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# Isolation of a fungal calcineurin A mutant suggests that amoebae can counter-select virulence attributes of microbes

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## Abstract

Evolutionary selection pressures that resulted in microbes found within environmental reservoirs that can cause diseases in animals are unknown. One hypothesis is that predatory organisms select microbes able to counteract animal immune cells. Here, a non-pathogenic yeast, *Sporobolomyces primogenomicus*, was exposed to predation by *Acanthamoeba castellanii*. Strains emerged that were resistant to being killed by this amoeba. All these strains had altered morphology, growing as pseudohyphae. The mutation in one strain was identified: *CNA1* encodes the calcineurin A subunit that is highly conserved in fungi and where it is essential for their virulence in hosts including mammals, insects, and plants.

## Lay Summary

One hypothesis why some microbes cause disease in humans is that they have been exposed to selection pressures in the environment, like predation by amoebae. This study selected yeast strains resistant to amoeba. One is due to the loss of calcineurin, a protein required for disease.

**Keywords:** amoeba, *Cryptococcus*, mycoses, phosphatase, virulence

Fungi represent a highly successful lineage in terms of both their global distribution and their species richness having diversified into millions of species. A small number of fungi are problematic to human health. Some, like species in the genera *Candida*, *Malassezia*, *Pneumocystis*, or the dermatophytes species in the Onygenales, are tightly associated with animal hosts. Another set is acquired from environmental sources. Species in this second group are of interest to understand why these, of the many million possible fungi, are able to attack animal hosts and what in the environment selected this trait.[1] The evolution of pathogens is an important problem, highlighted by the recent emergence of pathogenic fungi such as *Batrachochytrium* species responsible for the decline of amphibians, *Pseudogymnoascus destructans* on bats, *Ophidiomyces ophidiicola* on snakes, or *Candida auris* on humans.[2]

An attractive hypothesis is that exposure of fungi to environmental predators may select species that, when they 'inadvertently' arrived in a human host, had the adaptations that enable them to escape immune responses.[3–6] In additional support for the 'amoeboid predator-fungal animal virulence' hypothesis, exposure of fungi to amoebae or the slime mould *Dictyostelium* increases their virulence upon subsequent infection in mammalian hosts.[3,7] Many of these studies have used strains in the *Cryptococcus neoformans* and *C. gattii* species complexes, yeast that are able to cause disease in multiple mammalian species.

Counter to the 'amoeboid predator-fungal animal virulence' hypothesis, the interaction of *C. neoformans* and *C. deneoformans* with amoebae resulted in the isolation of pseudohyphal strains. These strains had 'micro-evolved' to avoid predation by amoebae, but with a consequence of decreasing their virulence in mice.[8–10] The basis for the changes in these strains is mutations within genes encoding components of the Regulation of Ace2 and Morphogenesis (RAM) pathway.[8] It was unclear if the resistance to amoebal predation was due solely to a physical consequence of the altered morphology or other changes in the strains, such as cell wall composition changes. This was explored further with another *C. neoformans* strain able to alternate between morphologies, revealing an inability of macrophages to phagocytose elongated cells as well as a RAM pathway mutant.[11] Experiments using other fungal species with different morphology have made similar correlations, i.e., yeast forms being relatively easy to 'consume' whereas filamentous forms are more difficult.[12] In another example, *C. neoformans* was evolved over time with amoebae.[13] However, none of the evolved strains had altered responses to macrophages and the one strain inoculated into mice was less pathogenic. Recently, an analysis of *Cryptococcus* species and strains was unable to correlate the levels of resistance to predation by amoebae with their replication within macrophages or virulence in mice.[14]

The predation hypothesis was based on fungal species that have already evolved to cause disease. These species reflect the outcomes of selection, thereby making interpretations of how they might have interacted with amoebae or other predators challenging. Here, this study considered what would happen earlier, i.e., when non-pathogens are exposed

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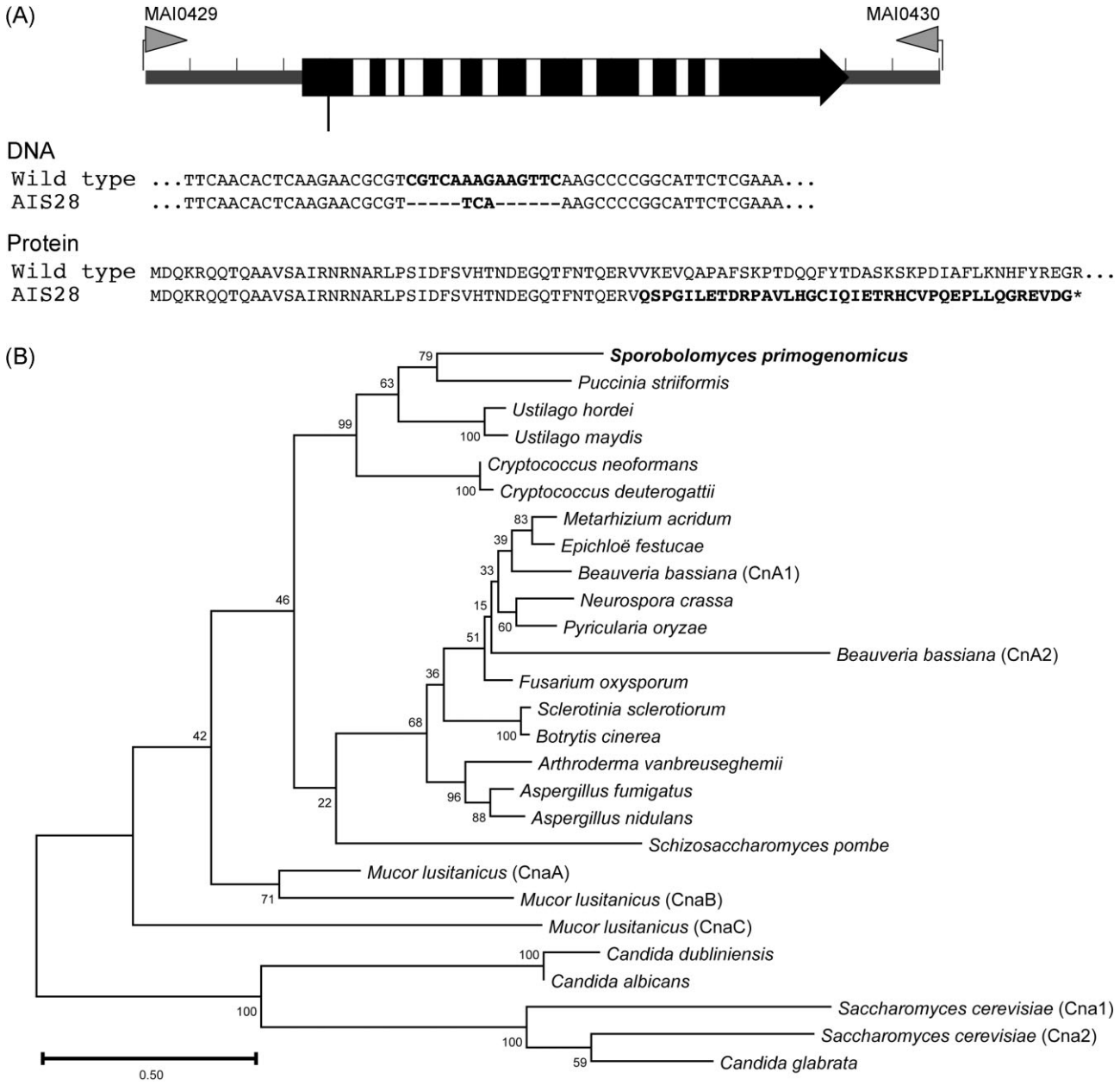


**Figure 1.** *S. primogenomicus* strain AIS28 has a mutation in the *CNA1* gene encoding calcineurin A. (A) Diagram of the structure of *S. primogenomicus CNA1,* with exons in black and introns in white boxes. The line indicates the site of the mutation in strain AIS28. The positions of primers MAI0429 and MAI0430 used to amplify the wild type copy for complementation are provided. The mutation in the DNA sequence, and its consequences on the predicted amino acid sequence of the protein are below. (B) Phylogenetic tree (maximum likelihood, LG + G model) of Cna1 homologs from fungal species selected due to the characterization of their homologs. Bootstrap support values as a percentage from 1000 reiterations are placed adjacent to the nodes.

to predators. *Sporobolomyces primogenomicus* is a free-living yeast, which was isolated from willow leaves, in the phylum Basidiomycota, and subphylum Pucciniomycotina. It is, therefore, in a different subphylum (Pucciniomycotina) than the *Cryptococcus* species (Agaricomycotina). Originally called *S. roseus*, DNA analysis revealed it is a distinct species, which was named *S. primogenomicus* as the first Pucciniomycotina whose genome was sequenced.[15] While there is only a single strain of *S. primogenomicus*, precluding genetic crossing, it can be manipulated by the transformation of exogenous DNA.[16]

The wild-type *S. primogenomicus* strain IAM 13481 was routinely cultured at 22°C on yeast extract peptone dextrose (YPD) or yeast nitrogen base (YNB) media supplemented with 2% glucose. The strain was plated as a cross on a 5% V8 juice pH 7 agar plate (9 cm diameter), a drop of *Acanathamoeba castellanii* strain ATCC 30234 was placed in the center, and the culture was allowed to develop over 2 months. Most fungal cells were cleared, but several patches of growth occurred. These were streaked out to isolate strains away from the amoebae, and examined for their morphologies. Of a dozen resistant strains that were isolated, all reproduced in a stable manner by growing as pseudohyphal cells. One of these strains, AIS28, was then examined in more detail.

A whole genome sequencing approach was undertaken to identify a genetic basis for the morphological change in strain AIS28. Ion torrent sequencing was performed by the Australian Genome Research Facility (AGRF). The sequencing

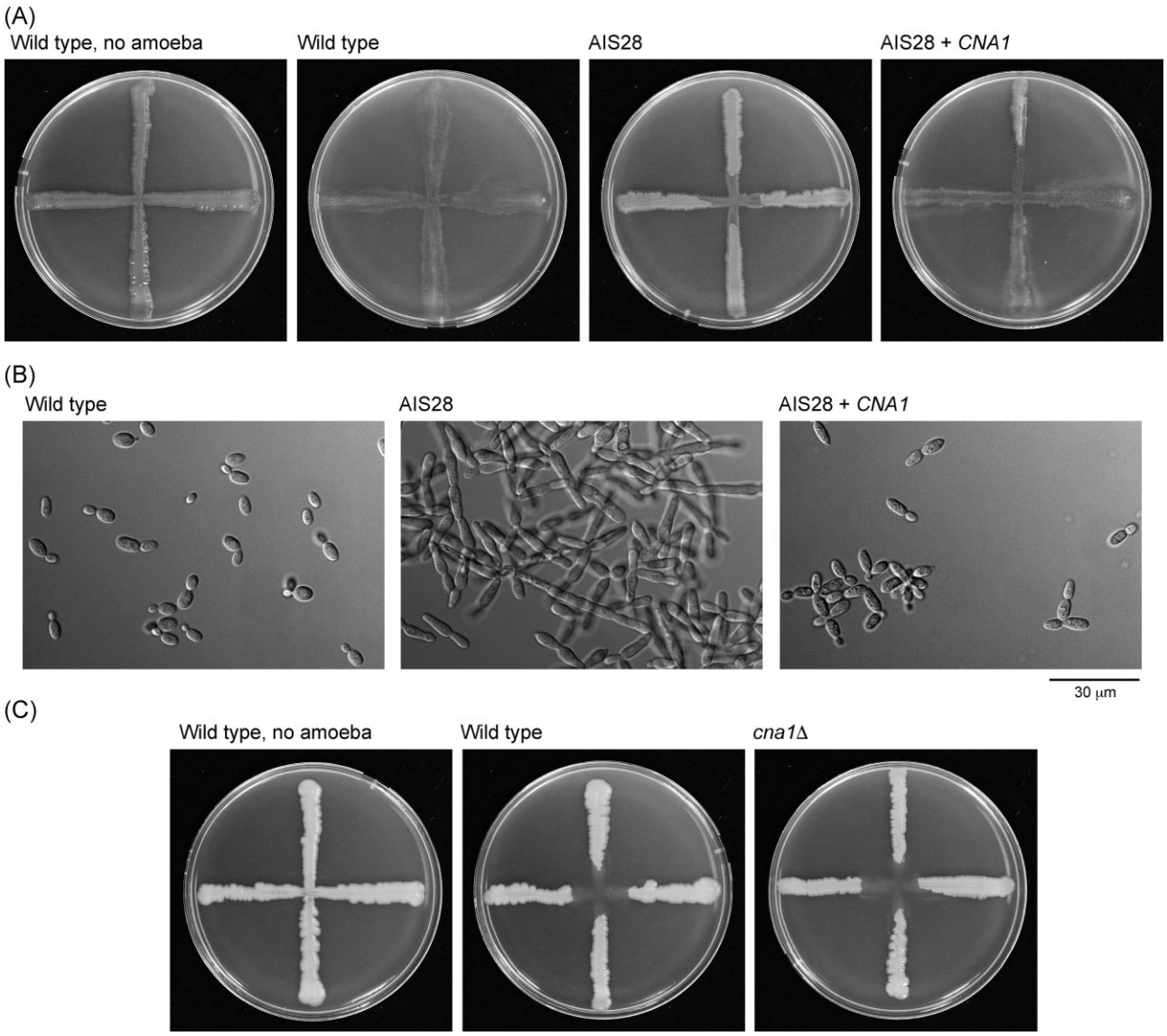


**Figure 2.** *CNA1* in *S. primogenomicus* is involved in resistance to predation by amoebae and cell morphology. (A) Growth of *S. primogenomicus* strains on 5% V8 juice agar plates with or without *A. castellanii* at 22°C after 25 days. Amoebae were placed at the intersection of the cross, where they consumed the yeast cells, migrating outwards and forming cysts in a diffuse pattern of a cross. (B) Overnight cultures in liquid YPD medium of the *S. primogenomicus* strains were examined by microscopy, showing cell elongation in strain AIS28 and a cell separation defect. (C) Growth of *C. neoformans* wild-type and *cna1* mutant with or without exposure to amoebae, grown concurrently as the *S. primogenomicus* plates in panel (A).

reads (GenBank BioProject PRJNA892711) were aligned using Geneious version 8.1.7 onto the genome sequence of strain IAM 13481 available from MycoCosm. Variants were detected using the criteria of minimum coverage of six reads, minimum similarity of 80%, and a maximum *P*-value of $1 \times 10^{-6}$. Polymorphisms were identified by visual inspection across each scaffold. The variants were then examined if those occurred within coding regions. No changes were found in the homologs of the RAM pathway. One mutation was found within the gene (called *CNA1*) encoding the putative catalytic subunit of calcineurin A (Fig. 1a). This mutation is a replacement of 14 nucleotides CGTCAAAGAAGTTC with the three nucleotides TCA in the first exon. The *S. primogenomicus CNA1* gene annotation was examined and then updated based on RNA-seq data,[17] which revealed that the second-last intron is slightly larger than the prediction at JGI (the revised sequence is GenBank accession ON094074). The change in DNA sequence in AIS28 is predicted to alter the amino acid sequence by 39 residues before truncating the protein (Fig. 1a). To provide evidence that this gene is calcineurin A, the protein sequences of homologs previously characterized in other fungi were obtained from GenBank, aligned with ClustalW and used to generate a phylogenetic tree in MEGA11.[18] This approach places the *S. primogenomicus* gene within this protein family and with strong support for the homologs in the related basidiomycete species (Fig. 1b).

To ensure that this mutation causes the phenotypes, a wild-type copy of *CNA1* was transformed into AIS28 to test for complementation. First, uracil auxotrophs in the AIS28 background were selected to generate strains able to be transformed. Strain AIS28 was cultured overnight in YPD, then plated onto media containing 5-fluoroorotic acid (1 g/l) dissolved in YNB supplemented with 20 mg/l uracil, which selects for spontaneous mutations in *URA3* or *URA5* gene. Two resistant strains with stable auxotrophic properties with mutations in *URA3* were used.

The wild-type version of *CNA1* was amplified off genomic DNA with primers MAI0429 (5′-GGGCGAATTCTT AATTAAGATGGATTCCCAAAGTCAAGATG-3′) and MAI 0430 (5′-TCCCCGGGTACCGAGCTCGATCACGTTCTCG CAACGTCCTC-3′), then cloned using Gibson assembly (New England Biolabs) into plasmid pAIS3 linearized with EcoRV. The plasmid was replicated in *Escherichia coli* strain NEB® 5-alpha, then electroporated into *Agrobacterium tumefaciens* strain EHA105 with selection on LB agar with kanamycin (50 $\mu$g/ml). This *A. tumefaciens* strain was used to transfer the *CNA1* and *URA3* genes into the two *cna1 ura3* strains using *Agrobacterium*-mediated transformation,[16] with selection based on *URA3* on YNB supplemented with cefotaxime to inhibit bacterial growth. This transformation largely restored AIS28 to the wild-type resistance to amoebae and cell morphology (Fig. 2a, b).

Calcineurin is a highly conserved serine/threonine protein phosphatase, regulated by calcium and the calmodulin protein, and impacting numerous downstream responses. It is required for fungi to cause disease, whether that be to mammal, insect, or plant hosts (as reviewed[19]). While highly conserved, calcineurin function differs between species. Of phylogenetic relevance to this study on *S. primogenomicus*, mutation of the calcineurin homolog also renders those strains pseudohyphal in the basidiomycetes *Ustilago maydis* and *U. hordei*,[20,21] both plant pathogens in the subphylum Ustilaginomycotina. However, the *cna1* mutant is not pseudohyphal in *C. neoformans*,[22] in the subphylum Agaricomycotina. To resolve the question if the resistance is due to the change in morphology or other impacts of the loss of calcineurin, wild-type *C. neoformans* and a *cna1* mutant[23] were compared for amoeba predation: mutation of *CNA1* did not lead to resistance (Fig. 2c), linking AIS28 resistance to its change in morphology.

In summary, while the 'amoeboid predator-fungal animal virulence' hypothesis is supported by experiments, it is also worth considering contrary evidence. Here, amoebae selected a strain with a mutation in the gene encoding one of the best-known virulence factors in pathogenic fungi. Thus, there is a continued need to explore the basis behind how pathogenic fungi cause disease and the selection processes that have led—and will likely continue to lead—to their evolution.

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## Declaration of interest

The author reports no conflicts of interest. The author alone is responsible for the content and writing of the paper.

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