Tao3 mediates a phenotypic switch between amoeba-adapted and mammalian-adapted forms of *Cryptococcus neoformans*

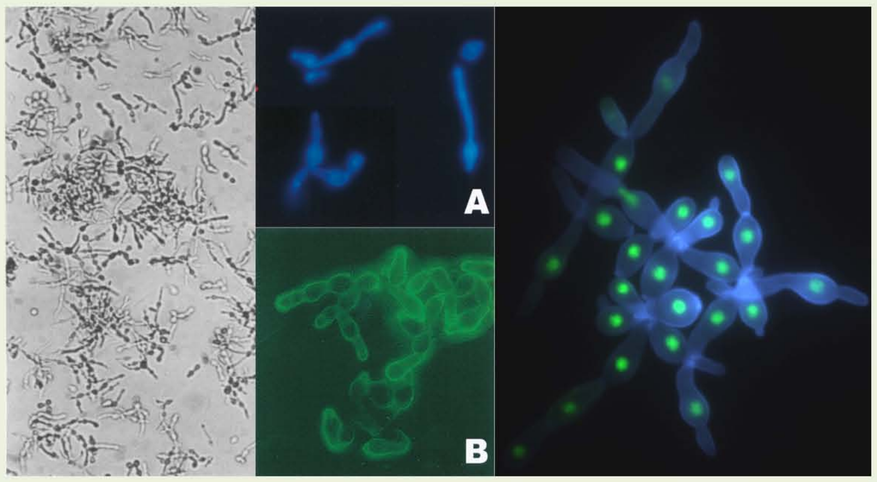
Denise A. Magditch, Alexander Idnurm
Division of Cell Biology and Biophysics, School of Biological Sciences, University of Missouri-Kansas City, Kansas City, MO 64110

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, locations in which the organism is exposed to microbial predators. Previous research showed that co-incubation of *C. neoformans* with amoeba causes a switch from yeast to a pseudohyphal form, enabling fungal survival in amoeba yet conversely reducing virulence in mammalian models of cryptococcosis. Here we identify the basis for pseudohyphal development in phenotypic-switched and amoeba-derived strains, showing that the *TAO3* gene, a component of the RAM pathway, bears point mutations. Reversion to wild type yeast morphology can occur through different mechanisms to suggest that underlying rates of spontaneous mutation control this process and thereby influence the pathogenic potential of an organism.

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 of desiccated yeast cells or the sexual basidiospores. The fungus is found worldwide and it causes disease predominantly 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 and 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 morphology 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 Ace2p activity and cellular Morphogenesis) pathway of genes identified by insertional mutagenesis (Fig. 1). We hypothesized that a RAM pathway gene is affected in strains undergoing switching.



Amoeba (1978) (1) Phenotypic switching (1999) (2) Insertional mutant (2006) (3)

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

ACKNOWLEDGEMENTS

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… Val Gly Thr Ile Lys Lys Glu Gly …

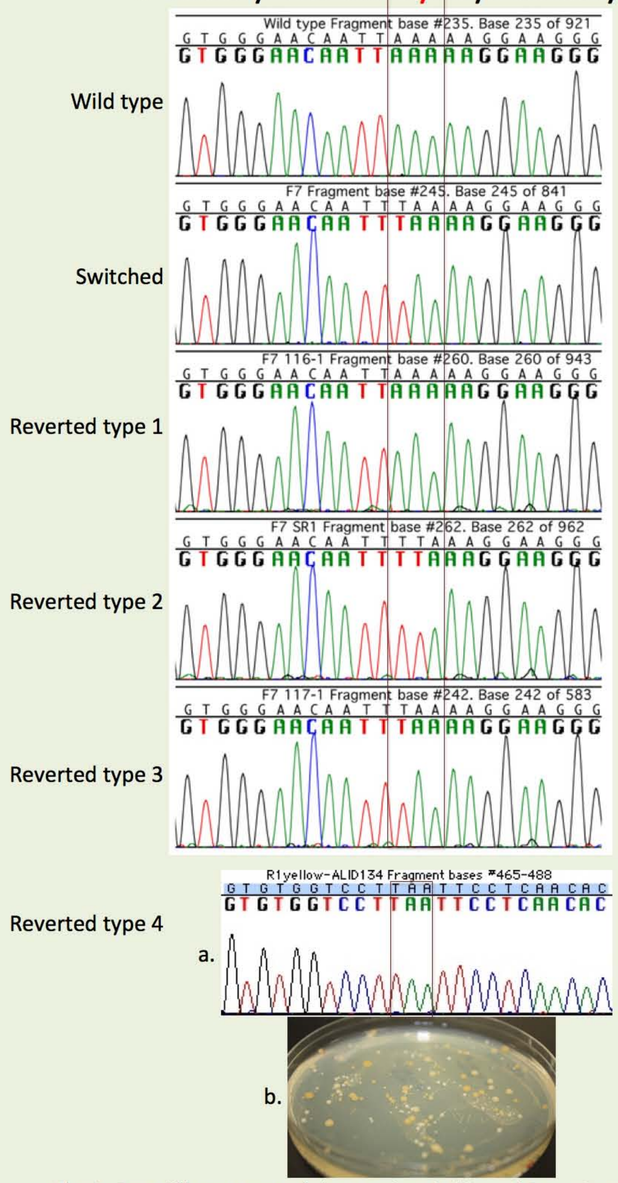

Fig. 2: Four different types of phenotypic switching. 1) Reversion to wild type. 2) Stop codon reverts to different amino acid. 3) Stop codon is still present. 4a) Similar to type 3, except its colony color reflects the media color. 4b) Reverted type 4 above shows an example of yellow colony growth.

Materials and Methods

***C. neoformans* strains.** Wild type strains used were KN99α (serotype A), Bulmer G (serotype A), ATCC 24067A (serotype D), and JEC21 (serotype D). "Historical" pseudohyphal strains were F7 (serotype D), and Bulmer C, D, and E (serotype A).

Sequence analysis to identify point mutations. Genomic DNA was extracted from strains, and the *TAO3* gene was amplified by PCR from strains Bulmer C, D, and E, and F7 and the PCR products were sequenced.

Gene complementation. Tests were performed on the three Bulmer strains and F7 using vectors that complement the deletion mutants of *mob2*, *cbk1*, *kic1*, and *sog2*. The *TAO3* gene in Bulmer D was reconstituted by homologous recombination. A BglII construct was generated by overlap PCR and introduced into Bulmer 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 *TAO3* gene, or *SOG2* gene, amplified by PCR and sequenced.

***TAO3* cDNA characterization.** The intron-exon boundaries of *TAO3* were confirmed by amplification from cDNAs reverse transcribed with Superscript III (Invitrogen) from RNA purified from wild type strain KN99α.

Deletion of *SOG2* and *TAO3*. The *TAO3* gene was deleted in the KN99α, 24067A and Bulmer G backgrounds. The *SOG2* gene was deleted in strain KN99α. The 5' and 3' flanks were amplified from genomic. Nouseothricin acetyltransferase was amplified from plasmid pAI3. 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.

RESULTS

We compared morphologies of the strains that had been exposed to amoeba (Bulmer C, D, and E) and a strain from a phenotypic switching study (F7) with strains known to have deletions of *cbk1*, *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.

We amplified by PCR the *TAO3* gene from the strains isolated from amoeba exposure and from the phenotypic switching experiment and sequenced it. All four isolates contained a mutation that results in a premature stop codon in the protein. We were able to rescue the wild type phenotype by insertion of a BglII-TAO3 construct which inserts a silent point mutation instead of a stop codon. It also inserts a new BglII restriction site, which helps determine reconstituted from reverted strains. To explore the basis of reversion, we sought out additional mutant strains by subjecting 24067A to UV light. We isolated five new spontaneous pseudohyphal strains, four of which had changes in the *TAO3* gene and one had a change in the *SOG2* gene. Two serotype A possible *TAO3* mutant strains from a previous study were examined, and changes within the *TAO3* gene were discovered.

We next sought to examine the rate of change for Bulmer D and F7 and did this by wild type selection on FK506, since the mutants are sensitive to this drug. We discovered a high rate of reversion for F7 (Bulmer D did not revert as quickly), but reversion was not necessarily back to wild type morphology. Four types of reversion were identified: 1) the original amino acid is present, 2) a different amino acid is present, 3) the stop codon is still present, and 4) the colony color reflects the media color with the stop codon still present (Fig. 2).

DISCUSSION

The RAM pathway is conserved in eukaryotes and has diverse and diverged functions. It includes six gene products, centered around the products of Cbk1 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 Cbk1 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 (Fig. 3).

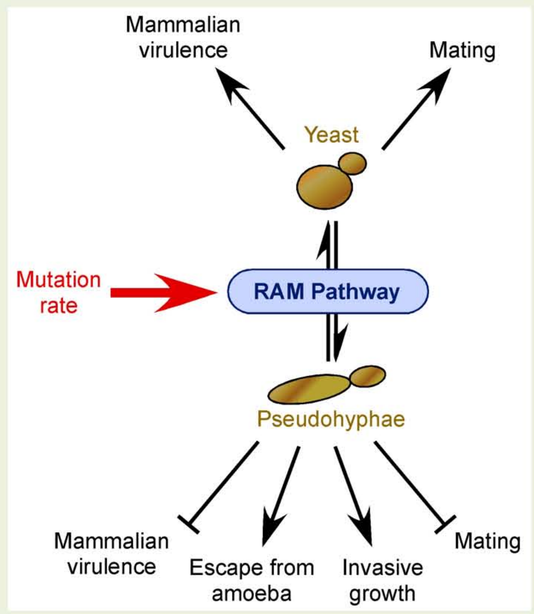

Fig. 3: The role of the RAM pathway in *C. neoformans* biology.