fungus. Previous research showed that C. neoformans has the ability to change phenotype in response to environmental factors, such as temperature and nutrient availability. This ability to change phenotype is not unique to C. neoformans, but is also found in many other species of fungi.

INTRODUCTION
The ability of microbes to cause disease comes from their success in adapting to the host environment. Many pathogens make committed changes at a genetically heritable level that occur at rates too high to be due to standard mutation that would be subject to natural selection. Cryptococcus neoformans is a fungal pathogen that is acquired directly from environmental exposure to desiccated yeast cells or the sexual basidiospores. The fungus is found worldwide and it causes disease predominately in immunocompromised individuals, especially AIDS patients, and the global mortality rate is estimated at 624,000 per annum. The closely-related species C. gattii causes disease in healthy individuals, and is responsible for an ongoing expanding outbreak of cryptococcosis in the Pacific Northwest since 1999. It has been proposed that this fungus may be pre-selected for virulence within mammalian animals because of interactions with environmental predatory microbes, such as amoeba and nematodes. A curious observation made in the 1970s was that C. neoformans exposed to amoeba changed from a yeast form to a pseudohyphal morphotype and became resistant to killing by amoeba. However, these pseudohyphal strains were less pathogenic in mouse models than the original wild type parents and the phenotype exhibited instability. Reports described these variants of the fungus as having a morphology similar to strains with mutations in the RAM (Regulation of Age2p activity and cellular Morphogenesis) pathway of genes that encode for pseudohyphal growth. We hypothesized that a RAM pathway gene is affected in strains undergoing switching.

RESULTS
We compared morphologies of the strains that had been exposed to amoeba (Bu1mer C, D, and E) and 1 strain from a phenotypic switching study (F7) with strains known to have deletions of ckb1, kic1, sog2, mob2, and tao3 genes. The strains were also compared for growth at elevated temperature (mammalian body temperature, 37°C) and resistance to the immunosuppressive agent FK506. Most strains were sensitive to a temperature increase, all were highly sensitive to FK506, and all shared a similar morphology. These results suggest the same genes or pathways are affected, and that this would be the RAM pathway.

DISCUSSION
The RAM pathway is conserved in eukaryotes and has diverse and diverged functions. It includes six gene products, centered around the products of Ckb1 and Kic1. The function of Tao3 is unclear, but it is thought to be a scaffold protein within the pathway, as it interacts with both Ckb1 and Kic1. We hypothesized that the pseudohyphal switch seems to be due to normal mutation rates in cells, especially since the rates are due to exposure to UV light. Since Tao3 is the largest gene in the pathway, it is more likely to be changed. Finding the change in the second largest gene, Sog2, also supports this hypothesis. In regards to pathogenesis, the pseudohyphal properties have not been fully explored. One speculation is that the pseudohyphal forms could allow the fungus to escape out of mammalian cells, in addition to the currently known ways it escapes from macrophages.

In summary, the RAM pathway, especially the gene Tao3, is responsible for the phenotypic switching of C. neoformans. The switch back to wild type involves at least four different processes and is strain-dependent. We predict the background rates of spontaneous DNA mutation are important for the frequency of switching.

Mammalian virulence
Escape from amoeba
Mating
Invasive growth
Mammalian virulence
Mating
Pseudohyphae
Mutation rate
RAM Pathway

Fig. 2: Four examples of pseudohyphal switching. 1) Reversion to wild type. 2) Stop codon reverts to different amino acid. 3) Stop codon is still present. 4x) Similar to type 3, except its colony color reflects the media color. 4b) Reverted type 4 above shows an example of yellow colony growth.

Fig. 3: The role of the RAM pathway in C. neoformans biology.

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REFERENCES

Fig. 1: Comparison of C. neoformans pseudohyphal strains from original experiments. Wild type cells are spherical in shape.

C. neoformans strains. Wild type strains used were KN99a (serotype A), Bu1mer G (serotype A), ATCC 24067A (serotype D), and YC21 (serotype D). “Historical” pseudohyphal strains were F7 (serotype D), and Bu1mer C, D, and E (serotype A).

Sequence analysis to identify point mutations. Genomic DNA was extracted from strains, and the TA03 gene was amplified by PCR from strains Bu1mer C, D, and E, and F7 and the PCR products were sequenced.

Gene complementation. Tests were performed on the three Bu1mer strains and F7 using vectors that complement the deletion mutants of mob2, ckb1, kic1, and sog2. The TA03 gene in Bu1mer D was reconstituted by homologous recombination. A BglII construct was generated by overlap PCR and introduced into Bu1mer D cells by biolistic transformation method using a PDS-1000/He Particle Delivery System (Bio-Rad) using standard methods.

Isolation of new pseudohyphal strains in the ATCC 24067A background. An overnight culture of strain ATCC 24067A was diluted, and ~18,000 cells in total distributed over 58 plates. Colonies with dry looking appearance were streaked to isolate single colonies. Genomic DNA was isolated, and the TA03 gene, or Sog2 gene, amplified by PCR and sequenced.

TA03 cDNA characterization. The intron-exon boundaries of TA03 were confirmed by amplification from cDNAs reverse transcribed with Superscript III (Invitrogen) from RNA purified from wild type strain KN99a.

Deletion of Sog2 and TA03. The TA03 gene was deleted in the KN99a, 24067A and Bu1mer G backgrounds. The Sog2 gene was deleted in strain KN99a. The S’ and S’ trans were amplified from genomic. Nourseothricin acetyltransferase was amplified from plasmid pA3. Overlap PCR was performed with the three pieces of DNA. These DNA molecules were transformed into yeast cells using the biolistic machine, cells allowed to recover, and transferred to YPD medium containing nourseothricin.