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Genetic and Genomic Approaches to Explore Virulence in Fungal Pathogens

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Cryptococcus neoformans causes life-threatening infections in immunocompromised people including AIDS patients and people receiving immunosuppressive therapy (1, 2). People acquire the fungus by inhalation of spores or yeast cells, and the initial pulmonary infection is often followed by severe meningitis (designated cryptococcosis). *C. neoformans* has emerged as a particular problem because of the AIDS epidemic, and the incidence of cryptococcosis can be as high as 30% in the ~25.8 million people with HIV/AIDS in sub-Saharan Africa (www.unaids.org; 1). Antifungal drugs (e.g., amphotericin B) suppress but do not cure cryptococcal meningitis, and there is a pressing need to develop more efficacious antifungal drugs and therapies.

The major virulence factors for *C. neoformans* include capsule and melanin formation, and the ability to grow at 37°C (2, 3). Capsule formation is a highly regulated process: e.g., the size of the capsule is influenced by iron and CO_2 levels, growth in serum, and host tissue location (3, 4). The phenoloxidase (laccase) for melanin synthesis is required for virulence, perhaps by mediating resistance to oxidative stress, and is also regulated by iron (5). A variety of signaling pathways control virulence in *C. neoformans*. For example, cell wall integrity is controlled by the *PKC1* pathway (3), temperature tolerance is mediated by the calcineurin pathway (3, 4), and the regulation of capsule size, mating, melanin formation and virulence is controlled by the cAMP pathway (3). In general, the host signals that influence these pathways are poorly understood, as are the changes in gene expression that occur as a result of host perception.

We are interested in the role of iron as a nutrient for *C. neoformans* and as a signal that allows the fungus to perceive the host environment. The importance of iron in microbial virulence is well documented and iron withholding is a host defense strategy to limit infection (6). The level of iron in host fluids is extremely low (10^{-18} M) due to the iron binding proteins transferrin (Tf) and lactoferrin (Lf). However, pathogenic microbes require 10^{-6} to 10^{-7} M iron and they must therefore liberate iron

from host proteins by binding ferrated Tf or Lf, by elaborating siderophores or by degrading hemoglobin or other iron-containing proteins. We have taken genomic and molecular genetic approaches to define the *C. neoformans* functions for iron acquisition in culture and during infection, and to identify the mechanism of iron sensing.

Initially, we employed the technique of serial analysis of gene expression (SAGE) to identify the transcriptional changes that occur during growth in low iron medium versus iron-replete medium (7). This approach revealed major changes in gene expression in response to different iron levels. For example, cells grown in iron-replete conditions had elevated transcripts for genes for iron storage, nitrogen metabolism, mitochondrial function and lipid metabolism. Cells grown in low iron conditions had elevated transcript levels for functions involved in iron acquisition. The iron acquisition genes encoded expected functions such as low and high affinity iron permeases/ferroxidases, ferric reductases, and siderophore iron transporters (e.g., *SIT1*). In addition, this analysis revealed that the transcript for a putative extracellular protein, Cig1, was the most abundant mRNA detected by SAGE and that this transcript was reduced 10 fold in iron replete cells. Subsequent gene disruption experiments confirmed that the *CIG1* gene, the *SIT1* gene and an iron permease gene all play roles in iron sensing and/or acquisition (7, 8).

The SAGE data provided iron-regulated genes to begin to define the mechanisms of iron sensing in *C. neoformans* and led us to characterize a GATA-type transcription factor that plays a major role in iron-mediated regulation of gene expression (9). This factor, designated Cir1 for Cryptococcus iron regulator, contains a zinc finger domain and is similar to iron regulatory proteins such as Urbs1, fep1, and SreA in other fungi. We deleted the *CIR1* gene and employed the resulting mutants to perform phenotypic tests and microarray experiments. The *cir1* mutants had elevated cell surface reductase activity and melanin production indicating a negative regulatory influence of Cir1. However, the mutants also were defective in capsule production and growth at 37°C thus indicating a positive role for Cir1. These and other phenotypic tests revealed that Cir1 controls all of the major known virulence factors and we found that *cir1* mutants were avirulent in a mouse model of cryptococcosis. Remarkably, our microarray analysis indicated that Cir1 regulates the expression of ~2000 genes in response to iron and that virtually all of the transcriptional response to iron is mediated by Cir1. We are now characterizing the downstream targets of Cir1 to further define the mechanisms of iron regulated gene expression and virulence in *C. neoformans*.

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