CHARACTERIZATION OF AN IRON PERMEASE IN

CRYPTOCOCCUS NEOFORMANS

by

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Date **Oct 30, 03**
Abstract

*Cryptococcus neoformans* is an opportunistic fungal pathogen with worldwide distribution. Pathogenesis is mediated by several virulence factors including growth at 37°C, melanin synthesis and capsule formation. Expression of the thick polysaccharide capsule is stimulated under iron-limiting conditions. Analysis of SAGE libraries of cells grown under iron-replete and iron-limited conditions identified orthologs of two components of the *Saccharomyces cerevisiae* high affinity iron uptake system, *FTR1* and *FET3*. These genes, encoding the high affinity iron permease (*FTR1*) and the multicopper oxidase (*FET3*), form a cluster of functionally related genes in *C. neoformans*.

Molecular genetic techniques were used to analyze the regulation of expression of *FTR1* and *FET3* by iron, copper and components of the PKA pathway. The results suggest that iron starvation stimulates transcription of *FTR1* and *FET3* regardless of genetic background and that maintenance of basal transcription of *FTR1* requires the activity of PKA catalytic subunits. Supplemental copper stimulates *FET3* expression but represses *FTR1* expression.

The contribution of *FTR1* to *C. neoformans* growth under iron-limited conditions and response to oxidative stress was investigated in *ftr1* mutants. The results suggest that *FTR1* is important for growth under iron-limiting conditions and that response to oxidative stress is also iron-dependent.

The high-affinity iron transport system of *C. neoformans* represents a key cellular strategy for acquisition of a limiting nutrient in the environment. Elucidation of the mechanisms by which *C. neoformans* obtains iron under iron-limited conditions aids
understanding of cell growth and proliferation inside the host and its potential contribution to virulence.
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Abbreviations and Conventions

Genes and Proteins of Interest

Aft1  iron-regulated transcription factor in *S. cerevisiae*
Arn1  ferrichrome transporter in *S. cerevisiae*
Arn2/Taf1  triacetyl fusaricine C transporter in *S. cerevisiae*
Arn3/Sit1  ferrioxamine B transporter in *S. cerevisiae*
Arn4/Enbl  enterobactin transporter in *S. cerevisiae*
CaARN1  gene encoding ferrichrome transporter in *C. albicans*
Cac1  adenyllyl cyclase
CaHMX1  gene encoding haem oxygenase in *C. albicans*
CCC2  gene encoding copper-loading protein in *S. cerevisiae*
CFL1  gene encoding ferric reductase in *C. albicans*
CPK1  gene encoding MAP kinase homolog in *C. neoformans*
CTA1  gene encoding catalase in *S. cerevisiae*
CTR1  gene encoding copper transporter in *C. neoformans*
CTT1  gene encoding catalase in *S. cerevisiae*
ExbB, D  components of ferrisiderophore transport complex in *Escherichia coli*
Fbp  iron binding protein
Fec A-E  ferric dicitrate transport genes in *E. coli*
FepB  enterochelin transporter in *E. coli*
Fes  ferrientochelin esterase
*FET3*  gene encoding a multicopper oxidase in *S. cerevisiae* and *C. neoformans*
FhuA  ferrichrome receptor in *E. coli*
FhuD  ferrihydroxamate transporter in *E. coli*
FpvA  pyoverdin biosynthesis gene in *P. aeruginosa*
*FRE1, 2, 3*  genes encoding ferric reductases in *Saccharomyces cerevisiae*
Frr  ferric reductases regulatory mutants in *C. neoformans*
*FTR1*  gene encoding an iron permease in *Saccharomyces cerevisiae* and *Cryptococcus neoformans*
Fur  ferric uptake regulator
Gap1  amino acid permease in *S. cerevisiae*
GAPDh  glyceraldehyde-3-phosphate dehydrogenase
GLUT5  gene encoding fructose transporter in rat intestine
Gpb1  β subunit of heterotrimeric G protein
Lbp  lactoferrin binding protein
Macl  copper regulated transcription factor in *S. cerevisiae*
MAP kinase  mitogen activated protein kinase
*MRS3/4*  gene encoding mitochondrial carrier protein of inner mitochondrial membrane
PKA  cAMP-dependent protein kinase
Gene designations for fungi follow the S. cerevisiae conventions for naming. Wild-type alleles are in upper-case italics for the wild-type allele and lower-case italics for the mutant allele. Fungal proteins are designated by non-italics in which the first letter is capitalized.

Amino acids are denoted by their one-letter code.

<table>
<thead>
<tr>
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<tbody>
<tr>
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</tr>
<tr>
<td>Arginine</td>
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<tr>
<td>Asparagine</td>
<td>N</td>
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<td>Aspartic acid</td>
<td>D</td>
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<td>Threonine</td>
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<td>W</td>
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<td>V</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
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<td>-----------</td>
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<td>BLAST</td>
<td>basic local alignment search tool</td>
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<tr>
<td>bp</td>
<td>basepair</td>
</tr>
<tr>
<td>cAMP</td>
<td>adenosine 3’ 5’ cyclic monophosphate</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethyl pyrocarbonate</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDDHA</td>
<td>ethylenediamine-N’, N’-bis (2-hydroxyphenylacetic acid)</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene-diamine-tetra-acetic acid</td>
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<td>GXM</td>
<td>glucuronoxylomannan</td>
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<td>kilobasepair</td>
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<td>LB</td>
<td>Luria-Bertani broth</td>
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<td>L-DOPA</td>
<td>3,4-dihydroxy-L-phenylalanine</td>
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<tr>
<td>MOPS</td>
<td>4-morpholinepropanesulfonic acid</td>
</tr>
<tr>
<td>NaCl</td>
<td>sodium chloride</td>
</tr>
<tr>
<td>NAD(P)H</td>
<td>nicotinamide dinucleotide phosphate</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>ORF</td>
<td>open reading frame</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>RNA</td>
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<td>SAGE</td>
<td>serial analysis of gene expression</td>
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<td>SCE</td>
<td>sorbitol, sodium citrate and EDTA buffer</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
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<tr>
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<td>Tris-Borate EDTA buffer</td>
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<tr>
<td>UV</td>
<td>ultra-violet</td>
</tr>
<tr>
<td>YNB</td>
<td>yeast nitrogen broth</td>
</tr>
<tr>
<td>YPD</td>
<td>yeast extract, peptone and dextrose media</td>
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</table>
Acknowledgements

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1 Introduction

1.1 An Introduction to *Cryptococcus neoformans*

*Cryptococcus neoformans* is a pathogenic fungus that is the most common cause of fungal meningitis in humans. It is classified as a basidiomycete yeast with a heterothallic mating system. As a basidiomycete the sexual hyphae of *C. neoformans* elaborate fused clamp connections and the eponymous basidium, a swollen terminal hyphal cell where the spores are produced in chains. The heterothallic mating system relies upon the fusion of cells of opposite mating types. In *C. neoformans* there are two mating types, MATa and MATα. Vegetatively, *C. neoformans* grows in a yeast-like budding form, but switches to hyphal growth when “mates” of opposite mating type encounter each other. After hyphal fusion to form the dikaryon, the cells continue to grow as filaments with the elongated cells connected to one another by the fused clamp connections characteristic of basidiomycete fungi. The terminal cell of the hypha eventually swells to form the basidium. In the basidium, the two nuclei fuse to form the diploid, which undergoes meiosis followed by serial mitoses to produce the haploid spores that begin the life cycle again. It is thought that humans inhale the spores. The α mating type can also “short-circuit” this cycle and undergo a process called haploid fruiting. During haploid fruiting, severe nitrogen limitation causes the haploid vegetative cells to grow filamentously. These hyphae are differentiated from sexual hyphae by the presence of unfused clamp connections between the elongated cells. Haploid spores are produced by mitosis and, like spores produced as the result of mating, may also initiate infection.
*C. neoformans* occurs in five different serotypes: A, B, C, D and AD. Serotype A, referred to as *C. neoformans* var. *grubii*, has a worldwide distribution and is the most common clinical isolate causing meningitis in immunocompromised individuals (Casadevall & Perfect, 1998). The environmental reservoir for *C. neoformans* var. *grubii* is not known, but yeast cells are frequently isolated from pigeon excreta. Until recently, no MATα strains of *C. neoformans* var. *grubii* were known, but Lengeler et al., described this type of strain (Lengeler *et al.*, 2000). In their original paper, the MATα strain was less virulent than the MATα strain and sterile. Since then, a mating reaction between MATα and MATα strains has been described (Nielsen *et al.*, 2003). Serotypes B and C are grouped together and are known as *C. neoformans* var. *gattii*. Historically these strains were believed to be geographically restricted to tropical climes and were found in association with the Australian red gum eucalyptus (*Eucalyptus camaldulensis*) (Casadevall and Perfect, 1998). *C. neoformans* var. *gattii* causes pulmonary disease in immunocompetent individuals. Recently there has been an outbreak of cryptococcosis on eastern Vancouver Island (Stephen *et al.*, 2002). Molecular typing indicates that the outbreak strains are *C. neoformans* var. *gattii* (S. Kidd, personal communication). The ecological niche of this temperate strain appears to be trees and soil in disturbed areas (K. Bartlett, conference proceedings). *C. neoformans* var. *neoformans* is serotype D. This serotype of *Cryptococcus* is attractive for laboratory study because there are congenic pairs of opposite mating type and this serotype is less virulent than *C. neoformans* var. *grubii*. The *C. neoformans* var. *neoformans* strains are similar enough to var. *grubii* to undergo mating reactions with a compatible serotype A strain (Cogliati *et al.*, 2001;
D'Souza et al., 2001; Heitman et al., 1999). The serotype AD strains are believed to be the results of such genetic crosses.

*C. neoformans* has several well-studied virulence factors including mating type, melanin synthesis and expression of the thick polysaccharide capsule that antigenically defines the serotypes. The vast majority of clinical isolates of *C. neoformans* are MAT\(\alpha\) strains and there is genetic evidence that mating type is linked to virulence (Kwon-Chung et al., 1992). Melanin is synthesized in the presence of polyphenol precursors such as L-DOPA. Melanin-deficient mutants are hypovirulent in the mouse model; other studies suggest that melanin contributes to virulence by protecting cells from cells of the immune system by interfering with phagocytosis and subsequent killing (Wang et al., 1995); (Salas et al., 1996). Recent studies have shown that melanin is deposited in the fungal cell wall over the course of a mouse pulmonary infection (Feldmesser et al., 2001). The most obvious virulence determinant is the polysaccharide capsule that *C. neoformans* synthesizes in response to iron-limitation and physiological levels of CO\(_2\) (Vartivarian et al., 1993). Acapsular mutants are hypovirulent in experimental models of cryptococcosis (Fromtling, Shadomy et al. 1982; D'Souza, Alspaugh et al. 2001). Complementation of the acapsular phenotype restores virulence (Chang & Kwon-Chung, 1994; Chang & Kwon-Chung, 1998; Chang & Kwon-Chung, 1999; Chang et al., 1996; D'Souza, et al., 2001; Wilder et al., 2002).

The combination of the worldwide distribution of *C. neoformans* and the increasing population of immunocompromised individuals (due primarily to the AIDS epidemic) situate *C. neoformans* as an emerging pathogen of global importance. Understanding the molecular basis of its virulence will hopefully yield new strategies for
combating it. The work described in this thesis deals with the molecular genetic analysis of iron uptake in *C. neoformans*. Iron is an important nutrient during infection and, interestingly, the availability of the metal influences capsule formation. This introductory chapter first describes iron uptake in bacterial pathogens, then considers parallel information in the model fungi *Saccharomyces cerevisiae* and *Candida albicans*. *C. neoformans* iron biology is then described in the context of this information.

1.2 Iron Uptake in Bacterial Pathogens

Inside the vertebrate host, iron is transported throughout the bloodstream complexed to the glycoprotein transferrin. The three major classes of transferrins are serum transferrin, lactoferrin and ovotransferrin. Transferrins are 80 kDa, single-chain glycoproteins with two iron binding sites that have an iron binding constant of about $10^{20}$ under physiological conditions. Aside from their high affinity for iron, another key feature of these glycoproteins is that they are never fully saturated. Because the binding capacity of the transferrins exceeds the amount of iron available in the vertebrate system, the free iron in circulation is negligible. Iron can also be stored complexed with ferritins. Found in animals, plants and microbes, these 24 subunit, 500 kDa proteins take the form of a sphere that can hold more than 4000 atoms of ferric iron.

Limiting the amount of iron available to invading microorganisms is a key mode of nutritional defense for the vertebrate host. The vast majority of bacteria require iron as a cofactor for various enzymes (such as RNA polymerase) or components of the electron transport chain. The fact that bacterial pathogens do manage to reproduce inside of the host indicates that these organisms possess mechanisms for acquisition of iron during
infection. Invading pathogens may access iron by direct contact with the source of iron (typically a transferrin) or by secretion of siderophores, compounds that can strip iron from host proteins or bind iron from insoluble ferric salts.

Many bacterial pathogens are capable of obtaining iron directly from host proteins. The transferrins of the vertebrate host represent an abundant source of iron. Many pathogenic bacteria synthesize transferrin binding proteins (Tbps) in response to iron starvation; these include *Neisseria gonorrhoea, Pasteurellaceae, Haemophilus influenzae, Moraxella (Branhamella) catarrhalis, Listeria monocytogenes, Actinobacillus pleuropneumoniae* and *Borrelia burgdorferi*. The Tbps were first characterized in *Neisseria* but the function and genetic organization of the Tbps is consistent across the different genera. Additionally, iron uptake from lactoferrin is accomplished by the lactoferrin binding proteins (Lbp). The genetic architecture of the operon and functions of its components is similar to that of the Tbps (Pettersson et al., 1998). This review will use the neisserial system as a model for receptor-mediated binding and uptake of iron from transferrin.

The *Neisseriaceae* genes TbpA and TbpB encoding the transferrin-binding proteins Tbp1 and Tbp2 are arranged polycistronically, that is they are transcribed together as a single transcript, and the upstream region contains a Fur regulatory binding region (Anderson et al., 1994; Cornelissen et al., 1992). The role of the Fur protein in regulation of genes involved in iron uptake will be discussed later. Each of these proteins is surface bound and capable of binding transferrin iron, but it is Tbp2 that discriminates between the ferrated and desferrated forms of the protein (Cornelissen & Sparling, 1996). The stoichiometry of the interaction of Tbp1 and Tbp2 with transferrin is 2:1 per
molecule of transferrin. Changes in trypsin digestion patterns suggest that both proteins undergo a conformational change once transferrin is bound (Cornelissen and Sparling, 1996). The Tbp1 protein is believed to act as the channel for passage of released ferric iron into the periplasmic space via a TonB-dependent mechanism in which TonB couples the protonmotive force across the cytoplasmic membrane to actively transport the ferric iron across the outer membrane (Cornelissen et al., 1997; Cornelissen, et al., 1992). Conformational determinants and iron-binding peptides at the N- and C- termini contribute to the iron-binding capabilities of Tbp2 (Renaud-Mongenie et al., 1997). The current model for neisserial iron acquisition from transferrin proposes that Tbp2 preferentially binds iron-charged transferrin passing it on to the Tbp1 dimer, at which point the transferrin iron is released (possibly by a conformational change) and is passed to the periplasm via TonB.

Once the iron has been released from transferrin, the iron must be transported across the periplasm and into the cytoplasm. This is accomplished via a periplasmic binding protein-dependent transport system composed of three Fe (III)-binding proteins (Fbp), FbpA, FbpB and FbpC (Khun et al., 1998). FbpA, the periplasmic binding protein, binds ferric ion and then associates with FbpB and FbpC, the other components of this ABC-type transporter (Adhikari et al., 1996). The physical characteristics of the Fbp system are structurally similar to transferrin because the components comprise a structure approximately the same size as one lobe of transferrin, are capable of binding one ferric ion per molecule, and binding is more efficient in co-ordination with bicarbonate (Nowalk et al., 1994). The events involved in binding and transport of transferrin are summarized in Figure 1-1.
The mechanism of transferrin iron transport in Gram-positive bacteria is not well characterized. *Staphylococcus* spp. express a transferrin-specific receptor of 42kDa that encodes a cell wall glyceraldehyde-3-phosphate dehydrogenase (GAPdH) (Modun et al., 1998). It is unknown whether the glycolytic activity of the enzyme or the reducing capabilities of the dehydrogenase are responsible for the removal of iron from transferrin. Internalization of the liberated iron is an active process.

Beyond direct uptake of iron from host proteins, many bacteria (pathogenic and non-pathogenic) secrete siderophores, low-molecular weight compounds that have a high affinity for iron. Bacteria, yeasts and fungi are all known to use siderophores and some organisms can use siderophores synthesized by other microbes. Siderophores have iron-binding constants ranging from $10^{22}$ to $10^{50}$ (Drechsel & Winkelmann, 1997). This high affinity for iron permits the removal of iron from ferritin and the transferrins, but does not permit removal of iron from heme proteins. Siderophores also bind iron from insoluble ferric salts, such as ferric hydroxide.

It has been proposed that siderophores, synthesized under iron-limiting conditions, are recyclable and that one siderophore molecule can be repeatedly charged with an iron atom in the environment and discharged of that iron atom by the cell. A cell could therefore require only a limited number of siderophore molecules in order to meet its iron needs. The balance of synthesis and repression of siderophores and other iron-regulated factors is dynamic. Once a minimum amount of iron has been recruited into the cell, the cell ceases to be iron deficient, and therefore represses synthesis of siderophores. This minimum amount of iron is quickly depleted by the cell, leading to de-repression of
siderophore synthesis; thus cells generally function on the verge of iron-depletion (Saritharan & Ratledge, 1990).

Figure 1-1 Transferrin binding in *Neisseria*

Tbp2 binds and presents holotransferrin to Tbp1. Ferric iron (filled circles) is transported across the inner membrane by the ABC-type transporter encoded by *fbpA*, *fbpB* and *fbpC*. Adapted from Ratledge and Dover (2000).
After the siderophore binds iron (an iron-charged siderophore is referred to as a ferrisiderophore), the efficient uptake of the siderophore and the release of the bound iron are of prime importance to the cell. In Gram-negative bacteria, the ferrisiderophore ligands are concentrated at the cell surface by interaction with specific high affinity receptor proteins. Like transport of transferrin-bound iron, the active translocation of the ferrisiderophore complex also uses a Ton transport system to couple the potential difference across the cytoplasmic membrane to transport of the ferrisiderophore across the outer membrane. The three proteins involved are TonB, ExbB and ExbD (Larsen et al., 1996; Larsen et al., 1999; Nicholson & Beall, 1999). At the outer membrane, the interaction between the ferrisiderophore receptors and TonB is probably mediated through the TonB box, a highly conserved region of the receptors’ N-termini. In studies with FhuA, the ferrichrome receptor in *E. coli*, receptor-ligand binding enhanced the interaction of the receptor with TonB, indicating that ligand binding induces a conformational change in the receptor that facilitates interaction between the receptor and TonB (Moeck et al., 1997). The crystal structure for FhuA shows a β barrel structure that is plugged by the N-terminal domain. The N-terminal domain of FhuA faces the external environment and contributes part of the ferrichrome-binding site (Ferguson et al., 1998). It is hypothesized that ligand binding induces a conformational change in the protein such that the N-terminal TonB box can interact with the TonB protein.

Like transport of the transferrins and lactoferrins, ferrisiderophore complexes are transported across the cytoplasmic membrane by an ABC transporter, consisting of a binding protein and a transmembrane diffusion complex of two hydrophobic domains and two hydrophilic membrane-associated domains that contain nucleotide-binding sites. In
*E. coli* there are several periplasmic ferrisiderophore-binding proteins known: FecB, which transports dicitrate, FepB, which transports enterochelin, and FhuD, which transports ferrihydroxamate (Elkins and Earhart 1989; Larsen, Thomas et al. 1999; Koster and Braun 1990). Although the role of each of these proteins has been elucidated within the context of a particular siderophore, each of these periplasmic binding proteins has broad substrate specificity. The mechanism of ferrisiderophore transport across the cytoplasmic membrane is incompletely characterized.

Protein-dependent uptake is also known in the Gram positive bacteria, with one important modification: the lack of an outer membrane in the Gram positive bacteria means that the binding protein must be anchored to the cell in order for this transport system to concentrate the ferrisiderophore complexes at the plasma membrane (Nikaido & Saier, 1992).

The ferric iron of the ferrisiderophore must be reduced in order to make the iron available in a soluble form. Such ferric reductases have been found in *Bacillus megaterium*, *Paracoccus (Micrococcus) denitrificans*, *E. coli*, *Pseudomonas aeruginosa*, *Legionella pneumophila* and *Mycobacterium smegmatis* (Arceneaux and Byers 1980; Tait 1975; Fischer, Strehlow et al. 1990; Cox 1980; Poch and Johnson 1993; Brown and Ratledge 1975). As with the ferrisiderophore binding proteins of the periplasm, the cytoplasmic ferric reductases have a broad substrate specificity. The reaction requires NAD(P)H as a source of reducing equivalents.
The large negative redox potential of many ferrisiderophore complexes has led some researchers to claim that these ferrisiderophores (for example, ferri-enterochelin, with a redox potential of −750 mV at pH 7) cannot be reduced by NAD(P)H, which has a redox potential of −324 mV at pH 7. The existence of Fes, an esterase active on ferri-enterochelin, has led to the proposal that ferri-enterochelin must first be hydrolyzed in order to reduce the attached ferric ion (Brickman & McIntosh, 1992). It is also possible that the equilibrium position could be shifted if the bacterium were able to efficiently remove the reduced ferrous ion.

There are positive and negative regulatory systems that control the expression of iron uptake systems in bacteria. The mechanisms can be roughly divided into those that operate under high iron concentrations ([Fe^{2+}] ≥ 100 μM) and those that operate under low iron concentrations ([Fe^{2+}] ≤ 10 μM). When the amount of iron in the environment is high, genes involved in the uptake of iron are repressed. Repression is mediated by direct binding of the Fur protein (ferric uptake regulator) to the target genes. When iron is limiting in the environment, this nutritional signal is transduced into the cell, and transcription is stimulated by a variety of mechanisms.

The ferric uptake regulator protein, Fur, was first discovered in *E. coli* mutants with constitutive expression of iron-regulated genes. When complexed to ferrous iron, the 148 amino acid protein can bind to DNA. Analysis of the genes involved in biosynthesis of the siderophore aerobactin indicated that Fur bound to a region of DNA adjacent to the promoter, indicating that it was a transcriptional regulator. Further studies with other iron-regulated genes showed that the consensus sequence for Fur binding was

Beyond the direct, negative regulation by Fur, there are also cases of positive regulation for iron uptake systems. One model for upregulation of iron-regulated genes under low iron conditions is the ferric dicitrate system of \textit{E. coli}. In \textit{E. coli}, ferric dicitrate induces iron uptake under low iron conditions (Frost & Rosenberg, 1973). The operon encoding the ferric dicitrate system is composed of the \textit{fecI}, \textit{fecR}, \textit{fecA-E} genes (Staudenmaier, Hove \textit{et al.} 1989; Pressler, Staudenmaier \textit{et al.} 1988). There are three transcripts of this operon and the first encodes \textit{fecI} and \textit{fecR}, which compose the regulatory component of the system. FecR transduces the nutritional signal; \textit{fecR} mutants don’t respond to citrate and have constitutive expression of all \textit{fec} genes (Kim \textit{et al.}, 1997). The sequence of \textit{fecI} bears a helix-turn-helix motif, suggesting that FecI acts as a transcription factor. The second transcript contains \textit{fecA}. Transcription of \textit{fecA} is repressed by iron, stimulated by citrate and is dependent on the presence FecI, lending more support to the hypothesis that FecI is involved in DNA binding and transcription of
The $fecA-E$ genes encoding the uptake machinery are present in the third transcript (Staudenmaier, et al., 1989). The proposed model for components of the $fec$ operon is that upon binding of ferric dicitrate, the N-terminus of FecA transduces the signal via a conformational change. This conformational change promotes interaction with FecR. FecR relays the signal to the transcription factor FecI and promotes transcription of the genes involved in ferric dicitrate transport ($fecA-E$); as such, FecR is the key integrative point, relaying the signal from FecA to FecI. Mutational analysis supports the hypothesis that the C-terminus of FecR is required for the citrate response, while the N-terminus interacts with FecI (Ochs et al., 1995). The TonB, ExbB and ExbD proteins have also been implicated in signal transduction and transport of ferric dicitrate across the outer membrane (Braun, 1995; Harle et al., 1995). The autoregulated ferric dicitrate system is shown in Figure 1-2.

*Pseudomonas aeruginosa* uses a variety of siderophores. Pyochelin and pyoverdin (also known as pseudobactin) are synthesized by *Pseudomonas*, enterobactin, ferrioxamine B and aerobactin are exosiderophores synthesized by other microbes. The receptors for ferric-pyochelin and ferric-pyoverdin are regulated by iron via Fur (O'Sullivan et al., 1994) and the presence of the corresponding siderophores (Gensberg et al., 1992). The $pup$ genes, homologous to the $fec$ genes of *E. coli*, regulate uptake of the *Pseudomonas* siderophores.
Figure 1-2 Autoregulation of the ferric dicitrate system in *E. coli*.

The ferric dicitrate binding event by FecA stimulates TonB-dependent uptake of iron from ferric dicitrate and promotes transcription of the ferric dicitrate biosynthesis genes by its action on FecR. Adapted from Crosa (1997).
Synthesis of the siderophore pyoverdin requires the products of the *pvdA, pvdD* and *fpvA* genes. These genes encode (in order) the L-ornithine N5 oxygenase responsible for formation of the hydroxamate ligands of the siderophore, the non-ribosomal machinery for assembly of the pyoverdin peptide, and the outer membrane receptor for ferri-pyoverdin (Merriman *et al.*, 1995; Visca & Orsi, 1994). The regulation of these three genes differs markedly from those previously described in that no Fur boxes have been identified (Georges & Meyer, 1995), (Marugg *et al.*, 1988).

Promoter analysis of *pvdA* indicates that −41 to −54 was required for iron-dependent regulation of *pvdA* (Rombel *et al.*, 1995). Within this region there is a G/CCTAAATCCC consensus sequence shared between *pvdA, pvdD* and *pvdE*. Mutations of the CTAAAT sequence reduced the activity of the promoter. It was also found that null *pvdA* mutations were silent unless the product of *pvdS* was present. In the presence of *pvdS*, the expression of *pvdA* was iron-regulated, suggesting that Fur might act on *pvdS* (Leoni *et al.*, 1996). A similar relationship exists for *pvdS* with *pvdD* and with *pvdE* (Cunliffe *et al.*, 1995).

The expression of the receptor, FpvA, is required for upregulation of pyoverdin receptor biosynthesis. This is a pattern of autoregulation is similar to that seen for the synthesis of the ferric-dicitrate receptor of *E. coli*.

### 1.3 Iron Uptake in *Saccharomyces cerevisiae*

The iron uptake system of the baker's yeast, *S. cerevisiae*, has been well studied and serves as a useful paradigm for the work described in this thesis. The roles of each of the components of the high affinity iron uptake system as well as their regulation have been elucidated.
Initial work in the yeast system indicated that ferric reduction was the first step in iron uptake. The addition of ferrous chelators inhibits iron uptake and the kinetics of ferric and ferrous iron uptake are different (Lesuisse et al., 1987). The proteins involved in this reduction, ferric reductases, have been isolated from various subcellular fractions: cytoplasm, mitochondria and plasma membrane (Lesuisse et al., 1990). Genetic evidence linking ferric iron reduction to iron uptake shows that mutants lacking ferric reductase are highly sensitive to iron deprivation (Dancis, Klausner et al. 1990, Dancis, Roman et al. 1992). Complementation of these mutants identified the FRE1 gene (Dancis, et al., 1990). Transcription of FRE1 follows the same pattern as ferric reductase activity. High levels of iron (≥100 μM) lead to decreased FRE1 transcription and decreased ferric reductase activity. Low levels of iron stimulate both FRE1 transcription and ferric reductase activity (Dancis, et al., 1990). While necessary for uptake of ferric iron, the FRE1 product is dispensable for uptake of ferrous iron (Dancis, et al., 1990). Another ferric reductase, encoded by FRE2 has also been cloned and characterized. FRE2 differs from FRE1 at the nucleotide level, but the structure of FRE2 retains several key features including the NADPH binding domain (Georgatsou & Alexandraki, 1994). NADPH supplies the reducing equivalents required for the action of the ferric reductase (Shatwell et al., 1996), (Lesuisse & Labbe, 1992).

Experiments to determine the environmental cues that stimulate ferric reductase activity showed that iron uptake in S. cerevisiae requires copper, which suggested a mechanistic link between copper and iron uptake (Dancis et al., 1994). Knockout of the plasma membrane copper transporter encoded by CTR1, or the post-Golgi P-type ATPase, an active Cu^{2+} transporter encoded by CCC2 yielded cells that were deficient in
iron uptake (Dancis, et al., 1994; Yuan, et al., 1995). The cytoplasmic copper-binding protein Atx1 transports copper from Ctr1 to Ccc2 (Lin et al., 1997). The target of copper imported into the cell was presumed to be Fet3, a multicopper oxidase. The nucleotide sequence of FET3 showed strong similarities to the family of blue multicopper oxidoreductases (Askwith et al., 1994). Analysis of the pattern of oxygen consumption showed a 4:1 relationship between substrate and oxygen also suggestive of a multicopper oxidase (DeSilva et al., 1995). By sequence analysis and immunoblotting it was determined that Fet3 was surface exposed (DeSilva, et al., 1995). The working hypothesis that Fet3 was part of a multimer involved in iron uptake was expanded upon when it was shown that Fet3 formed a complex with an iron permease, Ftr1, and that this complex was required for high affinity iron uptake in yeast (Stearman et al., 1996). It was also shown that Ftr1 was required for copper loading of Fet3. Correct localization of Ftr1 to the plasma membrane was dependent on the presence of Fet3. When Fet3 was absent MYC-tagged Ftr1 localized to the endoplasmic reticulum rather than the plasma membrane (Stearman, et al., 1996). Analysis of different FTR1 mutant alleles delineated the contributions of the different regions of Ftr1. Mutants lacking the last 76 amino acids could not function as permeases, but still allowed copper loading of nascent Fet3. Truncation of Ftr1 beyond the iron-binding consensus REGLE sequence eliminated Ftr1 function. Figure 1-3 is a cartoon of the iron uptake system in S. cerevisiae. Table 1-1 summarizes the roles of the different proteins involved in high affinity iron uptake.
Figure 1-3  High affinity iron uptake in *S. cerevisiae*

A cartoon showing the different components of the high affinity iron uptake system in *S. cerevisiae*. Proteins are shown in bold type.

Table 1-1  Proteins involved in high-affinity iron uptake in *S. cerevisiae*

<table>
<thead>
<tr>
<th>Protein Alias</th>
<th>Protein Function</th>
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<tbody>
<tr>
<td>Fre1</td>
<td>ferric reductase; reduces Fe$^{3+}$ to Fe$^{2+}$</td>
</tr>
<tr>
<td>Fet3</td>
<td>multicopper oxidase; oxidizes Fe$^{2+}$ to Fe$^{3+}$; forms dimer with Ftr1; required for expression of Ftr1 at plasma membrane</td>
</tr>
<tr>
<td>Ftr1</td>
<td>iron permease; transports Fe$^{3+}$ produced by Fet3 into the cell; required for copper loading of Fet3</td>
</tr>
<tr>
<td>Ctrl</td>
<td>copper transporter</td>
</tr>
<tr>
<td>Atx1</td>
<td>cytoplasmic copper-binding protein; transports copper from plasma membrane to Ccc2 at Golgi</td>
</tr>
<tr>
<td>Ccc2</td>
<td>loads copper onto apo-Fet3; required for function of Fet3 and high affinity iron transport system</td>
</tr>
</tbody>
</table>
The components of the high affinity iron uptake system are responsive to environmental signals. Transcription of the gene encoding the ferric reductase, \textit{FRE1}, is upregulated under conditions of iron and copper limitation (Dancis, et al., 1990). The activities of Fre1 and Fre2 are also temporally modulated, with the \textit{FRE1} product contributing to reductase activity during early growth, while the product of \textit{FRE2} is the predominant reductase during late exponential phase (Georgatsou and Alexandraki, 1994). Iron transport activity is also responsive to environmental iron levels (Eide \textit{et al.}, 1992). Regulation of \textit{FRE1}, \textit{FRE2}, \textit{FTR1} and \textit{FET3} is mediated by the iron-regulated transcription factor Aft1 (Yamaguchi-Iwai \textit{et al.}, 1995), (Yamaguchi-Iwai \textit{et al.}, 1996). The Aft1 consensus site, PyPuCACCCPu, is found in the 5' regions of all the above genes (Yamaguchi-Iwai, \textit{et al.}, 1996). There are other environmental cues involved in the regulation of high affinity iron uptake including ambient oxygen levels (Hassett \textit{et al.}, 1998).

The cAMP-dependent protein kinase also regulates iron uptake genes in \textit{S. cerevisiae}. Microarray analysis of genes regulated by different PKA subunits showed that Tpk2, one of three PKA catalytic subunits of \textit{S. cerevisiae}, represses transcription of genes involved in iron uptake (Robertson \textit{et al.}, 2000). Additionally, in \textit{S. cerevisiae} the ferrireductase activity of \textit{ras1} and \textit{ras2} mutants is not responsive to iron limitation, a phenotype that is relieved (in the \textit{ras2} mutant) by mutation in the regulatory subunit of Pka, \textit{bcyl}. This suggests that the ferrireductase component of the high affinity iron uptake system is at least partly controlled by cAMP-dependent protein phosphorylation (Lesuisse \textit{et al.}, 1991).
The high affinity iron transport system is not the only system available to *S. cerevisiae* for accumulation of iron. While *S. cerevisiae* does not synthesize its own siderophores, it does express transporters for uptake of xenosiderophores. The uptake of siderophore-bound iron can occur by reductive and non-reductive pathways (Yun *et al.*, 2001; Yun *et al.*, 2000). The ferric reductase components of the high affinity iron uptake system are required for reductive uptake. Genes of the major facilitator superfamily, *ARN1-4*, encode transporters involved in the non-reductive uptake of ferrioxamines, ferric triacetyl fusarinine C and ferric enterobactin (Heymann *et al.*, 2000), (Heymann *et al.*, 2000), (Yun *et al.*, 2000), (Heymann *et al.*, 1999), (Yun, *et al.*, 2000). The gene names, aliases and substrate specificity for the *ARN* genes are summarized in Table 1-2.

<table>
<thead>
<tr>
<th>Siderophore Receptor</th>
<th>Alias</th>
<th>Primary Substrate</th>
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<tbody>
<tr>
<td>Arn1</td>
<td></td>
<td>Ferrichrome</td>
</tr>
<tr>
<td>Am2</td>
<td>Taf1</td>
<td>Triacetyl fusarinine C</td>
</tr>
<tr>
<td>Arn3</td>
<td>Sit1</td>
<td>Ferrioxamine B (also ferrichrome)</td>
</tr>
<tr>
<td>Arn4</td>
<td>Enb1</td>
<td>Enterobactin</td>
</tr>
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</table>

Transport by each of the *ARN*-encoded proteins is not necessarily specific, though most of the transporters do have a preferred substrate (Lesuisse *et al.*, 2001).

Ferrioxamine B is preferentially taken up by Arn3. The product of *ARN2* is responsible for uptake of triacetyl fusarinine C. Uptake of the siderophore rhodotorulic acid appears to be by simple diffusion, not requiring any specific receptor, while ferrichrome uptake does not appear to depend on a single siderophore receptor (Lesuisse, *et al.*, 2001).
ARN1 encodes the ferrichrome transporter of *S. cerevisiae*. The mechanism of uptake via Arn1 has been studied in some detail. In the absence of ferrichrome, Arn1 is found predominantly in endosomes. However, in the presence of low concentrations of ferrichrome, Arn1 is relocated to the plasma membrane. At slightly higher ferrichrome concentrations, Arn1 present at the plasma membrane is rapidly endocytosed (Kim *et al.*, 2002). These observations support a system of uptake in which the presence of the ferrichrome stimulates membrane cycling of Arn1 between the endosome and the plasma membrane, supporting a mechanism in which surface binding of the siderophore is followed by endocytosis of Arn1. Signals that determine endosome sorting must be cytosolic, further suggesting that ferrichrome binding to Arn1 at the plasma membrane induces conformational changes to the cytosolic face of Arn1 (Kim, *et al.*, 2002).

Ferrioxamine uptake occurs by two distinct pathways in *S. cerevisiae* (Yun, *et al.*, 2000). At the cell surface the multicopper oxidase, Fet3, is sufficient for ferrioxamine uptake via a reductive mechanism. ARN3 encodes a permease found almost exclusively on the endosome; it is also sufficient for growth with ferrioxamine as the sole iron-source. The mechanism of uptake via Arn3 has yet to be elucidated although two mechanisms have been postulated. In the first, Arn3 is transiently expressed at the cell surface and is endocytosed upon ferrioxamine binding, similar to the mechanism seen for Arn1-mediated ferrichrome uptake. The second model proposes that after surface uptake the ferrisiderophore is transferred to the endosome by the permease. The localization of Arn3 has a pattern similar to that of Arn1, but is responsive to both ferrichrome and ferrioxamine B (Kim, *et al.*, 2002). The similarity of spatial re-organization of Arn3 and Arn1 in response to their respective siderophores suggests that the mechanism of uptake
of ferrioxamine B by Arn3 is similar to that for ferrichrome uptake by Arn1. Regulated endocytosis and exocytosis may be a general cell strategy for controlling the activity of membrane transport systems as it is also found for the amino acid permease Gap1. Localization of Gap1 to the plasma membrane or the late Golgi is dependent on the quality of the nitrogen source (Roberg et al., 1997).

Reductive uptake of siderophores is independent of the Arn family of transporters. Uptake of ferrioxamine B, ferrichrome, triacetyl fusarinine C and rhodotorulic acid can involve a reductase step. In wild-type cells this reduction is accomplished by the primary cell surface ferric reductases Fre1 and Fre2. A third ferric reductase, encoded by Fre3, is capable of reducing ferrioxamine B, tricacetyl fusarinine C, rhodotorulic acid (Yun, et al., 2001).

The iron-dependent transcription factor Aft1 is important for regulation of non-reductive siderophore iron uptake. AFT1 up mutants with constitutively active Aft1 accumulated such large amounts of ferric siderophores that the cells took on the rusty colour of the ferric siderophores, suggesting that Aft1 is involved in removal of iron from the siderophores (Lesuisse, et al., 2001). The global regulator Tup1 also regulates siderophore iron uptake. Disruption of Tup1 led to constitutive uptake of ferrichrome, ferricrocin and ferrioxamine B; uptake of triacetyl fusarinine C was not affected (Lesuisse, et al., 2001). Less is known about regulation of uptake of the other siderophores. Arn3, which encodes the ferrioxamine B receptor is responsive to iron levels, but is independent of the transcription factors Aft1 and Mac1 (Lesuisse et al., 1998).
1.4 Iron Uptake in *Candida albicans*

*Candida albicans* is an important opportunistic fungal pathogen and provides an interesting comparison with *C. neoformans* with respect to iron uptake. *C. albicans* normally exists as a commensal colonizing the gut and vaginal and oropharyngeal mucosa (Liu, 2002). In cases of immunosuppression, most often due to AIDS, *C. albicans* can cause systemic infection (Liu, 2002). Historically, genetic analysis of the molecular mechanisms of iron acquisition in *C. albicans* has been challenging because this organism exists as a diploid and lacks a sexual phase. Recently a mating reaction in *C. albicans* has been described (Hull et al., 2000; Magee & Magee, 2000). Many genetic studies of *C. albicans* have used complementation of *S. cerevisiae* defects in order to clone and identify *C. albicans* genes.

As a pathogen, acquisition of iron is required for *C. albicans* survival in the host. Homologs of components of the *S. cerevisiae* high-affinity iron uptake system have been described for *C. albicans*. These include a cell-associated ferric reductase that is induced under low iron conditions (Morrissey et al., 1996). As reported for *S. cerevisiae*, this ferric reductase activity is also growth stage-dependent, being most active during the early log phase (Georgatsou and Alexandraki, 1994; Morrissey, et al., 1996). This activity was later identified as the product of the *C. albicans* gene *CFL1*, which rescued the *S. cerevisiae frel* mutant (Hammacott et al., 2000). A multicopper oxidase gene from *C. albicans* has also been cloned and characterized (Eck et al., 1999). The *C. albicans* *FET3* homolog (*CaFET3*) differs from *S. cerevisiae FET3* in that *CaFET3* is not induced under low iron conditions. The *C. albicans fet3* mutant is as virulent as the wild-type in the mouse model of systemic candidiasis. The finding that the multicopper oxidase of the
The high-affinity iron uptake pathway is not the only route for iron uptake in *C. albicans*. Deletion of *CaCCC2*, the *C. albicans* ortholog of the *S. cerevisiae* CCC2, abrogates reductive iron uptake via the high affinity system, yet the mutant strain could grow on media supplemented with haem. This suggested that *C. albicans* could use haem as an iron source and that the uptake mechanism was independent of the high affinity system (Weissman *et al.*, 2002). This uptake pathway is mediated by a haem oxygenase encoded by *CaHMX1* and is strongly induced under iron-limiting conditions (Santos *et al.*, 2003). Overall, the regulatory machinery controlling iron uptake in *C. albicans* is not
fully understood. There is evidence that the Tup1 global regulator is involved in iron homeostasis (Knight et al., 2002).

1.5 Iron Biology of Cryptococcus neoformans

Iron uptake in C. neoformans is incompletely characterized. Work to date suggests that an uptake system similar to the reductive high-affinity system of S. cerevisiae exists in C. neoformans. A cell-wall associated ferric reductase activity requiring metabolic energy has been studied (Vartivarian et al., 1995). The activity of this component generates ferrous iron at the cell surface which is then internalized by the cell (Jacobson et al., 1998). The activity of the reductase is responsive to the amount of iron available to the cell and its activity is increased seven-fold when cells are starved for iron (Nyhus et al., 1997).

Iron uptake in C. neoformans is biphasic with one concentration-dependent increase in iron uptake at concentrations from one to 25 \( \mu \)M and another concentration-dependent increase in iron uptake at concentrations above 25\( \mu \)M (Jacobson, et al., 1998). Iron uptake is also dependent on both iron and copper. Iron repletion and copper limitation both decrease rates of iron uptake by the cell (Jacobson, et al., 1998).

No positive genetic identifications of the other components of the high affinity iron uptake system have been made, but four different ferric reductase regulatory (frr) mutants have been studied and possible roles in high affinity iron uptake suggested (Nyhus & Jacobson, 1999). Recent molecular characterization of frr1 identified it as a mitochondrial carrier protein of the inner mitochondrial membrane, designated MRS3/4, which is involved in iron acquisition and resistance to oxidative stress (Nyhus et al.,
2002). The mrs3/4 mutant has constitutive iron uptake and an increased sensitivity to oxidants. The frrl and oxyl mutations that are allelic to MRS3/4 code for a non-synonymous substitution of a conserved glutamate residue and a premature stop codon, respectively (Emery et al., 1994). Lack of molecular characterization of the other frrl mutants has prevented any positive assignment of function.

No siderophores have been detected for C. neoformans. However, spent media can reduce ferric iron (Nyhus, et al., 1997). It is suggested that this secreted reductant is 3-hydroxyanthranilic acid (3HAA), a compound found at concentrations up to 29 μM in iron-depleted culture supernatants but at less than 2μM in iron-replete culture supernatants. 3HAA is actively secreted by iron-limited C. neoformans cells. The activity of 3HAA and other secreted reductants contributes significantly to the iron uptake of iron-starved cells; washing the cells free of secreted reductants reduces iron uptake by nearly 50% (Jacobson, et al., 1998).

1.6 Cell Signalling in Cryptococcus neoformans

Sensing and responding appropriately to environmental signals is of key importance for organismal survival. C. neoformans expresses several easily quantified characters in response to defined signals: MATα strains will haploid fruit in response to nitrogen starvation, cells will produce melanin in the presence of diphenols, and capsule is produced under low iron conditions. These different environmental signals are transduced to the cell and stimulate the appropriate responses. The signaling pathways that accomplish this are an area of intense study. Currently there are three known cell-
signalling pathways in *C. neoformans*: the calcineurin pathway, the MAP kinase pathway and the cAMP-dependent protein kinase pathway.

The calcium-regulated protein phosphatase calcineurin has been cloned and disrupted in *C. neoformans* (Odom *et al.*, 1997). The calcineurin mutant is non-pathogenic and unable to survive at 37°C, physiological pH and dissolved CO₂ concentrations, conditions that mimic those of the host. The downstream targets of calcineurin are unknown.

Mating type-specific components of MAP kinase cascades have been identified in both cryptococcal mating types and their importance in regulating mating and virulence are areas of active research (Chang *et al.*, 2001; Wickes *et al.*, 1997). The β subunit of a heterotrimeric G protein, Gpb1, is involved in pheromone response during the early phase of mating. The early mating responses are mediated by a MAPK cascasde. For example, *gpb1* mutants are sterile and exhibit a cell fusion defect; these phenotypes are relieved by expression of the MAP kinase homolog *CPK1*. Overexpression of GPB1 causes increased conjugation tube formation and this has been shown to function in the pheromone response.

A cAMP-dependent protein kinase (PKA) pathway has been defined in *C. neoformans*. Characterization of the alpha subunit of the heterotrimeric G-protein, *GPA1*, indicates that it is required for mating and production of two of the most obvious virulence factors, melanin and capsule (Tolkacheva, McNamara *et al.* 1994; Alspaugh, Perfect *et al.* 1997). The melanin and capsule defects of the *gpa1* mutant are relieved when cells are supplied with exogenous cAMP, evidence that supports a role for cAMP signaling in *C. neoformans*’ response to nutritional signals (Alspaugh, *et al.*, 1997). The
role of Gpa1 is different from that of Gpb1. The Gα subunit senses nutrients and signals via cAMP while the Gβ subunit responds to pheromones and transduces signals via the MAP kinase pathway.

While these two signaling pathways, PKA and MAP kinase, use different G protein components to relay the signal, there is potential for cross-talk between the two pathways. When the C. neoformans RAS homolog, RAS1, is knocked-out in a serotype A strain, the mutant is unable to grow at 37°C, is defective in mating and is avirulent (Alspaugh et al., 2000; Alspaugh, et al., 1997). Melanin synthesis and capsule formation were unaffected. When ras1 mutants were supplemented with cAMP the mating defect was partially relieved. Overexpression of GPB1 in the mutant background completely relieved the mating defect, suggesting that Gpb1 functions downstream of Ras. Together, the data suggest that PKA and a MAP kinase pathway are involved in the regulation of mating and that Ras might provide a node for cross-talk between the two pathways. Figure 1-4 gives a schematic of these signaling pathways in C. neoformans.

The catalytic and regulatory components of the cAMP-dependent protein kinase have been cloned and disrupted in serotype A and D backgrounds of C. neoformans (D’Souza, et al., 2001). The serotype A null mutants for the catalytic subunit of PKA, PKA1, are acapsular, non-melanized and hypovirulent. Supplementing the cells with cAMP does not relieve the capsule and melanin phenotypes. Regulatory subunit mutants (pkr) are constitutively capsular, even under iron-replete conditions, melanize in the presence of diphenol precursors and are hypervirulent. Clearly, there are downstream targets of PKA that regulate these phenotypes.
Signalling pathways in *C. neoformans*

Schematic of the heterotrimeric G-protein-coupled signaling pathways in *C. neoformans*. The G-protein receptors are shown as open chevrons. The pheromone (MF) receptor is upstream of a MAP kinase cascade that is activated by the Gβ subunit of the G-protein. Nutrient signals are transduced by another G-protein-coupled receptor to the PKA pathway. The *C. neoformans* Ras homolog is a potential node for cross-talk between the MAP kinase and PKA pathways.
1.7 Objectives

Previous work in our laboratory involved the construction and sequencing of serial analysis of gene expression (SAGE) libraries of *C. neoformans* grown under iron-replete and iron-limited conditions. The SAGE technique uses type I and type II restriction enzyme digestion of cDNA in order to generate a 14bp tag that uniquely identifies each transcript in the cell. These tags are concatenated, cloned into a vector and then sequenced (Velculescu *et al.*, 1995). BLAST analysis of the sequenced tags against the genome sequence is used to identify the transcribed genes. Comparison of relative transcript abundance between the iron-limited and iron-replete SAGE libraries indicated that a putative *C. neoformans FTR1* homolog was expressed five-fold more under iron-limited conditions than under iron-replete conditions. The objectives of this thesis were to characterize the expression of *FTR1* and *FET3* in different serotypes and in response to different mutations of the PKA pathway and to characterize the role of the *FTR1* gene in *C. neoformans*’ biology by gene knockout.
2 Materials and Methods

2.1 Strains and Media

Plasmids were maintained in E. coli strain DH5α in LB media. C. neoformans strains H99 (serotype A, MATα), CDC2 (H99 pka1::ADE2), CDC8 (H99 pkr::URA5), JEC21 (serotype D, MATα), CDC99 (JEC21 pka2::ADE2), CDC40 (JEC21 pka1::URA5), CDC103 (JEC21 pka1::ADE2 pka2::URA5), CDC68 (JEC21 pkr::URA5), WM276 (serotype B) and ATCC 24067 (serotype D, MATα) used in expression analyses were grown in YPD followed by YNB and then in iron replete broth, iron-limited broth, and iron-limited broth supplemented with copper. Media recipes are given in the appendix. Table 2-1 summarizes the strains and genotypes used. Biolistically transformed cells were recovered on YPD agar supplemented with 1M sucrose. Nourseothricin-resistant recombinant strains were selected on YPD with 100 μg/mL nourseothricin (Werner BioAgents). Oxidant sensitivity assays used iron replete agarose and iron-limited agarose media.

Table 2-1 Summary of the C. neoformans strains used in this thesis.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Serotype</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>H99</td>
<td>A</td>
<td>wild type</td>
</tr>
<tr>
<td>CDC2</td>
<td>A</td>
<td>pka1::ADE2</td>
</tr>
<tr>
<td>CDC8</td>
<td>A</td>
<td>pkr1::URA5</td>
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<tr>
<td>JEC21</td>
<td>D</td>
<td>wild type</td>
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<td>CDC99</td>
<td>D</td>
<td>pka2::ADE2</td>
</tr>
<tr>
<td>CDC40</td>
<td>D</td>
<td>pka1::URA5</td>
</tr>
<tr>
<td>CDC103</td>
<td>D</td>
<td>pka1::URA5 pka2::ADE2</td>
</tr>
<tr>
<td>CDC68</td>
<td>D</td>
<td>pkr::URA5</td>
</tr>
<tr>
<td>WM276</td>
<td>B</td>
<td>wild type</td>
</tr>
</tbody>
</table>
2.2 Sequence Analyses *In silico*

The *FTR1* and *FET3* genes were identified by BLAST search of the SAGE tags against the genome sequence available for JEC21 from the *C. neoformans* Genome Project, Stanford Genome Technology Center, funded by the NIAID/NIH under cooperative agreement AI47087, and The Institute for Genomic Research, funded by the NIAID/NIH under cooperative agreement U01 AI48594. The sequence retrieved from the *C. neoformans* genome database was then used in BLAST search of the NCBI non-redundant nucleotide sequence database GenBank in order to assign a putative gene identification (Altschul *et al*., 1990). Searchable databases for the serotype A strain H99 available through the Cryptococcus neoformans Sequencing Project at the Whitehead Institute/MIT Center for Genome Research (http://www-genome.wi.mit.edu) and serotype B strain WM276 available through the British Columbia Cancer Agency Genome Sciences Centre as part of a Natural Sciences and Engineering Research Council-funded project were also mined for sequence. The retrieved sequences were used for DNA and protein comparison between the different *C. neoformans* serotypes. The databases for *S. cerevisiae* (Dolinski *et al*., 2003), *C. albicans*, *Histoplasma capsulatum* and *Ustilago maydis* were also searched and used for interspecific comparisons. The Saccharomyces Genome Database is available at http://www.yeastgenome.org/. Sequence data for *Candida albicans* was obtained from the Stanford Genome Technology Center website at http://www-sequence.stanford.edu/group/candida. Sequencing of *Candida albicans* was accomplished with the support of the NIDR and the Burroughs Wellcome Fund. *Histoplasma capsulatum* sequence was available through the Washington University
Genome Sequencing Centre at http://www.genome.wustl.edu/projects/hcapsulatum/. The Whitehead Institute is also responsible for the *Ustilago maydis* sequencing project.

Putative protein sequence for *C. neoformans* FTR1 and FET3 was determined by BLAST alignment against known protein sequence from *S. cerevisiae*.

2.3 Recombinant DNA Techniques

Basic DNA manipulations were performed according to standard protocols (Sambrook *et al.*, 1989). Restriction enzymes were from New England Biolabs or Invitrogen.

2.3.1 Northern Analysis of FTR1 and FET3 Under Iron-limited, Iron-Replete and Iron-Limited Copper-Replete Conditions

*C. neoformans* cells grown overnight in 4 mL YPD cultures at 30°C were used to inoculate 50 mL YNB broth at 1/1000 dilution. Cells were grown in YNB overnight, then harvested and washed three times with 20 mL of low-iron water. Cells were counted using a hemacytometer and 50 mL of iron-replete media, iron-limited media or iron-limited copper replete media was inoculated with 1X10⁹ cryptococcal cells. Cultures were incubated with shaking at 30°C for six hours. Cells were harvested and flash frozen in a dry ice/ethanol bath and lyophilized. The dried cells were then transferred to a 1.5 mL microfuge tube along with 4-6 2mm diameter glass beads. The cells were vortexed upright for three minutes on a bench-top vortexer (Baxter) and vortexed in the inverted position for another 15 minutes in a multi-tube vortexer (VWR). Powdered cells were transferred to a 15 mL screw-top tube, resuspended in 10 mL Trizol (Invitrogen), and
incubated at room temperature for five minutes. Chloroform (3 mL) was added to the tube. The tube was shaken by hand for 15 s and then incubated at room temperature for two minutes. The colloidal suspension was centrifuged at 3750 r.p.m. in a 4°C table-top centrifuge for 30 minutes (Beckman). The aqueous phase was then removed to a fresh 15 mL screw-top tube and an equal volume of chloroform was added. The tube was again shaken by hand for 15 s and incubated at room temperature for two minutes. The suspension was centrifuged at 3750 r.p.m. for 15 minutes at 4°C. The aqueous phase was then transferred to a baked, 30 mL Corex tube. RNA was precipitated by addition of 7.5 mL isopropanol and incubated at room temperature for 10 minutes. The balanced tube was centrifuged at 12000 g for five minutes at 4°C. After the supernatant was decanted and the pellet washed in 10 mL 75% ethanol (100% ethanol diluted in diethylpyrocarbonate-treated water), it was centrifuged at 7500 g for five minutes at 4°C. The pellet was air dried for 10 minutes and dissolved in RNAse-free water. To remove contaminating polysaccharide, the RNA was re-precipitated in 10M diethylpyrocarbonate-treated LiCl (200 µL/mL RNA solution) and incubated overnight at 4°C. The RNA was recovered by centrifugation at 15 000 r.p.m. for 15 minutes in a 4°C microcentrifuge. The pellet was washed in 1 mL of 75% ethanol and centrifuged at 15000 rpm for 5 minutes in a 4°C microcentrifuge. The supernatant was removed and the pellet dried for 10 minutes at room temperature. The RNA was dissolved in RNAse-free water and then stored at −75°C. For RNA manipulations, all glassware was baked for at least 4 hours at 180°C, all plasticware was treated with 3% H₂O₂ for 10 minutes and then rinsed three times with DEPC-treated water. For electrophoresis RNA (1-5 µg) was mixed with 2 µL 5X MOPS buffer, 3.5 µL 12.3 M formaldehyde and 10 µL formamide
and incubated at 65°C for 15 minutes. Formaldehyde gel loading buffer was used to load
the denatured RNA onto the formaldehyde gel. To prepare the formaldehyde gel, 1 g of
agarose was dissolved in 62 mL of DEPC-treated water. In the fumehood 20 mL of 5X
MOPS buffer and 18 mL of formaldehyde were added to the melted agarose solution.
The gel was cast in H2O2-treated gel-tray. After polymerization, the gel was transferred
to a H2O2-treated gel-box with 1X MOPS running buffer. After electrophoretic
separation, RNA was transferred overnight to HyBond-N+ (Amersham Pharmacia
Biotech) nitrocellulose membrane by capillary action using 20X SSC as the solvent.
RNA was cross-linked to the membrane by UV cross-linking (1200 J). The cross-linked
membrane was then hybridized according to the Zeta-Probe GT hybridization protocol.
Membranes were pre-hybridized in 20 mL of pre-hybridization buffer for one hour at
65°C. The pre-hybridization solution was removed and replaced with 4 mL of fresh pre-
hybridization solution to which the labeled probe was added. The hybridization probe
(the 151 bp PCR product from primers FTR1 and FTR2) was labeled with 50 µCi 32P-
dCTP using the RediPrime II oligo-labelling kit (Amersham-Pharmacia).
Membranes were hybridized overnight at 65°C. All washes were at 65°C and were as
follows: 20 mL of wash #1 for 1 hour, 20 mL of wash #1 for 45 minutes, 20 mL of wash
#2 for 45 minutes, 20 mL of wash #2 for 30 minutes. The membrane was then wrapped
in plastic wrap and exposed to X-OMAT AR autoradiographic film (EastmanKodak) or a
phosphorimager cassette (Molecular Dynamics). Exposure times ranged from 4 hours to
7 days. Autoradiographic film was developed using an automated developer.
Phosphorimager exposures were developed using the STORM laser scanner and analyzed
using ImageQuant (Amersham Biosciences) software. Quantitative comparison using the
phosphorimager data was accomplished by normalization of the \textit{FTR1} and \textit{FET3} signals to the amount of RNA loaded, as a function of the intensity of signal for the actin loading control. The RNA loading was normalized to the signal for iron-replete wild-type cells. Comparison of the actin signals to the iron-replete wild-type cells generated a normalization co-efficient used for the \textit{FTR1} and \textit{FET3} signals.

\subsection{2.3.2 Construction of Plasmids pMS3 and pMS4}

A 1.4 kb \textit{FTR1} fragment was PCR amplified using primers FTR3 and FTR4 and ligated into the pCR2.1 vector (Invitrogen) to yield pMS3. Tables 2-2 and 2-3 show the primers and primer combinations used throughout this work. pMS4 was generated by exploiting a unique \textit{XbaI} site at nucleotide position 1116 in pMS3 for insertion of the \textit{Spel/XbaI} fragment of pCH233 carrying the nourseothricin resistance cassette with a heterologous actin promoter. Figure 2-1 shows the cloning scheme to generate pMS4. Figure 2-2 shows detailed sequence analysis of the cloned region. The sequence and insert orientation of the resulting plasmids were confirmed by restriction enzyme digestion and sequencing. Figures 2-3, 2-4 and 2-5 show maps for plasmids pMS3, pCH233 and pMS4.

Table 2-2 Primers used for PCR amplifications in this thesis

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>FTR1</td>
<td>CGAGCAGCTTATGGCCGC</td>
</tr>
<tr>
<td>FTR2</td>
<td>CGACAGCTGTACCCGCCC</td>
</tr>
<tr>
<td>FTR3</td>
<td>TCCCCCCGGGGGGGACGAGCAGCTTATGGCCGC</td>
</tr>
<tr>
<td>FTR4</td>
<td>CCAATCGATTTGTTCTGCAGTTATGCCCAGGGTGGCCG</td>
</tr>
<tr>
<td>FTR7</td>
<td>ATCCACGGTGACCGGCG</td>
</tr>
<tr>
<td>FTR9</td>
<td>ATTTAGCTTCGCTCGGC</td>
</tr>
</tbody>
</table>
Table 2-3 Primer combinations used in this thesis

<table>
<thead>
<tr>
<th>Primer Combination</th>
<th>Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>FTR1 &amp; FTR2</td>
<td>Probe for Southern analysis</td>
</tr>
<tr>
<td>FTR3 &amp; FTR4</td>
<td>Fragment of FTR1 cloned into pMS3</td>
</tr>
<tr>
<td>FTR7 &amp; FTR9</td>
<td>Diagnostic for disruption of FTR1</td>
</tr>
</tbody>
</table>

Figure 2-1 Construction of pMS4

Scheme for construction of pMS4, the plasmid used to disrupt FTR1. pMS3 is the parental plasmid carrying 1.4 kb of FTR1 sequence. Plasmid pCH233 carried the nourseothricin resistance cassette (NAT) under control of the actin promoter.
Figure 2-2  Predicted amino acid sequence for FTR1.

The one-letter amino acid code is shown beneath the second nucleotide of the codon. The binding sites for primers FTR3 and FTR4 are shown in boxes. There are ten predicted exons. The XbaI restriction enzyme site used for insertion of the nourseothricin resistance cassette, located in the sixth intron, is shown as ▲.
Figure 2-3 Map of plasmid pMS3

The PCR product carrying the first 1400 nucleotides of FTR1 sequence was cloned into the parent plasmid (pCR2.1). Shown is the XbaI site used for insertion of nourseothricin resistance cassette.
Figure 2-4  Map of plasmid pCH233

Plasmid pCH233 carries the nourseothricin resistance cassette (NatR). Restriction sites for XbaI and SpeI were used for insertion of the nourseothricin resistance cassette at the XbaI site in pMS3.
Figure 2-5  Map of plasmid pMS4

The parent plasmid for pMS4 is pMS3. The unique XbaI site was used for insertion of the XbaI/Spel-digested pCH233 nourseothricin resistance cassette (NAT). pMS4 is the disruption construct used for biolistic transformation.
2.3.3 Southern Analysis of *ftrl* Mutants

Genomic DNA from putative *ftrl* mutants was isolated using a spheroplasting protocol (Casadevall and Perfect, 1998). YPD starter cultures (4 mL) grown at 30°C were diluted 500-fold into fresh YPD media and incubated at 30°C for 16 hours. Cells were pelleted at 3000 r.p.m. in a table-top centrifuge (Beckman). The pellet was then washed three times with 50 mL of a 0.5 M NaCl, 50 mM EDTA solution. The washed cells were resuspended in 9.5 mL of sterile water and 0.5 mL of β-mercaptoethanol was added. The solution was incubated at 37°C for one hour with occasional mixing. The cells were then centrifuged at 3000 r.p.m. in a table-top centrifuge for 5 minutes. The supernatant was decanted and the cells resuspended in 4 mL of SCE solution (1M sorbitol, 0.1 M sodium citrate, pH 5.8, 0.01 M EDTA). Lysing enzyme (5 g) from *Trichoderma harzianum* (Sigma) was dissolved in 1 mL SCE and added to the cells so that the final concentration of lysing enzymes was 1 mg/mL. This suspension was incubated at 37°C for 45 minutes with occasional mixing. The cells were then pelleted by centrifugation at 1000 r.p.m. for 10 minutes in the table-top centrifuge. Two washes with 5 mL of SCE solution were followed by resuspension in 2 mL of lysing solution (4% sodium dodecyl sulfate, 50 mM EDTA). Lysis proceeded for 5 minutes at room temperature. The mixture was then transferred to a 15 mL polypropylene tube and extracted twice with an equal volume of phenol:chloroform (1:1). The aqueous phase was transferred to a Corex glass tube and precipitated by addition of 1/10 volume of 3 M sodium acetate, pH 5.2 and 2 volumes of 100% ethanol. DNA was allowed to precipitate
overnight at -20°C. DNA was pelleted at 10000 r.p.m. for 30 minutes at 4°C and resuspended in 2 mL of 30 mM Tris-Cl. The DNA was then treated with 100 µg of RNase A per mL DNA solution and incubated at 37°C for 30 minutes. The RNase was removed by addition of 100 µg proteinase K per mL of DNA solution and incubated at 37°C for 30 minutes. The solution was then transferred to a 15 mL polypropylene tube and extracted with an equal volume of phenol:chloroform (1:1). The aqueous phase was extracted twice more with equal volumes of chloroform:isoamyl alcohol (24:1). The aqueous phase was transferred to a Corex tube and the DNA precipitated by addition of 1/10 volume of 3M sodium acetate, pH 5.2 and 2 volumes of 100% ethanol. Precipitation proceeded overnight at -20°C. DNA was pelleted at 10000 r.p.m. for 30 minutes at 4°C and then washed with 500 µL of 70% ethanol. DNA was resuspended in 30 mM Tris-Cl and stored at -20°C. DNA was separated on a 0.8% agarose gel in 0.5X TBE. The DNA was visualized using UV fluorescence of ethidium bromide-stained gels. Capillary transfer and UV cross-linking of DNA to nitrocellulose membrane and the hybridization protocol for Southern analysis was the same as that described here for Northern analysis, with the exception that the probe used was a 480 bp fragment from EcoRI digestion of pMS3 as the probe.

2.3.4 Northern Analysis of ftrl Mutants

RNA from ATCC 24067 and ftrl mutants grown under low iron conditions was isolated and probed as for Northern analysis of FTR1 transcript under iron-replete, iron-limited and iron-limited-copper replete conditions.
2.4 Techniques for Cryptococcus

2.4.1 Disruption of FTR1 in ATCC 24067 by Biolistic Transformation

The wild-type copy of FTR1 was disrupted in C. neoformans strain ATCC 24067 by biolistic transformation with pMS4. ATCC 24067 was grown overnight in 50 mL YPD. The cells were harvested and resuspended in 10 mL sterile distilled water. A 200 μL aliquot of cell suspension was then spread-plated onto dry YPD+1 M sorbitol agar. The cells were transformed using 1.2 mg of 0.6 μm gold beads carrying 1 μg of circular pMS4. The particles were delivered using the Biolistic PDS-1000 Particle Delivery System (DuPont Co.) with 1350 p.s.i. burst caps. After transformation, cells were allowed to recover at room temperature overnight. Plates were then scraped and the cells diluted and plated onto YPD supplemented with nourseothricin for selection.

2.4.2 Colony PCR of Putative ftr1 Cryptococcus neoformans Mutants

The putative ftr1 nourseothricin-resistant transformants were screened using colony PCR. Fresh cells from agar plates were added to 25 μL Platinum Taq (Invitrogen) PCR reactions. Primers FTR7 and FTR9 were used for diagnostic PCR screening of the transformants. FTR9 binds to FTR1 outside of the cloned region of FTR1 included in pMS4. The second pair of primers is diagnostic for a disruption of FTR1. The diagnostic PCR product is 1.4 kb. The scheme for colony PCR is shown in Figure 2-6.
Figure 2-6 Colony PCR screening scheme

Schematic of colony PCR screen using primer FTR7 and FTR9. The cloned region of *FTR1* is shown in orange. The primer binding sites for FTR7 and FTR9 are shown as blue arrows with nucleotide location in parentheses. FTR7 binds inside the nourseothricin resistance cassette (NAT, shown in burgundy). FTR9 binds outside of the cloned region of *FTR1*. The 1.4 kb product of FTR7 and FTR9 is diagnostic for a homologous recombination event at the *FTR1* locus.
2.4.3 Growth in Iron-Replete Medium and Iron-Limited Medium

The growth of the \textit{fhr1} mutants, an ectopic integrant and wild-type cells in iron-replete and iron-limited media was followed spectrophotometrically. Actively growing YPD cultures were harvested and counted. At $T_0$, 50 mL of iron-replete and iron-limited media were inoculated with $5 \times 10^7$ cells. Growth was followed spectrophotometrically at $\lambda=600\ \text{nm}$.

2.4.4 Oxidant Sensitivity

Sensitivity of cryptococcal cells to oxidants was measured as a function of growth inhibition by H$_2$O$_2$ by a modification of the protocol of Nyhus and Jacobson (Nyhus and Jacobson, 1999). Cells were grown overnight in YPD, harvested, washed and resuspended in 0.9% NaCl solution. Cells were spread-plated on iron-replete agarose or iron-limited agarose. A 6 mm disk of filter paper (Whatman) in the centre of each plate was saturated with 10 $\mu$L of 30% H$_2$O$_2$. Plates were incubated overnight at 30$^\circ$C and diameters of the zones of clearing around the filter paper disk were measured. Statistical analysis was by ANOVA.

2.4.5 India Ink Staining

The capsular phenotype of \textit{C. neoformans} was determined by India ink staining. Cells were spotted onto microscope slides and mixed with 2 microlitres of India ink.
Stained cells were observed at 1000X magnification on a Zeiss photomicroscope and images captured using NorthernEclipse software.
3 Results

3.1 A Gene Cluster Containing FTR1 and FET3

The preliminary identification and characterization of iron-regulated genes was the primary focus of initial work after the construction and sequencing of the SAGE libraries. The 14-nucleotide SAGE tags that showed the greatest fold-induction between the cells grown in iron-replete medium and iron-limited medium were used in a BLAST search against the serotype D strain B3501A C. neoformans genome database at Stanford Genome Technology Center. This analysis identified orthologs of the S. cerevisiae FTR1 and FET3 genes and these genes were clustered in the same region of contiguous genomic sequence (T. Lian, personal communication). Further study revealed that these two open reading frames were transcribed divergently. The two genes are separated by 1050 bp, and the region contains consensus binding sequences for the copper-regulatory transcription factor Mac1 (5'- TTTGC(T/G)C(A/G)-3') and the iron-regulatory transcription factor Aft1 (5'-(C/T)(G/A)CACCC(G/A)-3') as shown in Figure 3-1 (McDaniels et al., 1999; Yamagucho-Iwai, et al., 1996). The progress of the sequencing efforts for C. neoformans serotype A (H99) and serotype B (WM276) allowed a BLAST analysis of these genomes as well. The sequence homology and overall gene architecture are preserved in these other serotypes. The sequence identity between serotype A and serotype D is over 87% at the nucleotide level. The serotype B sequence, based on a contig constructed using VectorNTI Contig Express, is 65.3% identical to the serotype D sequence. The greater dissimilarity between serotype D and B may be the result of greater evolutionary distance between these two serotypes. However, the sequencing
Figure 3-1 Genetic architecture of *FTR1* and *FET3*

In this schematic the start and end points for *FET3* and *FTR1*, relative to position 1 (at the left end of the sequence) are in parentheses. The intergenic region is 1050 bp. Putative consensus binding sites for copper regulatory elements (CuRE) (5'-TTTGC(T/G)C(A/G)-3') and the iron-regulated transcription factor Aft1 5'-(C/T)(G/A)CACCC(G/A)-3' are shown in blue. The exact sequence locations for the CuRE and Aft1 binding sites are in parentheses.
effort for serotype B has not reached the 10X genome coverage considered necessary for a finished genome, and the similarity between serotype D and B may increase in the future as more sequence is finished.

Alignment of the *C. neoformans* iron permease predicted protein sequence with the *S. cerevisiae* homolog showed significant sequence similarity (48.5%) and over 30% identity (32.2%). Of note, the REGLE motif (the glutamic acids of which are thought to interact directly with iron) was conserved in the *C. neoformans* sequence (Stearman, et al., 1996).

Alignment of the putative *FET3* amino acid sequence with that for the *S. cerevisiae* homolog showed lower but still significant similarity (38.3%) and identity (24.1%) scores. The *FET3* sequence retains a putative multicopper oxidase signature that shows 76% similarity to that reported for *S. cerevisiae* (DNPGWVFFHCHIEWHLLQGLGLVLV) (Askwith & Kaplan, 1998). The retention of this signature is in accordance with the postulated role of Fet3 in ferrous iron oxidation. The protein alignments for *FTR1* and *FET3* with the *S. cerevisiae* sequences are shown in Figures 3-2 and 3-3.

In conclusion, homologs of *S. cerevisiae* *FTR1* and *FET3* were identified as iron-regulated genes in *C. neoformans* by BLAST analysis of SAGE tags from iron-limited and iron-replete libraries.
Figure 3-2  Protein alignment of Ftrl with \textit{S. cerevisiae} sequence

The \textit{S. cerevisiae} iron permease (Sc Ftrl) is aligned with the putative sequence for \textit{C. neoformans} iron permease (Cn Ftrl). The sequence similarity between the two proteins falls off steeply after Cn Ftrl residue 309. Identical residues are shown in red. Blocks of similar sequence are shown on a grey background. The conserved REGLE motif implicated in direct interaction with iron is boxed.
Figure 3-3  Protein alignment of Fet3 with *S. cerevisiae* Fet3 sequence

The incomplete sequence for *C. neoformans* Fet3 (CnFet3) is compared against the *S. cerevisiae* homolog (Sc Fet3). The putative multicopper oxidase signal is boxed.
3.2 Regulation of *FTR1* and *FET3* Transcription in *C. neoformans*

3.2.1 Transcriptional Control of *FTR1* and *FET3* by Iron and PKA Pathway Components

Northern hybridizations were performed in order to confirm the results from the SAGE library showing that *FTR1* and *FET3* were regulated by iron. In order to determine if the transcriptional response to iron-limitation was conserved among *C. neoformans* strains of different serotypes, Northern analysis of RNA from the serotype D strain JEC21, the serotype B strain WM276 and the serotype A strain H99 under iron-replete and iron-limiting conditions was carried out. As can be seen in Figure 3-4, the increase in *FTR1* transcript level in response to iron starvation observed for the serotype D strain JEC21 was also observed in the H99 serotype A strain and the WM276 serotype B strain. The fainter signal for the serotype B hybridization is probably a function of the fact that the probe was specific to serotype D. The sequence alignment between the serotypes, discussed in Section 3.1, showed that there was less sequence similarity between serotypes D and B (65.3% identical) than between serotype D and A (87% identical).

As mentioned earlier, components of the PKA pathway have been implicated in the regulation of genes associated with iron uptake in *S. cerevisiae* (Robertson, et al., 2000). In *C. neoformans* iron starvation stimulates capsule synthesis, a phenotype shared by cells with constitutive PKA pathway activity (D'Souza, et al., 2001; Robertson, et al., 2000). Northern analysis of *FTR1* and *FET3* transcription in PKA mutants was performed to test the hypothesis that components of the PKA pathway were involved in cell-signalling events mediating *C. neoformans'* response to iron-limitation.
Figure 3-4  Northern analysis of FTR1 transcription in response to iron in the C. neoformans serotype D, B and A (H99) backgrounds.

The loading control was the methylene blue-stained rRNA on the membrane.

RNA was isolated from the following serotype D strains of C. neoformans:

JEC21 (wild-type), CDC99 (pka2), CDC40 (pka1), CDC103 (pka1 pka2) and CDC68 (pkr). Cells were grown in iron-limited media and iron-replete media and RNA from these cells was analyzed by Northern blot. The RNA on the membrane was hybridized in succession with an FTR1 specific probe, an actin probe (as a loading control) and a FET3 specific probe. Phosphorimager analysis allowed normalization and quantitation of the fold-differences in expression between the different strains and the different treatments. The results of the three hybridization experiments are shown in Figure 3-5.
Figure 3-5  Northern analysis of FTR1 and FET3 transcription in PKA mutants.
Transcript levels of FTR1 (top panel), FET3 (middle panel) in serotype D PKA mutants grown under iron-replete (+Fe) and iron-limited (-Fe) conditions. The bottom panel is an actin loading control. On a qualitative level, the results of this Northern are representative of the many replicates done, however, this was the only experiment that generated quantitative data.

Qualitatively, the pattern of expression is the same for the two different genes in the various genetic backgrounds. FTR1 and FET3 transcript levels are elevated in cells that are starved for iron, regardless of the genotype of the cells. This confirms the SAGE result that showed a five-fold increase in FTR1 transcript level and a 1.3-fold increase in FET3 transcript level in iron-limited compared to iron-replete conditions.
Visual inspection of the hybridization results also shows that the catalytic subunits of PKA, Pkal and Pka2, are redundant for maintaining a basal level of expression for both \textit{FTR1} and \textit{FET3}. Deletion of both \textit{PKA1} and \textit{PKA2} appears to greatly reduce \textit{FTR1} and \textit{FET3} transcription under iron-replete conditions. The fold-differences in transcription of \textit{FTR1} and \textit{FET3} under iron-limited compared to iron-replete conditions are shown in Table 3-1.

### Table 3-1
Fold-difference of \textit{FTR1} and \textit{FET3} expression in iron-limited media compared to iron-replete media

<table>
<thead>
<tr>
<th>Strain</th>
<th>\textit{FTR1} Fold Difference (Iron Limited):(Iron Replete)</th>
<th>\textit{FET3} Fold Difference (Iron Limited):(Iron Replete)</th>
</tr>
</thead>
<tbody>
<tr>
<td>JEC21, wild-type</td>
<td>3.4</td>
<td>7.4</td>
</tr>
<tr>
<td>CDC99, pka2</td>
<td>2.1</td>
<td>1.1</td>
</tr>
<tr>
<td>CDC40, pka1</td>
<td>3.9</td>
<td>1.8</td>
</tr>
<tr>
<td>CDC103, pka1 pka2</td>
<td>26.4</td>
<td>7.6</td>
</tr>
<tr>
<td>CDC68, pkr</td>
<td>3.5</td>
<td>2.5</td>
</tr>
</tbody>
</table>

Comparison of basal transcript levels under iron-replete conditions shows that the effects of Pkal and Pka2 on basal \textit{FTR1} transcription are roughly additive. When \textit{PKA1} alone is knocked out, basal transcription of \textit{FTR1} under iron-replete conditions drops to 58% of the wild-type level. When \textit{PKA2} alone is knocked out, basal transcription of \textit{FTR1} is 66% that of wild-type. When both \textit{PKA1} and \textit{PKA2} are knocked out, the basal transcription level of \textit{FTR1} is 5% that of the wild-type. These results, summarized in Table 3-2, suggest that maintenance of basal transcript levels of \textit{FTR1} requires at least one of the Pka catalytic subunits.
Table 3-2 Comparison of basal transcript levels of FTR1 and FET3 in PKA mutants compared to wild-type.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Basal FTR1</th>
<th>Basal FET3</th>
</tr>
</thead>
<tbody>
<tr>
<td>JEC21, wild-type</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>CDC99, pka2</td>
<td>0.66</td>
<td>2.04</td>
</tr>
<tr>
<td>CDC40, pka1</td>
<td>0.58</td>
<td>4.14</td>
</tr>
<tr>
<td>CDC103, pka1 pka2</td>
<td>0.06</td>
<td>0.57</td>
</tr>
<tr>
<td>CDC68, pkr</td>
<td>0.64</td>
<td>2.53</td>
</tr>
</tbody>
</table>

The pattern for maintenance of basal levels of FET3 expression appears to be different from that for FTR1. Knockout of PKA2 increases transcription of FET3 two-fold above wild-type. The FET3 transcript level is four-times that of the wild-type in the PKA1 mutant. These two results suggest that the catalytic subunits of PKA act to repress FET3 transcription under iron-replete conditions, an effect opposite to that observed for basal transcription of FTR1. Surprisingly, the double catalytic knockout has basal FET3 transcript levels that are about half that of wild-type. This result suggests that the catalytic subunits act to maintain basal FET3 transcript levels. Finally, the regulatory mutant has basal FET3 transcription that is more than two times greater than the wild-type, a result that is consistent with a role for the catalytic subunits in stimulation of FET3 transcription under iron-replete conditions. In conclusion, there does not appear to be a coherent pattern to the transcriptional effects of PKA pathway components on FET3.

3.2.2 Divergent Regulation by Copper

The effect of copper repletion on the transcription of FTR1 and FET3 was analyzed, as iron-limited media is also copper-restricted and copper is known to influence
the expression of genes involved in iron uptake (Vartivarian, et al., 1993) (Lin, et al., 1997; Yuan et al., 1995). JEC21 was grown in iron-limited media and iron-limited media supplemented with 5μM CuSO₄. RNA was isolated and the transcript levels of FTR1 and FET3 were determined by Northern hybridization. The hybridization results are shown in Figure 3-6 and demonstrate that copper-repletion reduces FTR1 transcript levels and elevates FET3 transcript levels. This is different from the pattern seen in S. cerevisiae, where both FTR1 and FET3 are upregulated in response to copper repletion (Askwith, et al., 1994; Gross et al., 2000). These results suggest that homologous components of C. neoformans and S. cerevisiae high-affinity iron uptake systems are subject to different patterns of copper regulation.

Figure 3-6 Northern analysis of FTR1 and FET3 response to copper.

FTR1 expression (top panel) and FET3 expression (middle panel) in JEC21 grown under inducing (low-iron) conditions with (+Cu) and without (-Cu) supplemental copper. The lower panel shows the methylene blue-stained rRNA loading control on the membrane that was subsequently hybridized.
3.2.3 Construction of FTR1 Disruption Mutants

The role of the iron permease homolog, FTR1, in *C. neoformans* biology was further characterized by generating a mutant carrying a truncated FTR1 allele. The disruption construct carried the 5' region of FTR1 that encodes the first 220 amino acids of the iron permease, including the conserved REGLE domain implicated in direct interaction with iron (Stearman, et al., 1996). A nourseothricin resistance cassette for positive selection was inserted 28 nucleotides downstream from the REGLE-containing exon. The resulting plasmid, pMS4, was used for biolistic transformation of the serotype D strains JEC21 and ATCC 24067. Nourseothricin-resistant transformants were screened by colony PCR using primers FTR7 and FTR9 which give a diagnostic 1.6 kb product for strains in which homologous recombination at the FTR1 locus has occurred. Strains identified by colony PCR as putative mutants were confirmed by Southern blot of BglII-digested genomic DNA. Figure 3-7 shows the schematic for the Southern analysis. The probe is expected to hybridize to a 1.6 kb band in the wild-type strain. BglII-digested genomic DNA from mutants that are the result of a homologous recombination event will lack the 1.6 kb signal, having a signal at 3.4 kb instead.

Figure 3-8 shows the Southern analysis of the putative mutants in the ATCC 24067 background. The wild-type strains had the expected hybridization pattern. In the collection of transformants in the ATCC 24067 background, the probe hybridized to a ~3.4 kb fragment in strains A3-54, A3-56, A3-63 and A3-65 only, suggesting that these represented homologous integrants. Strains A3-27, A3-43, A3-53 and A3-68 represented mutants in which the disruption construct was integrated ectopically; they retained the
Figure 3-7  Scheme for Southern analysis of *ftrl* mutants

This screen exploits differences in the *BglII* digestion pattern between wild-type and mutants that are the result of a homologous recombination event at the *FTR1* locus. The probe is shown as a black bar. The top scheme shows the 1.6 kb fragment from the wild-type that the probe will hybridize to. The bottom scheme shows 3.4 kb fragment that the probe will hybridize to in homologous integrants.
Figure 3-8  Southern analysis of firl mutants in the ATCC 24067 background

*BglIII*-digested genomic DNA from putative firl mutants was analyzed by Southern hybridization. Strains A3-54, A3-56, A3-60, A3-63 and A3-65 represent homologous integrants. In the other strains ectopic integrations have occurred.

wild-type copy of the allele with addition of other, higher molecular weight species that hybridized to the *FTR1*-specific probe.

Strains A3-54, A3-56, A3-60, A3-63 and A3-65 from the ATCC 24067 background were selected for further characterization. Strain A3-27 was retained as an ectopic transformant control.

The JEC21 transformants, shown in Figure 3-9, were all either ectopic integrants or homologous integrants carrying co-incident ectopic integrations of the construct.
3.3 Phenotypes of *C. neoformans ftr1* Mutants

3.3.1 Transcription of the Truncated *FTR1* Transcript in *C. neoformans ftr1* Mutants

The transcript length of the putative *ftr1* mutants was confirmed by Northern blot analysis of RNA from ATCC 24067 and the *ftr1* mutants grown in iron-limited media.

Figure 3-10 shows the Northern blot of all five strains. Strains A3-54, A3-63 and A3-65
expressed the expected truncated \( FTR1 \) transcript. Strain A3-56 expressed a transcript approximately the same size as the wild-type transcript and this strain was eliminated from further characterization. Of interest was the fact that the signal intensities for all of the \( ftrl \) mutants (even the disqualified A3-56) exceeded those of the ATCC 24067 wild-type. Given that all strains were grown under \( FTR1 \)-inducing conditions and that RNA loading was approximately equal, it is possible that this difference in signal intensity was caused by increased level of iron-starvation in \( ftrl \) mutants. If so, then this result, aside from confirming the reduced transcript size, also supports the assertion that the \( ftrl \) mutants are impaired in their ability to import iron into the cell. However, there are other possible explanations for the observed increase in transcript level. For example it is possible that the truncated transcript lacks degradation signals or is more stable than the wild-type transcript.

![ATCC A3-54 A3-56 A3-63 A3-65 24067](image)

Figure 3-10 Transcription of \( FTR1 \) in \( ftrl \) mutants under iron-limiting conditions

The top panel shows the Northern blot of \( ftrl \) mutants grown under iron-limiting conditions and probed with \( FTR1 \) probe. The lower panel shows the methylene blue-stained membrane prior to hybridization. Transcript level for each of the mutants is much higher than that for the wild-type.
3.3.2 Capsule Phenotype

Iron limitation stimulates expression of capsule in *C. neoformans* and since the *ftr1* mutants are thought to be iron deficient, the capsule phenotype of the *ftr1* mutants was determined by India ink stain. Figure 3-11 shows examples of encapsulated and unencapsulated cells as visualized by India ink stain. For the assay the *ftr* and wild-type strains were grown in iron-replete and iron-limited media, stained and observed by light microscopy. All strains from all conditions expressed thick polysaccharide capsule, as seen in Figure 3-12. Expression of capsule by the wild-type strain under iron-replete conditions suggests that ATCC 24067 has constitutive expression of capsule. The pattern of capsule expression in ATCC 24067 obscured any changes in capsule expression in the mutants. From these results, the conclusion is that the disruption of iron transport does not interfere with capsule expression in the ATCC 24067 background.

Figure 3-11 Encapsulated and unencapsulated *C. neoformans* cells

The heavily encapsulated cells on the left show the characteristic “halo” around the cell where the polysaccharide excludes India ink particles. On the right are India ink-stained unencapsulated cells. No halo is present.
India ink staining was used to determine capsule phenotypes of wild-type (ATCC 24067) and $ftrl$ mutants (A3-54, A3-63 and A3-65) grown in iron-replete media and iron-limited media.

Figure 3-12  Capsule phenotypes of $ftrl$ mutants

India ink staining was used to determine capsule phenotypes of wild-type (ATCC 24067) and $ftrl$ mutants (A3-54, A3-63 and A3-65) grown in iron-replete media and iron-limited media.
3.3.3 Iron-Dependent Growth

The effect that disruption of the high-affinity iron transport system had on cell growth and reproduction was evaluated by growing the confirmed \textit{ftrl} mutants in iron-replete and iron-limited media. Growth was followed by spectrophotometric measurement of the optical density of cultures over time. Figures 3-13 and 3-14 show the growth of the wild-type (ATCC 24067), \textit{ftrl} mutant (A3-54, A3-63 and A3-65) and ectopic integrant (A3-27) strains in iron-replete and iron-limited media. There was no statistically significant difference in growth of the wild-type and ectopic integrant strains in iron-replete and iron-limited media. For the \textit{ftrl} mutants, there were differences in growth between the two media treatments. Qualitative comparison of the growth curves within the two different treatments showed that in iron-replete media, the \textit{ftrl} mutants have a longer lag phase and longer generation time than the wild-type strain; however, the \textit{ftrl} mutants do reach a final cell-density identical to that of the wild-type. Growth of the \textit{ftrl} mutants in iron-limited media is grossly impaired. The cells show no discernible log phase and final cell densities are a fraction of that seen for wild-type.
Figure 3-12  Growth of $ftrl$ mutants in iron-replete media

Growth of wild-type ATCC 24067, ectopic mutant A3-27, and $ftrl$ mutants A3-54, A3-63 and A3-65 in iron replete media. This graph shows the results of one experiment representative of three separate trials.
Figure 3-13  Growth of *ftrl* mutants in iron-limited media

Growth of wild-type ATCC 24067, ectopic integrant A3-27, and *ftrl* mutants A3-54, A3-63 and A3-65 in iron-limited media. This graph shows the results of one representative experiment from three replicates performed.
3.3.4 Oxidant Sensitivity

Reactive oxygen species (ROS) are an important component of host cellular response to invading microbes. The ROS produced by phagocytic cells are used to kill phagocytosed microbial cells. Intracellular pathogens have defenses against ROS, one of which is the expression of haem-containing enzymes like catalases and peroxidases which catalyze the degradation of hydrogen peroxide into water and oxygen. Iron-limitation could therefore affect a cell's sensitivity to oxidizing species like hydrogen peroxide, assuming that the cell expresses catalases or peroxidases. A BLAST search of the \textit{C. neoformans} genome database at the Stanford Genome Technology Center (http://www-sequence.stanford.edu/group/C.neoformans/) using sequence for the \textit{S. cerevisiae} catalase genes revealed two putative catalase genes in the \textit{C. neoformans} genome. Oxidant sensitivity of the \textit{ftr1} mutants under iron-replete and iron-limited conditions was measured using a plate assay to determine the zone of inhibition in response to \textit{H}_2\textit{O}_2 (Nyhus and Jacobson, 1999). Figure 3-15 shows an example of the assay. Figure 3-16 shows the error-bar chart of the mean zone of inhibition for the different strains under iron-replete and iron-limited conditions.

![Oxidant sensitivity plate assay](image)

Figure 3-15 Oxidant sensitivity plate assay
An example of the plate assay used to determine oxidant sensitivity.
Figure 3-16  Mean zone of inhibition in response to oxidative stress

Error bar chart showing mean zone of inhibition for wild-type (ATCC 24067), ectopic integrant (A3-27) and fir1 mutant (A3-54, A3-63 and A3-65) cells in oxidant sensitivity assays under iron replete (IRM) and iron-limited (LIM) conditions. Cumulative results from three separate trials are shown. Error bars show the 95% confidence interval for the measurements.

Statistical analysis of the data focused on three kinds of comparison, comparison of means between strains for each treatment, comparison within strains between the two treatments and a comparison between strains under different treatment regimens.

Comparison between strains for each treatment showed that there was not a statistically significant difference between the mean zones of inhibition seen for the wild-type and
ectopic integrant. The mean zone of inhibition observed for ATCC 24067 in low-iron media is indistinguishable from that seen for A3-27, the ectopic integrant, in low-iron media. The mean zones of inhibition for the wild-type and ectopic integrant under iron-replete conditions show no significant difference. Comparison of means between the wild-type and the mutants showed a statistically significant difference under both media regimes. Comparison within strains showed a significant difference between the iron-replete and iron-limited treatments for every strain except A3-54. Using a pooled estimation in which the three ftr1 mutants are treated together as one category, the differences in mean diameter between iron regimens are significantly different for all three strain classes (wild-type, ectopic integrant and ftr1 mutant). This suggests that iron-status of the media influences the response of all cells, regardless of genotype, to oxidative stress. Interestingly, the comparison of means between the iron-limited wild-type and the iron-replete mutants does not yield a statistically significant difference. This result suggests that the iron-replete ftr1 mutants behave like the iron-starved wild-type cells; i.e., the ftr1 mutants starve for iron amidst ferric abundance. Iron supplementation does not completely relieve the mutant phenotype, a pattern similar to that seen for growth of the mutants in iron-replete media. Disruption of the high-affinity iron uptake system seriously impairs the cells’ ability to take up iron. The contribution of the constitutively active low-affinity iron uptake system is not sufficient to restore the wild-type phenotype to iron-replete ftr1 mutants. The lack of a difference in phenotype between the iron-limited wild-type and the iron-replete ftr1 mutants also suggested that, at least under the conditions of this assay, iron availability via import is a key determinant
of *C. neoformans*’ response to oxidative stress. This finding is in agreement with the suggestion that iron-containing enzymes could mediate *C. neoformans*’ response to oxidative stresses.
4 Discussion

*C. neoformans* includes serotypes that are both primary and opportunistic pathogens. The recent outbreak of cryptococcosis on Vancouver Island and the ongoing AIDS epidemic in sub-Saharan Africa highlight the local and global importance of this pathogen (Bogaerts et al., 1999; Stephen, et al., 2002). *C. neoformans* expresses several virulence factors, a thick polysaccharide capsule being the most obvious. Capsule expression in response to iron limitation has been well established and this observation prompted us to initiate an analysis of iron regulated gene expression in *C. neoformans*. To this end, the construction of SAGE libraries for cells grown under iron-limiting and iron-replete conditions identified a homologue of *S. cerevisiae* FTR1 that was expressed five-fold higher under iron-limited conditions (T. Lian, in preparation). Interestingly, the multicopper oxidase FET3 that is the other component of the iron transport heterodimer is in a cluster with FTR1. The work described in this thesis investigated the regulation of *C. neoformans* FTR1 and FET3 by components of the PKA pathway, compared the genetic arrangement of the components of the iron transport complex with the architecture of these genes in *S. cerevisiae* and *C. albicans*, and examined the molecular role of the FTR1 gene product in the response of *C. neoformans* to a low iron environment.

4.1 The FTR1 and FET3 Genes Are Part of a Gene Cluster in *C. neoformans*

Application of the SAGE technique, combined with the progress of the genome sequencing efforts for *C. neoformans* allowed identification of the iron-regulated FTR1 and FET3 genes in *C. neoformans* and determination of their arrangement in the genome.
In *C. neoformans* *FTR1* and *FET3* are transcribed divergently and separated by 1 kb. Close clustering of other genes in *C. neoformans* has been reported previously. For example, three of the capsule associated genes identified in the laboratory of K. J. Kwon-Chung are tightly linked to other genes. *CAP60* is convergently transcribed with a gene similar to *CEL1*, the cellulose growth-specific gene of *Agaricus bisporus* (Chang and Kwon-Chung, 1998). *CEL1* and *CAP60* are separated by 478 base pairs. *CAP64* is convergently transcribed with a gene similar to the yeast proteasome subunit *PRE1*. These two genes are separated by 193 bp and there is evidence that the 3' cDNA ends of these two genes may even overlap (Chang, et al., 1996). The *CAP59* gene is convergently transcribed with *L27*, a gene coding for a ribosomal protein. Like *CAP64* and *PRE1* these two genes are separated by about 200 bp and the cDNA ends of these genes probably overlap (Chang *et al.*, 1995). The characteristics of the genes linked to the *CAP* genes are summarized in Table 4-1.

<table>
<thead>
<tr>
<th>CAP Gene</th>
<th>Linked Gene</th>
<th>Identity of Linked Gene</th>
<th>Intergenic Distance, bp</th>
<th>Arrangement</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAP59</td>
<td>L27</td>
<td>homolog of yeast ribosomal protein</td>
<td>234</td>
<td>CAP59 → L27</td>
</tr>
<tr>
<td></td>
<td>CEL1</td>
<td>homolog of <em>A. bisporus</em> cellulose-specific growth</td>
<td>476</td>
<td>CEL1 → CAP60</td>
</tr>
<tr>
<td>CAP64</td>
<td>PRE1</td>
<td>homolog of yeast proteasome subunit</td>
<td>193</td>
<td>PRE1 → CAP64</td>
</tr>
</tbody>
</table>
The intergenic distance between \textit{FTR1} and \textit{FET3} is in line with that reported for these other linked genes in \textit{C. neoformans}. Given that the gene functions are not known for the \textit{CAP} genes, it is impossible to determine whether the genes linked to the respective \textit{CAP} genes form part of a functional group.

The functional relationship between \textit{FTR1} and \textit{FET3} has been clearly illustrated in \textit{S. cerevisiae} (Stearman, et al., 1996). Cross-species comparisons of \textit{C. neoformans} with \textit{S. cerevisiae}, \textit{C. albicans}, \textit{S. pombe}, \textit{Ustilago maydis} and \textit{Histoplasma capsulatum} were performed using the available genome databases. Searches of the \textit{U. maydis} and \textit{H. capsulatum} databases did not yield \textit{FTR1} or \textit{FET3} homologs. For the species that could be compared (\textit{S. cerevisiae}, \textit{C. albicans} and \textit{S. pombe}) the results were illuminating with respect to overall genetic organization of genes encoding the high affinity iron-uptake system. There are some striking differences and some informative similarities, summarized in Table 4-2. Compared with \textit{S. cerevisiae} and \textit{C. albicans}, \textit{C. neoformans} is unusual in possessing only one \textit{FET3} gene. \textit{S. cerevisiae} has five \textit{FET} homologs and \textit{C. albicans} has four. For \textit{S. cerevisiae} the relatively close clustering of the \textit{FET} genes is probably the result of the whole-genome duplication that occurred in \textit{S. cerevisiae} about 100 million years ago (Wolfe & Shields, 1997). \textit{S. pombe}, unlike \textit{S. cerevisiae} and more similar to \textit{C. neoformans}, has only a single \textit{FET} gene. Each of these fungi has only one known iron permease. In \textit{S. cerevisiae} the permease is on a different chromosome than the oxidases, possibly as a result of recombination events subsequent to genome duplication that have disrupted previously linked groups of genes (Karkhoff-Schweizer, et al., 1994). In \textit{C. albicans} these genes are separated by nearly 6 Mb; sequencing efforts
for *C. albicans* have not yet determined the chromosomal boundaries. In contrast, the *C. neoformans* iron permease and multicopper oxidase are clustered together, separated by just 1 kb of sequence. The close clustering of the permease and multicopper oxidase seen in *C. neoformans* also appears in *S. pombe*, in which the two genes are separated by about 1.1 kb. Analysis shows that for both *C. neoformans* and *S. pombe* the permease and oxidase are divergently transcribed. Molecular phylogeny of the ascomycetes and basidiomycetes suggests that these two groups diverged from a common ancestor about 400 million years ago (Berbee & Taylor, 1993). The similarity in genetic architecture of the *FTR1* and *FET3* genes between *S. pombe*, an archaeascomycete and *C. neoformans*, a basidiomycete, suggest that the close genetic arrangement of *FTR1* and *FET3* might have been the ancestral condition. However, the *FTR1* and *FET3* cluster represents only one incidence of this preservation of genetic architecture between an ascomycete and a basidiomycete, so arguments about ancestral conditions remain hypothetical. A bioinformatic analysis of the respective genomes to determine preservation of gene linkage groups between *S. pombe* and *C. neoformans* would be required to test this hypothesis. The arrangement of *FTR1* and *FET3* in *C. neoformans* reported here differs from the arrangement of *FTR1* and *FET3* in *S. cerevisiae* and *C. albicans*, and is the first incidence of clustering of functionally linked genes in *C. neoformans*.
Iron import can be considered a virulence factor. The transferrin-binding proteins of *Actinobacillus pleuropneumoniae* have been characterized as virulence factors (Baltes et al., 2002). In *C. albicans* the iron permease is necessary for virulence in mice (Ramanan and Wang, 2000). If such a relationship also holds for *C. neoformans* then the clustering of *FTRl* and *FET3* represents a clustering of virulence determinants.

Pathogenicity islands in uropathogenic *E. coli* contain (among other things) iron uptake systems that contribute to the pathogenicity of these bacteria (Oelschlaeger et al., 2002). The *Yersinia* high pathogenicity island carries genes involved in the synthesis of siderophores and is found across multiple bacterial genera including *E. coli, Klebsiella* and *Salmonella* [Carniel, Microbes and Infection #159]. In *Streptococcus pneumoniae* two putative iron transporters located within a putative pathogenicity island are required for iron uptake from haemoglobin (Brown et al., 2001).
Clustering of genes that encode pathogenicity determinants has also been reported in other fungal pathogens. In *Aspergillus fumigatus* pigmentation of conidia is an important virulence determinant in the mouse model (Langfelder et al., 1998). Pigment biosynthesis in conidia of *A. fumigatus* is developmentally regulated by a cluster of at least six genes located within a 42.5 kb stretch of DNA. These genes encode some of the enzymes required for dihydroxynaphthalene melanin biosynthesis (Tsai et al., 1999). In *C. albicans* the *NAG* gene cluster is required for growth on N-acetylglucosamine. *NAG1*, *NAG2* and *NAG5* are required for N-acetylglucosamine catabolism (Yamada-Okabe et al., 2001). In this group, *NAG1* is transcribed divergently from *NAG2* and *NAG5*. Disruption of the glucosamine-6-phosphate deaminase (Nag1), N-acetylglucosamine-phosphate deacetylase (Nag2) and N-acetylglucosamine kinase (Nag5) cause changes in morphology (a lack of germ-tube formation in response to N-acetylglucosamine and hyperfilamentation in response to nutrient stress) and hypovirulence in a mouse model of systemic candidiasis (Singh et al., 2001). Mutation of the other genes of the cluster, *NAG3*, *NAG4* and *NAG6*, alter drug sensitivity and virulence, but the precise roles of these genes are not clearly known (Yamada-Okabe & Okabe, 2002). Interestingly, *NAG6* in this cluster is also transcribed divergently from *NAG3* and *NAG4*.

In summary, review of the literature shows that the individual characteristics of the *FTR1* and *FET3* cluster (clustering of genes in *C. neoformans*, clustering of iron uptake genes, clustering of functionally linked genes for virulence determinants and divergent transcription of genes in the cluster) are not uncommon. However, while incidence of each of the attributes of the putative *C. neoformans* iron-uptake cluster has
been reported, the occurrence of a cluster of functionally linked, iron uptake genes with a putative role in virulence has not previously been described in *C. neoformans*.

### 4.2 Regulation of *FTR1* and *FET3* by Iron and Copper

Based on the high sequence similarities between the *FTR1* and *FET3* genes of *C. neoformans* and *S. cerevisiae*, these genes probably represent the high affinity iron-uptake system of *C. neoformans*. The severe iron-limitation encountered in the host suggests that this plasma membrane complex is likely to be an important determinant of cell survival inside the host. As such, the regulation of this complex is of paramount importance to the cell. Transcription of the permease and oxidase that make up the iron transport complex in *C. neoformans* are regulated in response to iron just as in the high affinity iron uptake system of *S. cerevisiae*. In *S. cerevisiae*, iron uptake is regulated by environmental iron levels (Eide, et al., 1992). Transcription of the yeast permease and oxidase is stimulated under iron-limited conditions (Askwith, et al., 1994; Stearman, et al., 1996). In all of the *C. neoformans* serotypes tested the same pattern of transcription is seen for *FTR1* and *FET3*. In *S. cerevisiae* *FET3* and *FTR1* are also regulated by copper; transcription being stimulated when cells are grown under copper-rich conditions (Askwith, et al., 1994; Gross, et al., 2000). The effects of copper on expression of *FTR1* and *FET3* in *C. neoformans* are different. The multicopper oxidase of *C. neoformans* is upregulated in response to copper repletion, but the permease component of the complex is repressed under iron-replete conditions. The divergent regulation of the two components of the high affinity iron transporter by copper marks an unexpected difference in regulation of the iron transport systems of *C. neoformans* and *S. cerevisiae*. 
4.3 Regulation of \( FTR1 \) and \( FET3 \) by Components of the PKA Pathway

Components of the PKA pathway also influence transcription of the permease and oxidase. In \( S. \) \textit{cerevisiae}, microarray analysis of PKA mutants showed that one of the catalytic subunits of PKA, Tpk2, repressed genes involved in iron uptake, among them \( FTR1 \) (Robertson, et al., 2000). Northern analysis of \( C. \) \textit{neoformans} \( FTR1 \) and \( FET3 \) expression in various PKA mutant backgrounds showed that the catalytic subunits of PKA influenced the transcription of \( FTR1 \) and \( FET3 \) under iron-replete conditions. The two known catalytic subunits of PKA were redundant for maintenance of basal levels of transcription of \( FTR1 \). Mutant strains lacking one or the other of the PKA catalytic subunits maintained base-line levels of transcription under iron-replete conditions. In the mutant strain lacking both PKA catalytic subunits, transcription of \( FTR1 \) was negligible under iron-replete conditions. Under iron-limiting conditions the transcription of \( FTR1 \) in all PKA mutants (including the regulatory mutant) was somewhat reduced compared to wild-type. The lack of basal transcription in the double catalytic knockout skews the comparison of fold-induction between iron-replete and iron-limited conditions for the double mutant. Thus it is not so much that expression of \( FTR1 \) under low iron conditions is so much higher than that observed for the other strains, but that \( FTR1 \) transcription is so much lower in the double catalytic mutant than in the other strains under iron-replete conditions.

The pattern of expression for \( FET3 \) in the various PKA mutant backgrounds is somewhat different. Here, Pka1 appears to contribute significantly to the transcription of \( FET3 \) under iron-limited conditions. The transcription of \( FET3 \) in \( pka1 \) and \( pka2 \) mutants
grown in iron-replete media is higher than that of the wild-type except for the PKA catalytic subunit double knockout. This result is curious in that it simultaneously suggests that the catalytic subunits act together to repress basal \textit{FET3} transcription, as seen by the greater than wild-type level of transcription when one or the other subunit is absent and that the catalytic subunits are required for basal transcription of \textit{FET3}, as indicated by the reduced level of basal transcription in the double catalytic mutant and the increased \textit{FET3} transcription seen for the regulatory mutant in which the kinases are constitutively active.

From these results there are two clear conclusions. The first is that transcription of \textit{FTR1} and \textit{FET3} is responsive to iron levels in all backgrounds; iron limitation leads to an increase in transcription of \textit{FTR1} and \textit{FET3}. This suggests that the PKA pathway does not play an immediate downstream role in transduction of this nutritional signal. The second finding is that it appears that components of the PKA pathway are involved in more subtle regulation of transcription of \textit{FTR1} and \textit{FET3}, especially for maintenance of basal \textit{FTR1} transcript level.

In \textit{C. neoformans} the PKA pathway has been implicated in the control of various virulence factors, including the expression of melanin and capsule (D'Souza, et al., 2001). The similarity in capsular phenotype between cells grown in iron-limited conditions and regulatory mutants of the PKA pathway suggested a possible link between the response to the nutritional signal and the PKA pathway. Components of the PKA pathway have been shown to be involved in transmission of nutritional signals that regulate cell morphology (Lengeler \textit{et al.}, 2000). The \(\alpha\) subunit of the only known heterotrimeric G-protein in \textit{C. neoformans} has been implicated in the detection of glucose, a nutritional
signal important in the regulation of mating and melanization (Alspaugh, et al., 1997). With respect to cellular response to limiting levels of iron, it is likely that this signal is intracellular. In *S. cerevisiae* nuclear localization of the iron-regulated transcription factor Aft1 is determined by iron levels inside the cell (Yamaguchi-Iwai *et al.*, 2002). Response to intracellular iron stores is the most reasonable way for cells to regulate iron-responsive genes, as iron levels in the environment could exceed the cellular need. An excess of bio-available iron in the environment is unlikely as iron is often fixed in insoluble ferric hydroxides outside the host and complexed to proteins like heme or transferrin inside of the host. However, should a glut of iron be available, cells can still exploit this resource through the activity of the constitutively expressed low-affinity iron uptake system (Nyhus, *et al.*, 1997). Given this information, a role for PKA as the transducer of an extracellular iron signal seems unlikely.

The data do support a model in which the PKA pathway is necessary for maintenance of a basal level of transcription of *FTR1* and *FET3*. The requirement of PKA catalytic subunits for maintenance of basal transcription has been observed for other genes, including the gene encoding tyrosine hydroxylase in rats. This enzyme catalyzes the reaction that converts L-tyrosine to L-DOPA (Kim *et al.*, 1994). In addition, transcription of the GLUT5 gene encoding a fructose transporter in the intestine is stimulated by cAMP. Blocking cAMP signaling with addition of a PKA inhibitor causes rapid degradation of the GLUT5 mRNA even though basal transcription remains constant (Gouyon *et al.*, 2003). A similar relationship between PKA signaling and transcript stability under iron-replete conditions could explain the current results for *FTR1* and *FET3* in *C. neoformans*. In this case, PKA might be required for maintenance of the
$FTRI$ transcript levels. Deletion of both PKA catalytic subunits might not change the basal level of transcription of $FTRI$ but could influence the stability of the transcript under iron-replete conditions. Transcript stability for $FTRI$ or $FET3$ was not evaluated in this thesis.

### 4.4 Intra-Serotype Variation in Transformation Efficiency

Biolistic transformation is the primary technique for gene disruption in $C. neoformans$ and this method was used to disrupt $FTRI$ (Davidson et al., 2000; Toffaletti et al., 1993). Prior to the use of biolistic technology, transformation was accomplished by electroporation of spheroplasts. Gene silencing using RNA interference has also been reported (Liu et al., 2002). It has already been documented that there are differences in transformation efficiency between electroporation and biolistic transformation (Davidson, et al., 2000). Generally, homologous recombination during biolistic transformation occurs at a frequency of between 1% and 50%, while electroporation yields homologous transformants at a rate of between 0.001% and 0.1%. Transformation efficiency also varies between serotypes. Gene disruption in serotype A strains occurs at frequencies between 2% and 50%. Gene disruption in the congenic serotype D strains JEC20 and JEC21 has been reported at frequencies of 1% to 4%. Other researchers have also been plagued with low transformation efficiencies or incidences in which the transforming DNA is maintained extrachromosomally or is otherwise unstable (Chang and Kwon-Chung, 1994; Chang and Kwon-Chung, 1998; Chang, et al., 1996). Observational data from this thesis research indicates that there may be intra-serotype variations in transformation efficiency as well. Over the course of two separate biolistic
transformations there were no incidences of a sole homologous recombination event in the serotype D strain JEC21. Each time that a homologous recombination event occurred there were co-incident ectopic integrations of the introduced DNA. By comparison, the serotype D strain ATCC 24067 was readily transformed to obtain mutants. The first biolistic transformation yielded three mutants that carried a single targeted copy of the introduced DNA. The cause of this strain difference is open to speculation. The serotype D strain JEC21 is the product of a back-crossing experiment and has been passaged many times in the laboratory (Heitman, et al., 1999). The ATCC strain is a clinical isolate that has less widespread use as an experimental strain. Laboratory passage can change virulence attributes like melanin synthesis, capsule size and structure and change chromosome size (Franzot et al., 1998). Genotypic differences between the ATCC 24067 and JEC21 strain backgrounds could even extend to chromosome number (Wickes et al., 1994). Ultimately, the direction of the research presented in this thesis was determined by the genetic idiosyncrasies of these strains and \textit{ftr1} mutant phenotypes were examined in the ATCC 24067 background.

4.5 Phenotypic characterization of \textit{ftr1} Mutants

4.5.1 Iron-Deficiency in \textit{C. neoformans} \textit{ftr1} Mutants

Mutants carrying a truncated \textit{FTR1} gene were generated and characterized. The most obvious mutant phenotype was a severe growth defect in iron-limited media. Wild-type cells grew to the same final density regardless of the iron-status of the media, while the mutants grew to less than a tenth of the final density of wild-type cells or mutant cells supplemented with iron. The mutant phenotype in iron-limited media was partially
relieved by supplementation with iron. In iron-rich media the final cell density of the mutants was equivalent to that of the wild-type cells. However, the mutants’ lag phases and doubling times were approximately twice that of the wild-type.

The argument that mutant cells experienced iron starvation was further supported by Northern analysis performed to confirm that the mutants expressed a truncated \( FTRI \) transcript. For cells grown under inducing conditions for six hours, transcription of \( ftr1 \) in the mutants was much higher than in the wild-type, suggesting that the mutant cells were experiencing a greater degree of iron starvation than the wild-type cells. This result also suggests that responses to iron-starvation of the mutant cells are detectable (by Northern analysis if not by densitometric measurements of growth) within the first six hours of iron-limited growth.

Overall, these results reveal an important role for Ftr1 in the ability of \( C. \) neoformans to grow under iron starvation conditions.

4.5.2 Increased Oxidant Sensitivity in \( C. \) neoformans is a Function of Iron Limitation

Iron limitation is not the only adverse environmental condition encountered by \( C. \) neoformans in the host and invading fungal cells may be engulfed by phagocytic cells of the immune system. After phagocytosis the invading cells may be killed by oxidative or nitrosative means. The phenomenon of the oxidative burst in the lysosome of phagocytic cells generates the superoxide anion (\( O_2^- \)) by action of an NADPH oxygenase and hydrogen peroxide (\( H_2O_2 \)) by action of a dismutase. These reactive oxygen species can damage the DNA, proteins and lipids of a cell.
Oxidizing molecules have several deleterious effects on cells. Reactive oxygen species (ROS) can oxidize DNA bases, the guanine base being particularly susceptible to such damage. There is a baseline level of DNA oxidation experienced by cells and cells do have mechanisms for repair of these damaged bases. Problems occur when these bases are not quickly repaired. Oxidized purines and pyrimidines can cause problems including decreased hydrogen bonding, conformational changes in the DNA and effects on the fidelity of RNA and DNA polymerases (Kehrer, 2000). Lipid damage by ROS is especially nefarious as it generates a chain-reaction. Abstraction of protons from unsaturated fatty acid double-bonds generates new radical species that react with the molecular oxygen diradical. The lipid peroxyl radical thus formed can then remove a hydrogen atom from another fatty acid double bond, beginning the cycle of oxidation and radical formation all over again (Kehrer, 2000). Reactions between proteins and ROS can generate reactive and stable products. Reactive oxygen species have been implicated in oxidative killing of Blastomyces deratitidis and Aspergillus fumigatus (Sugar et al., 1983; Washburn et al., 1987). There are also pathogens that are relatively immune to the products of the oxidative burst. These include Histoplasma capsulatum, Burkholderia pseudomallei and Mycobacterium tuberculosis (Egan & Gordon, 1996; Kurita et al., 1991; Manca et al., 1999). For M. tuberculosis one of the main cellular defenses is the expression of catalase-peroxidase. Catalase-peroxidase, a haem protein encoded by the KatG gene, catalyzes the hydrolysis of hydrogen peroxide to oxygen and water (Zhang et al., 1992).
The deleterious effects of ROS are partially mediated by iron. The Haber-Weiss reaction describes the iron-catalyzed reaction between the superoxide anion and hydrogen peroxide (Haber & Weiss, 1934).

\[
\text{Fe}^{3+} + \text{O}_2^- \rightarrow \text{Fe}^{2+} + \text{O}_2 \\
\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^- + \text{OH}^-
\]

\[
\text{O}_2^- + \text{H}_2\text{O}_2 \rightarrow \text{O}_2 + \text{OH}^- + \text{OH}^-
\]

The net Haber-Weiss reaction shows that superoxide anion reacts with hydrogen peroxide to form oxygen, hydroxide anion and the hydroxyl radical.

In biological systems, free radicals are destroyed by catalysis. Superoxide dismutases catalyze the redox reaction that produces hydrogen peroxide from superoxide and hydrogen. The hydrogen peroxide produced by this reaction is then broken down by the action of catalase. The net reaction is shown below:

\[
\text{O}_2^- + \text{O}_2^- + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2 \\
2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2 \\
4\text{O}_2^- + 4\text{H}^+ \rightarrow 2\text{H}_2\text{O} + 3\text{O}_2
\]

As long as catalase is available to break down the products of superoxide dismutase activity on the superoxide anion, the reactions catalyzed by these two enzymes represent an effective defense against oxidizing species like superoxide and hydrogen peroxide (McCord, 2000).

In addition to growth changes, another phenotype of the fnrl mutants is an increased sensitivity to oxidants. In plate assays with and without supplemental iron, the diameter of the zone of clearing was measured as a rough estimate of oxidant sensitivity.
For all but one of the strains tested, the mean diameter was significantly larger when grown under iron-limiting conditions. Comparison of mutant and wild-type cells showed that mutants were significantly more sensitive to oxidants than the wild-type, regardless of the iron status of the media. The ftr1 mutants presumably retain activity of the ferric reductase genes, in which case there would be free ferrous iron at the cell surface. Reaction with hydrogen peroxide and molecular oxygen could then generate hydroxyl radicals capable of interfering with lipids and proteins at the yeast cell surface.

It is also possible that the increased sensitivity of the ftr1 mutants is due to lack of cellular iron for synthesis of the haem containing catalases and peroxidases. BLAST analysis of the C. neoformans genome database (Stanford Genome Technology Centre) using the S. cerevisiae catalase genes, CTA1 and CTT1, identified two putative C. neoformans catalase genes. The top NCBI BLAST hits for the putative CTA1 and CTT1 C. neoformans homologues are the catalases from Colletotrichum gloeosporioides, a plant pathogen that infects papaya, and Cladosporum fulvum, a tomato pathogen. The presence of putative iron-containing catalase genes in C. neoformans suggests that the increased sensitivity of ftr1 mutants to oxidative stress could be the result of a paucity of enzymes that catalyze the destruction of ROI. The fact that iron-supplementation partially relieves the oxidant-sensitive phenotype of the mutants argues that catalase and peroxidase could play a role in response to H2O2. Under iron-replete conditions the constitutively active low-affinity iron-transport system could supply enough iron to the cell for synthesis of these oxidant-destroying enzymes. The effect of iron repletion does not restore the wild-type phenotype to the ftr1 mutants; iron-repleted mutants have a phenotype statistically indistinguishable from iron-starved wild-type cells, which could
point to a contributing role for Haber-Weiss generated radicals causing oxidative damage. Under iron-replete conditions, the abundance of catalyst for generation of the hydroxyl radical could partially negate the effects of increased enzymatic breakdown of H₂O₂.

4.5.3 Capsule Phenotypes of C. neoformans ftr1 Mutants

The established literature indicates that iron-limitation stimulates C. neoformans capsule production (Casadevall and Perfect, 1998; Vartivarian, et al., 1993). It is surprising that the capsule phenotype of the ftr1 mutants did not differ from wild-type, since expression data from Northern analysis and growth in iron-limited media indicated that ftr1 mutants were unable to efficiently import iron into the cell and were experiencing severe iron limitation. The C. neoformans strain used in this study, ATCC 24067, is a serotype D clinical isolate. It was constitutively capsular under the conditions tested. The ftr1 mutants were also constitutively capsular and did not appear to express more or less capsule than the wild-type strain. It could be that the conditions tested, six hour incubation in iron-limited media, while appropriate for detection of capsular differences in the laboratory serotype D strain JEC21 (as determined by T. Lian (personal communication)) were not appropriate for detection of a difference in capsule synthesis for ATCC 24067. The original paper describing the regulation of capsule by iron used the serotype D National Institutes of Health strains B-3501 and B-3500 (parent strains for the congenic JEC21 and JEC20 serotype D strains), American Type Culture Collection strain 4189 (a serotype A clinical isolate) and two clinical strains of unknown serotype (Vartivarian, et al., 1993). This thesis work used the nutrient-poor, defined media of Vartivarian, et al. (1993). Experiments in our laboratory showed that a complex but iron-
limited medium (YPD supplemented with the ferrous iron chelator EDDHA (Sigma)) failed to stimulate capsule formation, even after incubation for four days (Lian, personal communication). Perhaps the effect of iron on capsule formation is only detectable when other nutrients are also limiting. Effects of other nutrients or environmental signals on capsule formation have been documented. Physiological levels of CO₂ also increase capsule formation under iron-limited conditions (Vartivarian, et al., 1993). In the SAGE libraries that provided the basis for this thesis, a copper transporter showed the greatest fold induction between iron-replete and iron-limited conditions. This does not mean that copper is necessarily involved in capsule formation in _C. neoformans_, but it does argue that the media used for the libraries (and for the original work on iron’s contribution to capsule formation) is deficient for various nutrients and that the synthesis of capsule might be a complex, integrated response to various environmental signals, not just iron.

4.6 Summary

The molecular genetic mechanisms governing iron uptake in _C. neoformans_ are incompletely characterized. Previous work in our laboratory involved construction of SAGE libraries in order to compare patterns of gene transcription under iron-replete and iron-limited conditions. Data from those libraries identified two genes transcriptionally regulated by iron, _FTR1_ and _FET3_, homologs of components of the high-affinity iron uptake system in _S. cerevisiae_. This thesis work has focused on the genetic architecture of these two genes, which represents the first incidence of a cluster of functionally related, divergently transcribed genes with a proposed role in virulence in _C. neoformans_.

The phenotypic similarity in capsule size between PKA regulatory subunit mutants and cells grown under iron-limited conditions and research in *S. cerevisiae* that implicated PKA pathway components in control of iron uptake genes suggested a role for PKA in control of *FTR1* and *FET3* expression. This was examined by Northern analysis of *FTR1* and *FET3* expression in PKA catalytic and regulatory mutants grown under iron-limited and iron-replete conditions. Results of these analyses show that *FTR1* and *FET3* transcription responds to environmental iron levels, regardless of PKA background; transcription is repressed under iron-replete conditions compared to iron-limited conditions. Comparison of the transcription of *FTR1* under iron-replete conditions showed that the catalytic subunits of PKA were required for maintenance of the basal level of *FTR1* transcription. The contributions of the PKA catalytic subunits were additive and the mutant lacking both PKA catalytic subunits had negligible *FTR1* transcription under iron-replete conditions. The influence of PKA on *FET3* transcription is less straightforward and there is no clear pattern of regulation by the PKA components.

Signals other than those from the PKA pathway are implicated in transcriptional control of *FTR1* and *FET3*. Northern analysis to determine the effect of copper on transcription of these two genes showed that copper had divergent effects on transcription of *FTR1* and *FET3*. The multicopper oxidase, *FET3*, was upregulated in response to copper-repletion of the iron-limited media. The opposite pattern was observed for the iron permease; addition of copper caused repression of *FTR1* transcription. The divergent regulation of *FTR1* and *FET3* by copper was unexpected, as reports in *S. cerevisiae* indicated that both *FTR1* and *FET3* were upregulated in response to copper-
repletion. It is possible that \textit{FET3} but not \textit{FTR1} has a role in acquisition of copper, and thus is upregulated in response to copper while \textit{FTR1} is not.

The \textit{ftr1} mutants expressed phenotypes that included growth defects in iron-limited media and an increased sensitivity to oxidants. In iron-limited media \textit{ftr1} mutants exhibited a severe growth defect that was partially relieved by iron repletion. When cells were grown in iron-rich media the \textit{ftr1} mutants reached the same final cell density as the wild-type cells but had a longer lag phase and longer generation time during log phase. Mutants were also significantly more sensitive to oxidative stress, as measured by response to \( \text{H}_2\text{O}_2 \). This sensitivity was partly relieved by iron-repletion. It is possible that this effect is due to the activity of haem-containing catalases, the synthesis of which in \textit{ftr1} mutants would be aided by iron supplementation.

Expression of the primary virulence factor, capsule, is influenced by environmental iron levels. ATCC 24067 is constitutively capsular (or at least constitutively capsular under the conditions tested). Despite being iron-starved, the \textit{ftr1} mutants did not express any more capsule than the wild-type cells. Anecdotal evidence also suggests that while iron may be the primary nutritional signal influencing capsule production, other nutritional signals may be important under different conditions.

\section*{4.7 Future Directions}

While components of the \textit{C. neoformans} high-affinity iron-uptake system appear to mirror the system found in \textit{S. cerevisiae}, there are some surprising differences, especially the divergent regulation by copper of the \textit{FTR1} and \textit{FET3} components of this system. Further elucidation of \textit{C. neoformans}' responses to copper might be
illuminating. Workers in other laboratories have begun some of this analysis, especially with respect to the activity of the copper transporter encoded by CTR1 (Lian, personal communication).

The role of the high affinity iron permease in *C. neoformans* virulence has yet to be definitively illustrated. Work in our laboratory is ongoing for reconstitution of the mutant strains with a wild-type copy of *FTR1*, followed by animal studies. It’s role in virulence along with potential virulence determinants that are a result of interactions with the other component of the high-affinity iron uptake heterodimer, *FET3*, also remain to be determined.

As a surface-exposed protein Ftr1 is a potential antigenic determinant of *C. neoformans* and as such could be a vaccine target. Presently most of the efforts for development of a *C. neoformans* vaccine have focused on the glucuronoxylomannan (GXM) component of the polysaccharide capsule (Casadevall *et al.*, 2002). Expression of the *FTR1*-encoded protein and vaccination to determine efficacy would be the next step towards development of a vaccine based on the iron-permease.

The genetic arrangement of *FTR1* and *FET3* is a novel one in *C. neoformans*. Bioinformatic analysis could be used to reveal other clusters of functionally related genes. While such findings would be interesting from a molecular biological point of view, cross-species comparisons might also illuminate evolutionary relationships among the fungi.
5 References


6 Appendices

6.1 Media for *Cryptococcus neoformans*

**YPD broth**

For 1 litre

Yeast extract 20 g
Bacto Peptone (Difco) 10 g
Dextrose 20 g

pH to 7.2

**YPD agar**

For 1 litre

Yeast extract 20 g
Bacto Peptone (Difco) 10 g
Dextrose 20 g
Agar 15 g

**YNB**

For 1 litre

Yeast nitrogen base (Difco) 1.45 g
Ammonium sulfate 5 g
Glucose 5 g

pH to 7.2.

**Iron Replete Media**

For 1 litre

Glucose 5 g
L-asparagine 5 g

Dissolve in 100 mL distilled water and chelate iron by batch purification with 0.5 g Chelex (BioRad). Add:
K\textsubscript{2}HPO\textsubscript{4} 0.4 g  
CaCl\textsubscript{2} 0.25 g  
MgSO\textsubscript{4}.7H\textsubscript{2}O 80 mg  
HEPES buffer 5 g  
NaHCO\textsubscript{3} 1.9 g  
FeEDTA (C\textsubscript{10}H\textsubscript{12}N\textsubscript{2}O\textsubscript{8}Fe(III)Na) 36.7 mg

Add Chelex-treated water to a final volume of 1 L. pH to 7.2. Autoclave. Add 1 mL 1000X salt solution.

**Iron Replete Agarose**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>5 g</td>
</tr>
<tr>
<td>L-asparagine</td>
<td>5 g</td>
</tr>
</tbody>
</table>

Dissolve in 100 mL distilled water and chelate iron by batch purification with 0.5 g Chelex (BioRad).

K\textsubscript{2}HPO\textsubscript{4} 0.4 g  
CaCl\textsubscript{2} 0.25 g  
MgSO\textsubscript{4}.7H\textsubscript{2}O 80 mg  
HEPES buffer 5 g  
NaHCO\textsubscript{3} 1.9 g  
FeEDTA 36.7 mg  
Agarose 15 g

pH to 7.2. Autoclave. Add 1 mL 1000X salt solution.

**Iron-Limited Media**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
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<tbody>
<tr>
<td>Glucose</td>
<td>5 g</td>
</tr>
<tr>
<td>L-asparagine</td>
<td>5 g</td>
</tr>
</tbody>
</table>

Dissolve in 100 mL distilled water and chelate iron by batch purification with 0.5 g Chelex (BioRad).

K\textsubscript{2}HPO\textsubscript{4} 0.4 g  
CaCl\textsubscript{2} 0.25 g  
MgSO\textsubscript{4}.7H\textsubscript{2}O 80 mg  
HEPES buffer 5 g  
NaHCO\textsubscript{3} 1.9 g

pH to 7.2. Autoclave. Add 1 mL 1000X salt solution.
Iron-Limited Agarose

For 1 litre

Glucose
5 g
L-asparagine
5 g

Dissolve in 100 mL distilled water and chelate iron by batch purification with 0.5 g Chelex (Bio-Rad).

K$_2$HPO$_4$
0.4 g
CaCl$_2$
0.25 g
MgSO$_4$$\cdot$7H$_2$O
80 mg
HEPES buffer
5 g
NaHCO$_3$
1.9 g
Agarose
15 g

pH to 7.2. Autoclave. Add 1 mL 1000X salt solution.

Iron-Limited Copper Replete Media

For 1 litre

Glucose
5 g
L-asparagine
5 g

Dissolve in 100 mL distilled water and chelate iron by batch purification with 0.5 g Chelex (BioRad).

K$_2$HPO$_4$
0.4 g
CaCl$_2$
0.25 g
MgSO$_4$$\cdot$7H$_2$O
80 mg
HEPES buffer
5 g
NaHCO$_3$
1.9 g
CuSO$_4$, 0.1 M
50 µL

pH to 7.2. Autoclave. Add 1 mL 1000X salt solution.

1000X Salt Solution

For 1 L

CuSO$_4$
5 mg
ZnSO$_4$
2 g
MnCl$_2$
10 mg
Na$_2$MoO$_4$$\cdot$2H$_2$O
460 mg
H$_3$BO$_3$
57 mg
6.2 Molecular Techniques

5X TBE

Tris-Borate 0.45 M
EDTA, pH 8.3 0.01 M

Dissolve components in water. Dilute 1/10 for use as running buffer.

5X MOPS Buffer

For 1 L
MOPS, pH 7.0 20.93 g
Sodium acetate 82.03 g
EDTA, pH 8.0 5 mM

Dissolve MOPS in water and pH to 7.0. Add sodium acetate, EDTA and adjust volume to 1 L with water.

Formaldehyde Gel Loading Buffer

For 10 mL
Glycerol 5 mL
EDTA, pH 8.0 1 mM
Bromophenol 25 mg
Xylene cyanol FF 25 mg

20X SSC

For 1 L
Sodium chloride 175.3 g
Sodium citrate 88.2 g

Dissolve sodium chloride and sodium citrate in water, adjust to pH 7.0. Make up to 1 L with water.
Zeta Probe (BioRad) Hybridization Solutions

Pre-Hybridization Buffer

For 1 L

Na₂HPO₄  71 g
SDS       70 g

Dissolve Na₂HPO₄ in water and pH to 7.2. Add SDS and adjust volume to 1 L with water.

Wash Solution 1

For 1 L

Na₂HPO₄  5.68 g
SDS       50 g

Dissolve Na₂HPO₄ in water and pH to 7.2. Add sodium dodecyl sulfate and adjust volume to 1 L with water.

Wash Solution 2

For 1 L

Na₂HPO₄  5.68 g
SDS       10 g

Dissolve Na₂HPO₄ in water and pH to 7.2. Add sodium dodecyl sulfate and adjust volume to 1 L with water.

6.3 Cryptococcus Methods

Isolation of DNA from *C. neoformans*

SCE Solution

For 1 L

Sorbitol  182.2 g
Sodium citrate, pH 5.8  21.4 g
EDTA      0.01 M

Dissolve sodium citrate in water, adjust pH to 5.8. Add sorbitol and EDTA, adjust volume to 1 L with water.
### Lysing Solution

<table>
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<th>Amount</th>
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<tr>
<td>EDTA</td>
<td>50 mM</td>
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</tbody>
</table>

### C. neoformans Colony PCR

#### Platinum Taq Colony PCR Reaction Mix

<table>
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<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X PCR buffer</td>
<td>2.5 μL</td>
</tr>
<tr>
<td>dimethyl sulfoxide</td>
<td>1.25 μL</td>
</tr>
<tr>
<td>100 mM dNTP</td>
<td>1.0 μL</td>
</tr>
<tr>
<td>20 μM forward primer</td>
<td>1.0 μL</td>
</tr>
<tr>
<td>20 μM reverse primer</td>
<td>1.0 μL</td>
</tr>
<tr>
<td>Platinum Taq polymerase (5U/μL)</td>
<td>0.2 μL</td>
</tr>
<tr>
<td>sdH₂O</td>
<td>18 μL</td>
</tr>
</tbody>
</table>

#### 10X PCR buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>(NH₄)₂SO₄</td>
<td>166 mM</td>
</tr>
<tr>
<td>Tris-Cl, pH 8.8</td>
<td>670 mM</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>67 mM</td>
</tr>
<tr>
<td>β-mercaptoethanol</td>
<td>100 mM</td>
</tr>
</tbody>
</table>