Ferric Reductases in *Candida albicans*: Expression and Regulation.

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by

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Candida albicans is an opportunistic fungal pathogen of humans causing superficial mucosal infections and more serious systemic infections in immunocompromised individuals. It is found in both yeast and hyphal forms at sites of infection. In the human body levels of iron are extremely low, and an invading organism needs a way of sequestering iron. In *C. albicans* there is a high affinity iron uptake system in which iron is first reduced to the soluble ferrous form by ferric reductases. Reduced iron is then taken up into the cell by a complex of a multicopper oxidase protein with an iron transport protein. Multicopper oxidase proteins require copper to function and so high affinity iron and copper uptake is inextricably linked.

It has previously been demonstrated that CaFre10p is the major cell surface ferric and cupric reductase. It is shown here that CaFre7p also makes a significant contribution to cell surface ferric and cupric reductase activity. However, whereas *CaFRE10* is regulated in response to iron levels, *CaFRE7* is regulated in a copper responsive manner. The *CaFRE10* gene is regulated by the GATA-type transcriptional repressor Sfu1p and *CaFRE7* is not. We show that in a mutant containing a deletion of *SFU1* the expression of the major iron transport protein *CaFTR1* is increased and there is a corresponding increase in radioactive iron uptake. It is also shown here for the first time that expression of *CaFRE10* and *CaFRE7* is lower in hyphae compared to yeast and that this leads to a corresponding decrease in cell surface ferric, but not cupric reductase activity. This shows for the first time that two important virulence determinants, the acquisition of iron and the morphological form of *C. albicans*, are linked.

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Abbreviations

ATP	Adenosine triphosphate
BCS	Bathocuproine disulphonic acid
bp	Base pair
BPS	Bathophenanthroline disulphonic acid
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CuRE	Copper response element
dATP	Deoxyadenosine 5’-triphosphate
dCTP	Deoxycytidine 5’-triphosphate
DEPC	Diethyl pyrocarbonate
dGTP	Deoxyguanosine 5’-triphosphate
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide 5’-triphosphate
DTT	Dithiothreitol
dTTP	Deoxythymidine 5’-triphosphate
EDTA	Ethylenediaminotetra acetic acid
EMSA	Electrophoretic mobility shift assay
ENT	Enterobactin
ESCRT	Endosomal sorting complex required for transport
FAD	Flavin adenine dinucleotide
5-FOA	5-Fluoroorotic acid
FOB	Ferroxamine B
g	Grams

GlcNAc	*N*-acetylglucosamine
HCl	Hydrochloric acid
kb	Kilobase pairs
kDa	Kilodaltons
l	Litres
LA	Luria agar
LB	Luria broth
M	Molar
MD	Minimal defined
ml	Millilitres
µM	Micromolar
µg	Micrograms
µl	Microlitres
mM	Millimolar
MOPS	3-(N-morpholino) propanesulphonic acid
mRNA	Messenger ribonucleic acid
NADPH	Nicotinamide adenine dinucleotide phosphate
nM	Nanomolar
ng	Nanograms
ORF	Open reading frame
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PKA	Protein kinase A
pmol	Picomolar
OD	Optical density

RNA	Ribonucleic acid
RNase	Ribonuclease
RPM	Revolutions per minute
RT-PCR	Reverse transcriptase polymerase chain reaction
SAP	Shrimp alkaline phosphatase
SD	Synthetic defined
SDS	Sodium dodecyl sulphate
SSC	Standard saline citrate
TAFC	Triacetylfusarinine C
TE	Tris-EDTA buffer
TAE	Tris-Acetate-EDTA
Tris	Tris(hydroxymethyl)aminomethane
TMD	Transmembrane domains
v/v	Volume by volume
w/v	Weight by volume
YPD	Yeast peptone dextose medium
YPG	Yeast peptone glycerol medium

Chapter 1: Introduction

Candida albicans is a pleomorphic fungus, having yeast, hyphal and pseudohyphal morphological forms (Sudbery *et al.*, 2004). It is an opportunistic pathogen of humans which causes both superficial muscosal infections, such as thrush, as well as more serious systemic infections in immunocompromised individuals. These systemic infections have a fatality rate of 55% even with antifungal therapy (Weinberger *et al.,* 2005) and *C. albicans* is the 4th most frequently isolated microorganism from blood samples in hospitals in the US (Wisplinghoff *et al.*, 2004).

All organisms require iron to survive because it is an essential cofactor in a number of important proteins, and for pathogenic organisms iron is hard to acquire as the levels of free iron in the host are very low (Crichton & Pierre, 2001). This project aims to further characterize the mechanisms that *C. albicans* uses to sequester iron. This introduction starts with a brief review of the importance of iron, the background to the pleomorphic nature of *C. albicans* and its pathogenicity. High affinity iron uptake systems in fungi in the context of high affinity iron uptake in *C. albicans* is then discussed.

1.1: Iron and Copper:

Iron, after aluminum, is the second most abundant metal and is ubiquitous in the environment in its insoluble Fe(III) ferric form (Crichton & Pierre, 2001). Copper is very similar to iron in its chemistry, being another transition element that is found in the

environment in its insoluble cupric form, Cu(II). Iron has the ability to exist in two stable valences with a large difference in redox potential of -0.5 volts to +0.6 volts, almost the entire range found in the biological world. This large redox potential makes iron invaluable as a cofactor in electron transport (Pierre, *et al.*, 2002). Iron is used as a cofactor in a number of vital proteins including haemoproteins (e.g. cytochromes), iron-sulphur proteins (e.g. ferredoxins) and mononuclear non-heam proteins. Similarly copper is a useful cofactor for many enzymes, with a redox potential of +0.25 volts and +0.75 volts (Crichton & Pierre, 2001). Copper is essential for the correct functioning of some cytochromes and superoxide dismutase (Van Ho *et al.*, 2002). However, free iron and copper generate oxygen free radicals by reacting with reactive oxygen species via the Harber-Weiss-Fenton reaction (Figure 1.1).

These radicals are highly destructive, damaging DNA, lipids and proteins (Imlay & Linn, 1988). This presents any organism with the difficulty of acquiring sufficient quantities of copper and iron to allow it to survive, without taking up too much and suffering damage from reactive oxygen species (Crichton & Pierre, 2001).

1.2: *Candida albicans*; Morphology, Mating and Virulence

Candida albicans is an opportunistic pathogen of humans, causing superficial mucosal infections and systemic blood borne infections. Furthermore the ability to switch between yeast and hyphal forms is required for virulence (Sudbery *et al.*, 2004). *C. albicans* is an obligate diploid with key components of the meiotic pathway apparently missing (Bennett & Johnson, 2003; Bennett & Johnson, 2005). There is also natural heterozygosity in its

Harber-Weiss-Fenton Reactions

A. $Fe^{2+}\,(Cu^{+}) + H_2O_2 \longrightarrow Fe^{3+}\,(Cu^{2+}) + OH^{-} + OH^{\bullet}$

B. $Fe^{3+}\,(Cu^{2+}) + H_2O_2 \longrightarrow Fe^{2+}\,(Cu^{+}) + {}^{\bullet}OOH + H^{+}$

C. $Fe^{2+}\,(Cu^{+}) + O_2 \longrightarrow Fe^{3+}\,(Cu^{2+}) + O^{\bullet}_{2-}$

Figure 1.1: Harber-Weiss-Fenton Reactions: The oxidative reactions of both iron and copper produce toxic oxygen and hydroxyl free radicals. These radicals can damage most types of molecules within the cell including nucleic acids, lipids and proteins (Imlay & Linn, 1988).

genome (Jones *et al.,* 2004), which is made up of 8 chromosomes, 1-7 and R (Chibana *et al.,* 2000).

Morphology

When grown at 30°C or below pH 4 *C. albicans* grows in its yeast phase; a simple eukaryotic cell which reproduces by budding. When grown at 35°C, pH 6, in high phosphate conditions or in low nitrogen conditions *C. albicans* forms pseudohyphae, these are filaments made up of elongated yeast cells in a chain. Growing *C. albicans* at pH 7, 37°C, in the presence of *N*-acetylglucosamine (GlcNAc) or in the presence of serum (above 34°C) induces the formation of true hyphae. True hyphae are long, continuous filaments comprised of coencytic multinucleate compartments (Sudbery *et al.,* 2004). Regulation in response to this diverse set of environmental cues is a complex cascade and not all the components or signals are fully characterized. However, much headway has been made in understanding the regulation of the morphological switch in *C. albicans* (Biswas *et al.,* 2007).

Conditions which induce hyphal formation include nitrogen starvation, amino acid starvation, methionine and glucose levels, changes in pH, the presence of serum and the presence of *N*-acetylglucosamine (Sudbery *et al.,* 2004). The protein Gap1p has been identified as being required for sensing nitrogen starvation and *N*-acetylglucosamine. In a homozygous mutant hyphae are not formed in response to these inducing factors, yet hyphae are still formed in response to serum (Biswas *et al.,* 2003). The proteins Gcn4p and Mep2p are also involved in regulating morphogenesis in response to nitrogen starvation (Biswas & Morschhauser, 2005; Tripathi *et al.,* 2002). Changes in morphology

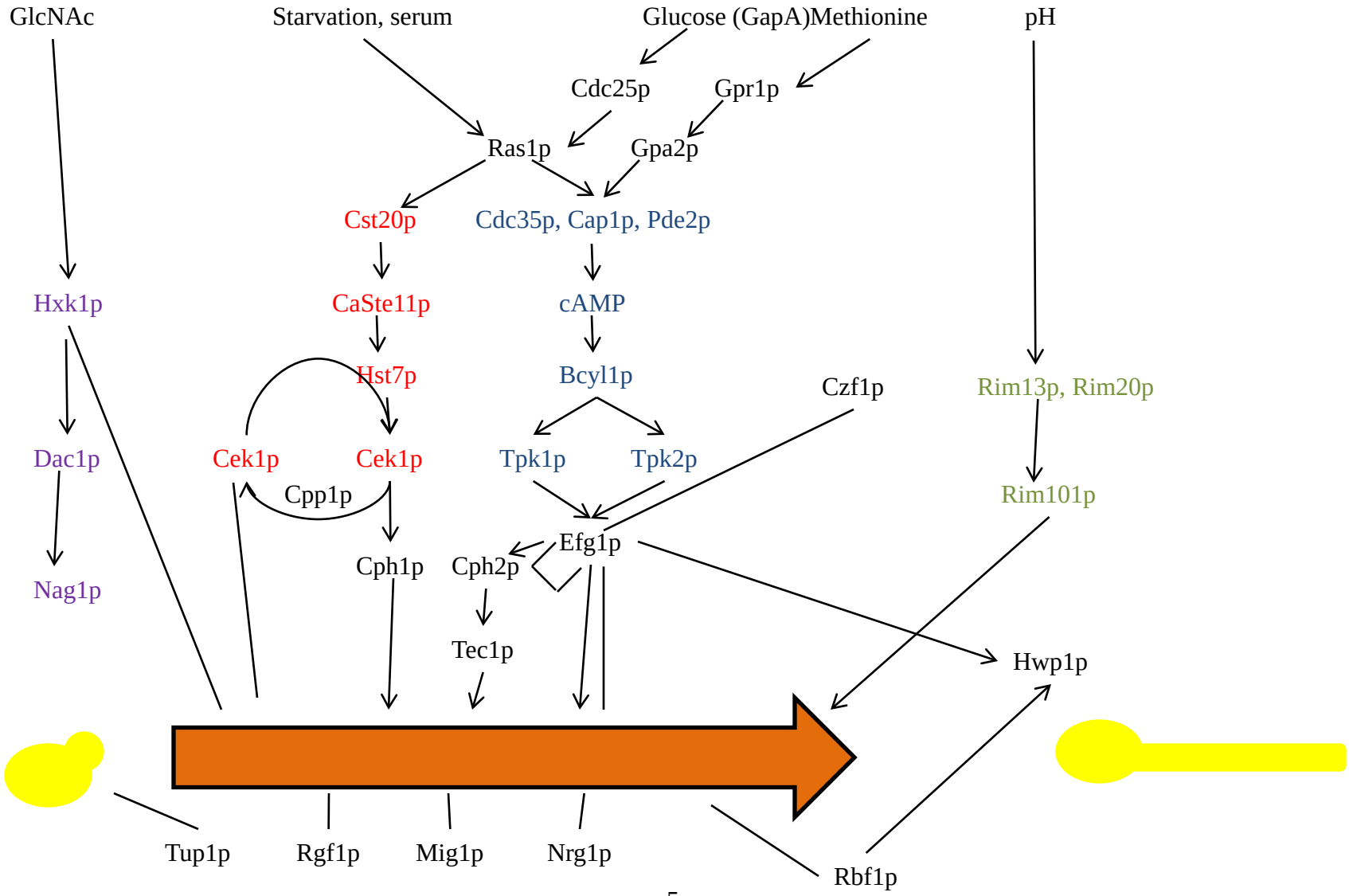

GlcNAc
Starvation, serum
Glucose (GapA)Methionine
pH
Cdc25p
Gpr1p
Ras1p
Gpa2p
Cst20p
Cdc35p, Cap1p, Pde2p
Hxk1p
CaSte11p
cAMP
Hst7p
Bcyl1p
Czf1p
Rim13p, Rim20p
Dac1p
Cek1p
Cek1p
Tpk1p
Tpk2p
Cpp1p
Rim101p
Efg1p
Cph1p
Cph2p
Nag1p
Tec1p
Hwp1p
Tup1p
Rgf1p
Mig1p
Nrg1p
Rbf1p

Figure 1.2: Regulation of Hyphal -Specific Genes: At the top of the diagram the factors which induce hyphae are given and the large orange arrow at the bottom of the diagram represents hyphal-specific genes which are involved in the switch from yeast to hyphae. In between are the inducers of hyphal formation and the numerous regulatory factors involved in this complex morphological switch. Arrows represent activation lines indicate repression. The *N*-acetylglucosamine (GlcNAc) catabolic pathway is shown in purple and deletion of its components prevents hyphal formation in response to GlcNAc. The MAPK cascaade that some factors feed into is shown in red, the cAMP-PKA pathway is shown in blue and the pH dependant pathway is shown in green. (Adapted from Biswas, *et al*, 2007).

in response to amino acid starvation is mediated by Cys1p cleavage of Stp1-2p and by Csh1p (Biswas *et al.,* 2007; Martinez & Ljungdahl, 2004; Martinez & Ljungdahl, 2005). Transition from yeast to hyphae in response to methionine is dependent on Gpr1p (Biswas *et al.,* 2007; Maidan *et al.,* 2005). Changes in morphology in response to pH are mediated by Rim101p; in acidic conditions Rim101p is found in its "long" form and in alkaline conditions it is cleaved by the processing proteins Rim20p and Rim13p to its active short form (Davis 2003), this is shown in green in figure 1.2. Czf1p and possibly Mkc1p may be involved in the morphology of *C. albicans* when it is embedded (Biswas *et al.,* 2007). Deletion of components of the *N*-acetylglucosamine catabolic pathway (Nag1, Dag1 and Hxk1), shown in purple in figure 1.2, also prevent the formation of hyphae in response to *N*-acetylglucosamine (Singh *et al.,* 2001).

Several of these environmental sensors feed into two main regulatory cascades, a Mitogen-activated protein kinase (MAPK) pathway, shown in red in figure 1.2 and a cyclic adenosine monophosphate-protein kinase A (cAMP-PKA) pathway, shown in blue in figure 1.2. Others, such as Rim101p and components of the *N*-acetylglucosamine catabolic pathway act directly (Biswas *et al.,* 2007). Upstream of both the MAPK pathway and the cAMP-PKA pathway is Ras1p, a major hyphal regulator which is acted on by Gpr1p via Gpa2p and glucose via Cdc25p as well as being effected by starvation and the presence of serum (Biswas *et al.,* 2007). The MAPK pathway involves a cascade of protein kinases downstream of which is the transcriptional regulator Cph1p. The cAMP-PKA pathway contains the adenylate cyclase coded for by *CDC35* downstream of Ras1p to generate cAMP (cyclic adenosine monophosphate). Downstream of cAMP are two protein kinase As (PKAs), Tpk1p which is active on solid media and Tpk2p which is active in liquid

media; these PKAs act on the transcriptional regulator Efg1p (Biswas *et al.*, 2007). As well as acting directly to regulate genes, Efg1p acts on the regulator Tec1p, which is also acted on by Cph2p independently of the cAMP-PKA pathway (Biswas *et al.*, 2007).

In addition to these activating pathways there are also a number of repressors of hyphal formation; CaTup1p binds to Nrg1p, Mig1p and Rfg1p to repress filamentation in non inducing conditions (Biswas *et al.*, 2007; Murad *et al.*, 2001a; Murad *et al.*, 2001b). Furthermore, the *HOG1* oxidative stress pathway represses induction of hyphae in response to serum, low temperatures, low pH and nitrogen starvation (Biswas *et al.*, 2007; Eisman *et al.*, 2006). There is also a number of other less well characterized repressors and activators of hyphal formation (Biswas *et al.*, 2007). It should also be noted that these pathways also regulate a number of virulence determinants such as adhesins and extracellular hydrolytic enzymes (Biswas *et al.*, 2007; Kumamoto & Vinces, 2005).

C. albicans yeast bud in an axial or bipolar pattern depending on the temperature; pseudohyphae bud in a unipolar pattern and the cells remain attached following cytokinesis. Pseudohyphae and yeast form septin rings at the neck of the bud and pseudohyphae have constrictions where the mother and daughter cells are attached (Berman, 2006). Hyphae are narrower than pseudohyphae and the first septum is formed inside the germ tube with the nucleus migrating into the germ tube before the first nuclear division, in yeast and pseudohyphae nuclei divide across the septal junction. Pseudohyphae have a longer G2 phase so that mother and daughter cells divide at roughly the same time, giving synchronicity of the cell cycles (Berman, 2006). In filamentous fungi polarized growth is directed by an organelle called a Spitzenkörper which mediates the direction of hyphal

growth; in budding yeast polarized growth is mediated by a polarisome which mediates clustering of actin cables at the bud site (Steinberg, 2007). In *C. albicans* both a Spitzenkörper and a polarisome can be detected at the hyphal apex in hyphal form and only the polarisome is present in the yeast and pseudohyphal forms; the presence of a Spitzenkörper in hyphae indicates a diverse mechanism of growth (Crampin *et al.*, 2005).

Mating

Until recently *C. albicans* was thought to be an obligate diploid, but lately a sexual cycle has been characterized (Bennett & Johnson, 2005). Mating in *C. albicans* shows some similarities to that found in the model organism *Saccharomyces cerevisiae*, but there are significant differences that may be adaptations for mating in human hosts. Mating in *C. albicans*, as in *S. cerevisiae*, is dependent on the *MAT* locus, which has two alleles, a and α. However, unlike *S. cerevisiae*, other genes are present at the *C. albicans MAT* locus (*PIK*, *PAK* and *OBP*) with different alleles for the mating types. In *S. cerevisiae* mating occurs in the haploid stage of the lifecycle. In the obligate diploid *C. albicans* a homozygous strains and α homozygous strains can mate and a/α heterozygous strains cannot, however in *C. albicans*, simply being *MAT* homozygous isn't enough to allow mating to occur, as it must also switch from white to the opaque form. Only *MAT* homozygous strains can make this switch (Bennett & Johnson, 2005). The White form of *C. albicans* produces a smooth, white, dome-shaped colony and the cells comprising it are round. The Opaque form of *C. albicans* produces large, grey, flat colonies, comprised of bean shaped cells that are twice the mass and three times the volume of white cells and are unable to form hyphae (Anderson & Soll, 1987; Slutsky *et al.*, 1987b). Furthermore, the levels of *HBR1*, which is upregulated in response to haemoglobin, can affect the expression

of a1, α1 and α2 in a/α heterozygous strains and can even induce white opaque switching (Pendrak *et al.,* 2004). Additionally, in *S. cerevisiae MATa1* and *MATα2* regulate sporulation and this is not the case in *C. albicans* (Rustad *et al.,* 2006). In *C. albicans* a-type cells a1 and a2 are present in the a allele and a2 activates a specific genes, in α-type cells α1 and α2 are present and α1 activates α mating. In a/α heterozygous strains a1 and α2 act together to repress white opaque switching and mating (Bennett & Johnson, 2005).

C. albicans also produces a mating pheromone called *MFα* that is processed by a serine protease, Kex2p, which is required for mating in α-type cells. *STE2* in *C. albicans* encodes a receptor required for mating in a type cells (Bennett *et al.,* 2003; Panwar *et al.,* 2003). The mating pheromone *MFA1* is required for mating in a type cells (Dignard *et al.,* 2007). These mating pheromones cause an arrest in the G_1 stage of the cell cycle and the production of projections for cell fusion in the opposite mating types (Bennett & Johnson, 2005; Zhao *et al.,* 2005). The mating pheromones cause a transcriptional response by activating the same MAPK cascade which is involved in regulating morphogenesis, indicating a close link between morphology and mating (Bennett & Johnson, 2005). In *S. cerevisiae* and most other fungi mating is directed by the genotype of the organism, however in *C. albicans* it seems that other factors may be involved (Bennett & Johnson, 2005).

Once mating has occurred in order to get back to a diploid state chromosome reduction is required. This can occur through meiosis in a sexual cycle or chromosome loss in a parasexual cycle. A parasexual cycle has been identified in *C. albicans*, however, it is unknown if meiosis occurs; some key homologs of the genes required for meiosis appear to

be missing from the *C. albicans* genome (Bennett & Johnson, 2003; Bennett & Johnson, 2005). The apparent lack of meiosis in *C. albicans* makes the classic yeast genetic techniques used in *S. cerevisiae* inappropriate to *C. albicans*. Initially it was uncertain whether *C. albicans* was able to mate in the host, as the opaque form, which is required for mating, is not stable at body temperature (Slutsky *et al.*, 1987a). It has since been shown that in anaerobic conditions the opaque form is stable and mating has been shown to occur in several mouse models. These include an anaerobic model of the gastrointestinal tract, the skin and in a tail vein systemic model (Hull, *et al.*, 2000; Lachke *et al.*, 2003; Dumitru *et al.*, 2007).

Virulence

The virulence of *C. albicans* is a well studied area due to its clinical relevance, however not all the virulence determinants have been fully characterized. This section contains a short review of some of the factors that contribute to *C. albicans* pathogenicity. For some fungi, including *C. albicans*, the condition of the host is often important for the switch from a commensal to a virulent infection (Latge & Calderone, 2002). For example, immunocompetent patients rarely develop *Aspergillus* infections, but these infections are much more common in patients who are immunocompromised due to radio therapy treatment or prescription of immune suppressant drugs to prevent organ rejection following transplant operations. In contrast, people who are immunocompromised as a result of HIV are more likely to suffer from *Pneumocystis carinii*, *C. albicans* and *Cryptococcus neoformans* infections (Latge & Calderone, 2002). These host differences are attributed to differences in the host immune response and this is borne out by research that suggests the interaction with dendritic cells and the production of cytokines in response has an effect on

whether the infection is virulent or commensal (Romani *et al.,* 2002; Casadevall, 2003). These observations demonstrate the interplay between host and pathogen virulence factors.

Secreted proteases are a feature found in saprotrophic fungi (e.g. *Aspergillus niger*) that help them utilize available nutrients, but in pathogenic fungi they also have a role in virulence (Naglik *et al.,* 2003). Proteinases have been shown to play a key role in the virulence of *C. albicans*; they have been implicated in digesting cell membranes to aid adhesion, digesting molecules for nutrient acquisition and damaging host immune cells (Naglik *et al.,* 2003). *C. albicans* contains 10 genes encoding secreted aspartyl proteases (SAP) and the virulence of *C. albicans* strains correlates with Sap activity *in vitro* (Naglik *et al.,* 2003).

Phospholipases are virulence factors in a number of fungi such as *Aspergillus fumigatus* as well as in pathogenic bacteria including *Clostridia sp., Pseudomonas sp., Mycobacterium tuberculosis* and others. Phospholipases act by digesting glycerophospholipids in the cell membrane. In *C. albicans*, two extracellular and two intracellular phospholipases have been characterized and implicated in adhesion, invasion and cell penetration (Schaller *et al.,* 2005). The expression of four other lipases (encoded by *LIP5, LIP6, LIP8* and *LIP9*) in the mouse model of infection also suggest they have a role in the virulence of *C. albicans* (Brown *et al.,* 2007).

Within the host, a number of mechanisms exist to prevent pathogens from colonizing surfaces, including urine flow, mucus secretion (followed by coughing and sneezing) and blood flow which all serve to wash away the invading pathogen. In bacteria, adherence to

surfaces is achieved by fimbriae, which are appendages protruding from the cell, or by polysaccharide components of the bacterial cell wall which interact with the host cell (Wilson *et al.,* 2002). Adherence is equally as important for pathogenic fungi and strains of *C. albicans* that are more adherent are more pathogenic (Mendes-Giannini *et al.*, 2005). Proteins with possible laminin, a component of host cell membranes, binding properties have been found in *A. fumigatus*, *Histoplasma capsulatum*, *Paracoccoidioides brasiliensis*, *Penicillium marneffi* and *C. albicans* (Mendes-Giannini *et al.,* 2005). Adherence is also thought to be achieved by expression of the agglutinin-like sequence (*ALS*) gene family, which code for cell surface glycoproteins. These proteins allow adherence to epithelial cell proteins including laminin and fibronectin (Mendes-Giannini *et al.,* 2005) and the ability to adhere to surfaces can be aided by phospholipases. To date nine *ALS* genes have been identified in *C. albicans* and heterologous expression of these genes in the non-adherent *S. cerevisiae* system results in *S. cerevisiae* becoming adherent (Hoyer, 2001).

It is not surprising that the ability of *C. albicans* to change its morphological form has been shown to be necessary for virulence (Braun & Johnson 1997; Lo *et al.,* 1997; Rocha *et al.,* 2001), as the regulatory networks that control the yeast-hyphal switch also regulate the expression of virulence factors that are not associated with the morphological change (Biswas *et al.*, 2007; Kumamoto & Vinces, 2005). The yeast form of *C. albicans* is essential for dissemination of *C. albicans* in the blood stream and the yeast-hyphal switch is essential for pathogenicity. Therefore it has been suggested that the different morphological forms are essential for different stages of infection, hyphae for tissue and cell penetration because of pressure the growing hyphal tip and yeast for dissemination (Kumamoto & Vinces; 2005; Gow *et al.,* 2002; Whiteway, 2004). Mating has been shown

to have an effect on virulence with homozygous strains being less virulent than a/α strains in a mouse model (Lockhart *et al.*, 2005; Wu *et al.*, 2007).

A further factor contributing to the success of *C. albicans* as an opportunistic pathogen is its ability to survive treatment with antifungal agents. *C. albicans* has a great deal of intrinsic resistance to antifungal agents, with fatality rates of 55% even with antifungal therapy (Weinberger *et al.*, 2005). This high mortality rate is in part attributable to intrinsic resistance, but can also be attributed to some acquired resistance which is observed to antifungals in *C. albicans*. The main types of *C. albicans* antifungals are polyenes (e.g. amphotericin B), which disrupt the lipid bilayers by binding to sterols; 5-florocytosine, which causes disruptions in nucleic acid synthesis; echinocandins, which have been implicated in inhibition of (1,3)-D-β-glucan biosynthesis and azoles, which inhibit the product of the *ERG11* gene, which codes for a component of the ergosterol biosynthetic pathway. Mutations in *ERG3* cause amphotericin B and azole resistance by lowering the amount of ergosterol in the membrane, although this is rare. *C. albicans* azole resistance is achieved by mutations in or upregulation of *ERG11*, changes in the biosynthesis of ergosterol and upregulation of multidrug efflux transporter genes *MDR1*, *CDR1* and *CDR2*. 5-florocytosine resistance is associated with mutations in the pyrimidine salvage enzyme Fur1p (Sanglard & Odds, 2002; Cannon *et al.*, 2007).

1.3: Iron and Virulence:

As is the case with all organisms, the mammalian host requires iron for survival but has to limit the amount of free iron as it is toxic, this is achieved by keeping iron tightly bound in

transferrin and lactoferrin and the iron storage molecule ferritin. The host also uses this sequestration of free iron as a defense against colonization by pathogens as pathogens also require iron to survive; when the host detects a pathogen there is a hypoferraemic response to further reduce the levels of free iron. In the mammalian host iron is absorbed in duodenum and the upper jejunum. Heme iron is taken up by heme carrier protein 1(HCP1) and oxidized in the cell by a heam oxyenase to acquire ferrous iron. Non heme iron is reduced by DcytB, a cytochrome b reductase, and taken up by DMT1, a ferrous iron transporter. Cellular ferrous iron can be exported from cells by hephaestin, a copper dependant ferroxidase in a mechanism involving ferroporin (figure 1.3). The iron bound to transferrin, lactoferrin or lactotransferrin, which are single chain glycoproteins containing two iron binding sites and are capable of binding two ferric iron ions, for transport in the blood stream. Iron enters the cells in which it is required via transferrin receptors where it is subsequently bound in haem, incorporated into proteins or stored in ferritin proteins, which can bind more than 4000 ferric iron ions (reviewed: Edison *et al.,* 2008). Iron homeostasis is controlled by hepcidin, which is secreted by the liver and in much smaller quantities by inflammatory macrophages and monocytes. Hepacidin expression is regulated by numerous factors including hypoxia and copper deficiency amongst others. Upstream of hepacidin are the HFE, HJV and TFR2 regulators, but their role in iron homeostasis is not yet fully characterized (Edison *et al.,* 2008).

The free iron levels in the human body are reduced even further by the hypoferraemic response to infection when a pathogen invades (Reviewed: Edison *et al.,* 2008). During infection cytokines induce an increase in hepacidin synthesis which in turn induces the loss of ferroportin, an iron export protein, from macrophages. Hepacidin also decreases the

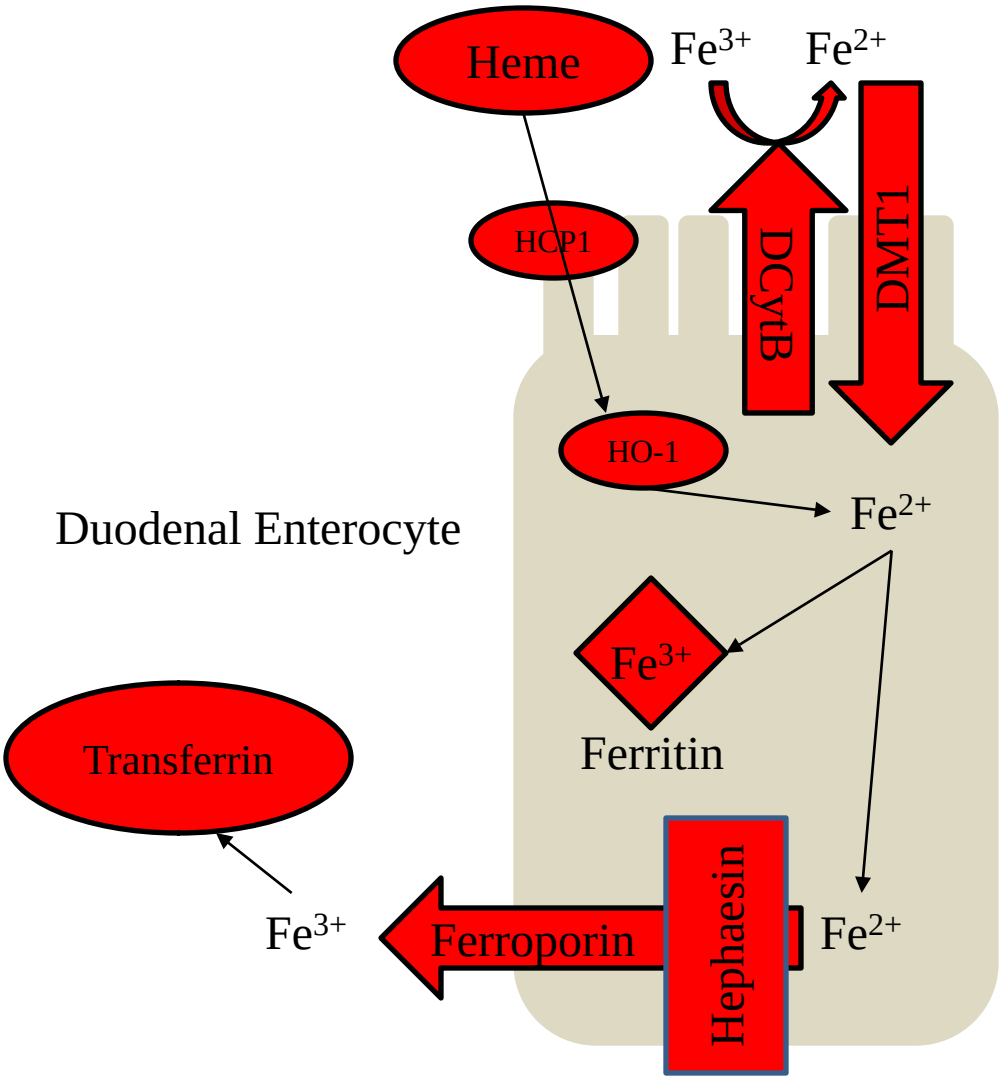


Figure 1.3: In mammals heme iron is taken up by Heme Carrier Protein 1 (HCP1), then oxidised by Haem Oxygenase 1 (HO-1) to release ferrous iron. Non-haem iron is reduced by DCytB, a cytochrome b reductase, at the cell surface, then taken up by DMT1, a ferrous iron transport protein. The ferrous iron taken up into the duodenal enterocyte is either stored in ferritin or trasported out of the cell by a complex of Ferroporin with Hephaesin, durin which it is oxidised back to ferric form. The ferric form is incorporated into transferrin for transport in the blood (adapted from Edison *et al.*, 2008).

production of iron containing red blood cells and by decreasing the growth and survival of the tissues which produce red blood cells. All this serves to reduce the level of free iron in the host by leaving it bound up in ferritin, the iron storage molecule and in a long-term infection this response can lead to anemia in the host (Nadahur *et al.*, 2008). The lack of free iron presents pathogenic organisms with the problem of acquiring iron once they have entered the host. The levels of free iron have been demonstrated to affect virulence of *E. coli* in a guinea pig model and *Vibrio vulnificus* in a mouse model. In these experiments, when free iron was injected into infected animals it significantly reduced their resistance to infection. Further evidence is provided for the importance of free iron in pathogen resistance by the finding that patients with leukemia suffering from iron overload are more susceptible to *C. albicans* infections (reviewed: Bullen *et al.*, 2005).

Iron in the host is stored away in ferritin molecules and when it is required it is transported around tightly bound up in transferrin or lactoferrin. All other iron is bound up in proteins, mostly in the mitochondria of cells or in erythrocytes (Edison *et al.*, 2008). Two major routes of iron uptake by pathogenic organisms within this iron limited host environment are the production and the uptake of iron chelators called siderophores (reviewed: Howard, 1999) and by the operation of a high affinity reductive iron uptake system which will be focused on in this study (reviewed Van Ho *et al.*, 2002). The uptake of iron has been shown repeatedly to be required for virulence in bacterial and fungal species, particularly the proteins involved in siderophore biosynthesis and uptake (Ratledge & Dover, 2000; Howard, 1999). Proteins involved in high affinity iron uptake such as *CaFTR1* in *C. albicans* are also required for virulence (Ramanan & Wang, 2000). Furthermore a low iron environment is characteristic of the host. In bacteria the Fur protein represses genes in

high iron conditions. Fur has been shown to regulate iron uptake systems as well as a number of virulence factors that are not involved in iron metabolism. In this way iron levels are used as an indicator of entry into the host to induce the expression of virulence factors (Escolar *et al.,* 1999).

C. albicans has five main systems of acquiring iron, a low affinity system, which is active in iron replete conditions and a high affinity system which is active in low iron conditions, such as the host. A haem iron acquisition and utilization system, a siderophore uptake system and a mechanism for acquiring iron from ferritin (Van Ho *et al.,* 2002; Almeida *et al.,* 2008). The focus of this project is the ferric reductase proteins, which are part of the high affinity iron uptake system. The most well characterized high and low affinity iron uptake systems are found in the model organism *Saccharomyces cerevisiae*. *S. cerevisiae* can also utilize siderophores, however it cannot utilize heam as an iron source (reviewed Van Ho *et al.,* 2002). The *C. albicans* high affinity iron uptake shows homology to that found in *S. cerevisiae*, but its regulation has been shown to be considerably different, presumably due to their different environmental niches (Woodacre *et al.,* 2008; Lan *et al.,* 2004).

1.4: Low Affinity Iron and Copper Uptake in *S. cerevisiae*:

In *S. cerevisiae*, it is the iron transport protein ScFet4p that is responsible for low affinity iron uptake after iron is reduced at the cell surface (Stearman *et al.,* 1996; Urbanowski & Piper, 1999; Dix *et al.,* 1994; Dix *et al.,* 1997). The protein ScFet4p is located at the

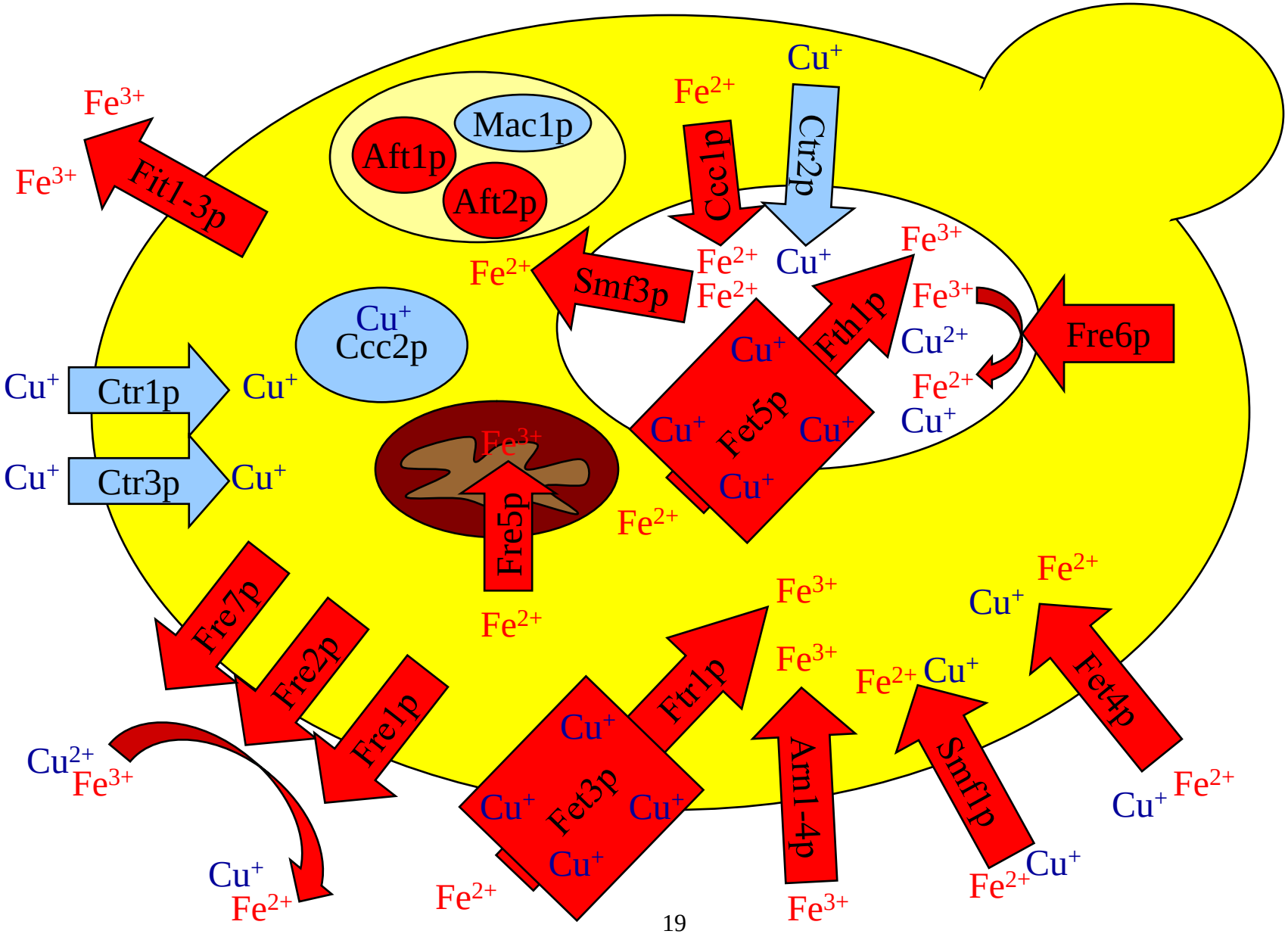

Fe3+
Fe3+
Fit1-3p
Mac1p
Aft1p
Aft2p
Cu+
Fe2+
Ccc1p
Ctr2p
Fe2+
Smf3p
Fe2+
Cu+
Fe3+
Fe2+
Fth1p
Fe3+
Cu2+
Fre6p
Fe2+
Cu+
Cu+
Ccc2p
Cu+
Ctr1p
Cu+
Cu+
Ctr3p
Cu+
Fe3+
Fre5p
Fe2+
Cu+
Cu+
Fet5p
Cu+
Cu+
Fe2+
Fre7p
Fre2p
Fre1p
Cu2+
Fe3+
Cu+
Fe2+
Cu+
Cu+
Fet3p
Cu+
Fe2+
Ftr1p
Fe3+
Fe3+
Arn1-4p
Fe3+
Fe2+
Cu+
Smf1p
Fe2+
Cu+
Fe2+
Cu+
Fet4p
Cu+
Fe2+

Figure 1.4: High Affinity Iron and Copper Uptake in *S. cerevisiae*: Copper uptake is shown in blue and iron uptake in red. The nucleus in pale yellow, the vacuole as white and the mitochondria in brown. Not to scale. In iron limiting conditions iron and copper are both reduced first at the cell surface by a ferric reductase protein, the major ferric reductase is encoded by *CaFRE1*, once reduced copper is then taken up by the copper transporter Ctr1p and iron is taken up by a complex of a multicopper oxidase (Fet3p) and an iron permease (Ftr1p). Once inside the cell copper is shuttled around by the copper chaperone Ccc2p. The Arn proteins are involved in the uptake of siderophores as an iron source, Smf1p and Fet4p are low affinity iron and copper transporters and the Fit proteins increase the association of iron with the cell surface. The vacuole is used as a store of iron, ferrous iron is transported into the vacuole in iron replete conditions by a complex of Fth1p and Fet5p or by Ccc2p. Iron is transported back to the cytosol when it is needed by Smf3p. Copper sensitive regulation is achieved by CaMac1p and iron sensitive regulation is achieved by Aft1p (Van Ho, *et al.* 2002).

plasma membrane and deletion of *ScFET4* does not affect high affinity iron uptake, but abolishes low affinity transport. Furthermore, overexpression of Scfet4p increases low affinity iron uptake (Dix *et al.,* 1994, Dix *et al.,* 1997). In addition to iron, ScFet4p is also capable of transporting nickel, cobalt, cadmium, zinc, copper and manganese (Hassett *et al.,* 2000; Portnoy *et al.,* 2001; Waters & Eide 2002). The low affinity iron uptake system is also active in anaerobic conditions since some components of the high affinity system require oxygen to function (de Silva *et al.,* 1997; Hassett *et al.,* 1998; Jensen & Culotta, 2002). Much like iron, copper has both high and low affinity transport systems in a number of organisms, including *S. cerevisiae.* In *S. cerevisiae* ScFet4p can transport copper and has been demonstrated to participate in low affinity copper transport (Hassett *et al.,* 2000). However, ScFet4p may not be the only protein responsible for low affinity copper transport as over expression of ScSmf1p, a low affinity metal transporter, causes copper toxicity (Liu *et al.,* 1997).

1.5: High Affinity Iron Uptake in Fungi:

When the high affinity iron uptake system is in use, in iron limited conditions, iron is first reduced at the cell surface by membrane spanning proteins called ferric reductases (Dancis *et al.,* 1990). Eukaryotic ferric reductase proteins contain flavin prosthetic groups and NAD(P)H binding sites and as such are similar to b-type cytochromes (Pierre *et al.,* 2002). The reduced iron is then taken up into the cell by a complex of an iron transport protein with a multicopper oxidase (Stearman *et al.,* 1996; Ardon *et al.,* 2001). Multicopper oxidases require copper to function and as a result of this iron and copper acquisition are inextricably linked as shown in figure 1.4 (Van Ho *et al.,* 2002).

Ferric Reductase Proteins

***S. cerevisiae*:** ScFre1p is found in the plasma membrane and reduces Fe^{3+} to Fe^{2+} as well as Cu^{2+} to Cu^{+}. Over expression of *ScFRE1* leads to copper sensitivity. Deletion of *ScFRE1* results in an 80% decrease in cell surface ferric reductase activity (Dancis *et al.,* 1990; Dancis *et al.,* 1992; Anderson *et al.,* 1992). The ScFre1 protein interacts physiologically with ScFet3p, a protein which oxidises Cu^{+} amongst other things, to maintain copper homeostasis (Shi *et al.,* 2003). The ScFre1 protein is, as well as being involved in copper homeostasis, also thought to be involved in ferric citrate reductase activity; indeed a *Scfre1Δ* mutant strain displayed reduced ferric citrate reductase activity (Eide *et al.*, 1992).

The expression of *ScFRE2* is regulated solely by iron, yet ScFre2p is both a ferric and cupric reductase, the deletion of *ScFRE2* in a *Scfre1Δ* mutant removes some but not all of the residual ferric reductase activity (Georgatsou & Alexandraki, 1994). Deletion of *ScFRE3* in a *Scfre1Δ/Scfre2Δ* strain resulted in a 73% decrease in ferroxamine B (FOB) siderophore reduction. When cells were grown in a rich media deletion of only *ScFRE3* decreased FOB iron uptake. *ScFRE3* is also implicated in ferrichrome and triacetylfusarinine C (TAFC) iron uptake as deletion of *ScFRE3* along with *ScFRE1* and *ScFRE2* results in failure to grow on ferrichrome or TAFC. The ScFre3 protein also facilitates the uptake of rhodotorulic acid (RA) iron as a *Scfre1Δ/Scfre2Δ/Scfre3Δ* mutant could not grow on RA as a sole iron source, but a *Scfre1Δ/Scfre2ΔScFRE3*$^{+}$ strain could. *ScFRE3* was localized to the plasma membrane by tagging with a triple HA epitope (Yun *et al.,* 2001).

All the ferric reductases are transmembrane proteins, but not all are found at the cell surface. This accounts for the majority of the reductase activity at the cell surface and siderophore uptake across the cell surface membrane being attributable to *ScFRE1-4.* Ferric reductases are presumably needed on intracellular membranes as iron is oxidized back to its ferric form when transported into the cytoplasm, so movement across intracellular membranes would require a further reduction. The ScFre5 protein has been found in the mitochondrial membrane (Sickmann *et al.,* 2003) and ScFre6p has been localized to the vacuole and is important for the functioning of the vacuolar copper transporter ScCtr2p (Huh *et al.,* 2003; Singh *et al.,* 2007; Rees & Thiele, 2007). Expression of *ScFRE7* is regulated solely by copper and its overexpression can rescue a *Scfre1Δ/Scfre2Δ* strain, indicating a role in cell surface ferric reductase activity (Georgatsou & Alexandraki, 1999; Martins *et al.,* 1998; Rees & Thiele, 2007; Labbe *et al.,* 1997).

In *S. cerevisiae* there are seven ferric reductase (*FRE*) genes involved in high and low affinity iron transport and in the reduction of siderophores produced by other microorganisims at the cell surface. Growth on media containing bathophenanthroline sulfonate (BPS), an iron chelator, causes markedly increased expression of *ScFRE1-6* and did not affect *ScFRE7* expression. The expression of *ScFRE7* and *ScFRE1* was induced by addition of the copper chelator, bathocupronine disulphonate (BCS). This indicates that *ScFRE2-6* are induced in low iron environments, *ScFRE7* is induced in low copper environments and *ScFRE1* is induced in both low iron and low copper environments (Bakel *et al.,* 2005; Georgatsou & Alexandraki, 1999; Martins *et al.,* 1998).

***C. albicans*:** Cell surface ferric reductase activity regulated by iron and copper levels in *C. albicans* was first demonstrated in our laboratory in 1996 (Morrissey *et al.*, 1996), since then sixteen putative ferric reductase genes have been identified in *C. albicans* through complementation studies (Hammacott *et al.*, 2000) and sequence analysis of the *C. albicans* genome (Jones *et al.*, 2004; Mason, 2006). A few of these have been investigated using microarrays and Northern blots and four knockout mutants have been made (*FRE1*, *FRE2*, *FRE5* and *FRE10*), but only one had a major phenotypic effect on cell surface ferric reductase activity (Lan *et al.*, 2004; Knight *et al.*, 2005; Mason, 2006). The major *C. albicans* ferric reductase is *CaFRE10* (also called *CFL95*); deletion of *CaFRE10* causes a 75% decrease in cell surface ferric reductase activity and a 77% decrease in the uptake of ferric iron (Mason, 2006). *CaFRE10* has been implicated in transferrin uptake, as the uptake of iron from transferrin is reduced by 75% in a *Cafre10Δ/Cafre10Δ* mutant. However, *CaFRE10* is not essential for transferrin iron uptake indicating that another reductase is at work (Knight *et al.*, 2005). *CaFRE1* (also called *CFL1*) and *CaFRE2* (also called *CFL2*) can complement an *S. cerevisiae fre1Δ/fre2Δ* mutant. A *Cafre2Δ/Cafre2Δ* mutant showed a small decrease in cell surface ferric reductase activity (Mason, 2006).

All of the ferric reductases identified in *C. albicans* have signal sequences indicating that they localize to the cell surface, however this was the case with sequence analysis of the *S. cerevisiae* ferric reductase genes and experimental evidence has shown that some of the proteins localize to other parts of the cell (Mason, 2006). In *C. albicans CaFRE5* (also called *CFL4*) has a mitochondrial signaling sequence when analyzed using some bioinformatics software packages. The expression of *CaFRE1*, *CaFRE2*, *CaFRE5* and *CaFRE31* (also called *CFL5*) was found in microarray studies to be increased in low iron

concentrations (Lan *et al.,* 2004). The expression of *CaFRE2, CaFRE7*, *CaFRE9* and *CaFRP1* was found to be increased in alkaline conditions (Bensen *et al.,* 2004). *CaFRE2* and *CaFRP1* are directly regulated by the pH dependant transcription factor RIM101p by binding of RIM101p to sites upstream in the promoters of these genes (Baek *et al.,* 2008). *CaFRP1* was also shown to be regulated by iron starvation independently of RIM101p; this regulation is achieved by the recruitment of Hap43 to the CBF complex, which binds the CCAAT motif, in iron starvation conditions (Baek *et al.,* 2008). *CaFRE1* was found to be inhibited by serum treatment (Lesuisse *et al.,* 2002). *CaFRE7* is regulated by copper showing that the ferric reductases are regulated by both copper and iron levels as well as being regulated by pH and serum (Woodacre *et al.,* 2008).

Other fungi: In the fission yeast *S. pombe*, *Frp1* encodes the ferric reductase and its product has an amino acid identity of 27% to *S. cerevisiae* Fre1p. Mutations in *Frp1* result in inability to utilize ferric iron and growth deficiencies in an iron limited medium (Roman *et al.,* 1993). Ferric reductases have been identified in a number of other species across the fungal kingdom by sequence homology, including 9 in *Aspergillus nidulans*, 18 in *Aspergillus fumigatus*, 9 in *Cryptococcous neoformans*, 7 in *Ustillago maydis* and 10 in *Neurospora crassa* amongst others (Mason, 2006). However, not a lot of experimental evidence for function is available.

Multicopper Oxidase Proteins

The multicopper oxidase proteins require copper to function and once they are loaded with copper form a complex with iron transport proteins to facilitate the transport of iron across

cellular membranes. Three multicopper oxidase genes (*ScFET3-5*) have been identified in *S. cerevisiae*. The protein encoded by *ScFET3*, forms a complex with ScFtr1p, a high affinity iron permease, to facilitate the transport of iron across the cell surface membrane (Stearman *et al.,* 1996). *ScFET5* is homologous to *ScFET3* and its overexpression in a *Scfet3Δ/Scfet4Δ* mutant in moderate iron limited conditions resulted in the restoration of wild-type growth, however it is less effective than *ScFET3* and *ScFET4* (Spizzo *et al.,* 1997). The ScFet5 protein has been found to form a complex with the iron transporter ScFth1p, which is localized to the vacuolar membrane (Urbanowski & Piper, 1999).

ScFet3p is also involved in copper homeostasis (Shi, *et al.,* 2003). Activity of ScFet3p is dependent upon the presence of Cl^- ions. The protein Gef1p is a voltage regulated chloride channel, in a *gef1Δ/gef1Δ* mutant in low copper conditions apo-Fet3p is not copper loaded and high affinity iron transport is not observed (Spizzo *et al.,* 1997). *ScFTR1* and *ScFET3* are both regulated at the transcriptional level by iron, but are also regulated at the post transcriptional level. At high iron levels fluorescently tagged ScFet3p and ScFtr1p were rapidly lost and in a mutant lacking a vacuolar protease (*pep4Δ*) and fluorescently tagged ScFet3p and ScFtr1p accumulated in the vacuole at high iron concentrations (Felice *et al.,* 2005).

There are homologues of *ScFET3* (CaFet3p has 55% identity to Fet3p in *S. cerevisiae*) and *ScFTR1* in *C. albicans* (Eck *et al.,* 1999). The gene *CaFET3* was not essential for virulence in the mouse model of systemic infection; however, adherence to mouse L929 fibroblasts was reduced in a *fet3Δ/fet3Δ* mutant (Eck *et al.,* 1999). Five other multicopper oxidases are present in the *C. albicans* genome; *CaFET31*, *CaFET33*, *CaFET34*, *CaFET35*

and *CaFET99* (Braun *et al.,* 2005). A homologue of *ScFET3* was also found in *Arxula adeninivorans* and the gene was found to be iron regulated and localized to the plasma membrane in both yeast and hyphal forms (Wartmann *et al.,* 2002).

Iron Transport Proteins

In *S. cerevisiae* ScFtr1p forms a complex with ScFet3p and is found on the plasma membrane, where it is involved in ferrous iron transport. In a *Scfet3Δ* mutant, ScFtr1p localized in the endoplasmic reticulum, suggesting that the movement of ScFtr1p to the plasma membrane depends on complex formation with a multicopper oxidase (Stearman *et al.,* 1996). A high affinity iron transporter in *S. cerevisiae,* encoded by *ScFTH1,* has also been identified and the protein forms a complex with ScFet5p. Deletion of *ScFET5* results in accumulation ScFth1p in the endoplasmic reticulum. The complex is localized to the vacuolar membrane and due to the fact that the oxidase domain of ScFet5p is luminal, it is thought that the complex is involved in utilization of intravacuolar iron stores. Further evidence that ScFth1p is found at the vacuole is that it does not undergo vacuolar degradation by Pep4p, a feature of proteins found on the vacuolar membrane (Urbanowski & Piper, 1999).

In *C. albicans* there are two *ScFTH1* homologs (*CaFTH1* and *CaFTH2*), along with *ScFTR1* and *ScFTR2* homologs, *CaFTR1* and *CaFTR2* respectively (Van Ho *et al.,* 2002). The CaFtr1 protein, like ScFtr1p found in *S. cerevisiae,* contains five iron-binding (Glu-Xaa-Xaa-Glu) motifs and disruption of one of these, the Glu-Gly-Leu-Glu sequence prohibited growth in iron limited conditions (Fang & Wang, 2002). *CaFTR1* has been

found to be essential for virulence in the mouse model of systemic infection caused by *C. albicans*, *FTR2* was not essential for virulence (Ramanan & Wang, 2000).

In *S. pombe* reduced iron is taken up by a system homologous to the ScFet3/Ftr1p system in *S. cerevisiae*, the multicopper oxidase is Fio1p and the iron transport protein is Fip1p (Askwith & Kaplan, 1997). Two homologs of *ScFTR1* were identified in the plant pathogenic fungus *Fusarium graminearum*, *FgFtr1* was found to be expressed at a higher level in low iron conditions and *FgFTR2* was expressed in an *fgftr1Δ* mutant. Both *FgFtr1* and *FgFtr2* were able to rescue a *Scftr1Δ* mutant and mutation of the REXXE motifs prevented this rescue from occurring, demonstrating the importance of these motifs in iron transport. Furthermore both FgFtr1p and FgFtr2p were shown to interact with ScFet3p to localize to the plasma membrane in *S.cerevisiae* (Park *et al.*, 2006). Further GFP-localisation experiments demonstrated that in *F. graminearum* FgFtr1p was found on the plasma membrane and FgFtr2p was found on vacuolar membrane. In addition to this two putative multicopper oxidase genes showed interaction of FgFtr1p and FgFet1p and FgFtr2p and FgFet2p (Park *et al.*, 2007). In the methylotrophic yeast *Pichia pastoris* another multicopper oxidase – iron transport protein complex has been identified (Paronetto *et al.*, 2001; Bonaccorsi di Patti *et al.*, 2005). In the pathogenic zygomycete *Rhizopus oryzae* the *rFTR1* expressed in an *S. cerevisiae ftr1Δ* mutant rescued the phenotype indicating that *R. oryzae* also possesses a high affinity iron uptake system (Fu *et al.*, 2004). There is also evidence for a copper dependant reductive iron uptake system in *Cryptococcus neoformans* (Jacobson & Vartivarian, 1992; Jacobson *et al.*, 1998; Nyhus & Jacobson, 1999). The wide distribution of multicopper oxidase dependent iron transport proteins indicates conservation of this system throughout the fungal kingdom.

Siderophore Uptake Proteins and Acquisition of Iron from Ferritin

Both *S. cerevisiae* and *C. albicans* can utilize siderophores produced by other organisms. The genes *ScARN1-4* in *S. cerevisiae* are members of the major facilitator family involved in taking up siderophores with varying specificity, and are closely regulated by iron levels (Philpott *et al.,* 2002). The ScArn1 protein transports a specific class of ferrichrome with anhydromevalonyl residues linked to N^{δ}-orthanine (Heymann *et al.,* 2000). The ScArn2 protein (also called Taf1p) can also transport ferrichrome to some extent (Philpott *et al.,* 2002) and transports triacetylfusarinine C (TAFC) (Heymann *et al.,* 1999). The ScArn3 protein (also called Sit1p) transports ferroxamine (Lesuisse *et al.,* 1998) and ScArn4p (also called Enb1p) transports enterobactin (Philpott *et al.,* 2002). The ScFet3 protein has been shown to be involved in the uptake of iron from enterobactin (ENT) (Yun *et al.,* 2001). There are also three facilitators of iron uptake (*ScFIT1-3*) which are involved in increasing the efficiency of uptake of ferroxamine B (FOB) and ferrichrome bound iron. In mutants where one or more of the *FIT* genes have been knocked out there is less iron uptake from these siderophores. The Fit proteins localize to the cell wall and appear to act by increasing the quantity of iron associated with the cell wall and periplasmic space, which provides more iron for uptake into the cell (Protchenko *et al.,* 2001).

C. albicans possesses a siderophore iron transporter with 46% identity to *S. cerevisiae* Arn1p and is specific for ferrichrome. The *C. albicans ARN1* gene is regulated by iron (Ardon *et al.,* 2001) and is repressed by CaTup1p and Rfg1p in high iron conditions. In a *CatupΔ/Catup1Δ* mutant siderophore transport is derepressed (Lesuisse *et al.,* 2002). Overexpression of *CaARN1* in *S. cerevisiae* caused siderophore iron uptake to be inhibited

indicating that CaArn1p interacts with *S. cerevisiae* proteins concerned with siderophore uptake but is inactive in *S. cerevisiae* (Lesuisse *et al.,* 2002). The CaArn1 protein is found typically in the plasma membrane, with some protein also found in the cytoplasm. It is thought that CaArn1p internalization may be a mechanism for ferrichrome iron transport since more CaArn1p was found in the cytoplasm at increased ferrichrome concentrations, although it could be due to a mechanism for recycling CaArn1p (Hu *et al.,* 2002). Iron transport via CaArn1p may be important for virulence as in reconstituted human epithelium a *Caarn1Δ/Caarn1Δ* mutant was defective in infection (Heymann *et al.,* 2002). However, the *Caarn1Δ/Caarn1Δ* mutant was fully virulent in a systemic mouse model (Hu *et al.,* 2002; Heymann *et al.,* 2002).

C. albicans can utilize ferritin, the mammalian iron storage molecule, as a sole iron source and *S. cerevisiae* cannot. A *Caftr1Δ/Caftr1Δ* mutant was unable to grow with ferritin as the sole iron source, indicating that ferritin utilization is dependent on the reductive pathway. Furthermore, only the hyphal form of *C. albicans* bound ferritin and that the yeast form did not and this binding of ferritin was dependant on the hyphal specific Als3p adhesin. Als3p dependant *C. albicans* ferritin binding occurs in epithelial cells and an *als3Δ/als3Δ* mutant did less damage to epithelial cells (Almeida *et al.,* 2008).

Heme Utilisation

A further strategy used by *C. albicans* to obtain iron is the utilisation of haem and haemoglobin as exogenous iron sources. The growth inhibition of *C. albicans* caused by iron deficiency in human serum can be rescued by the addition of haemglobin, haem and iron obtained from red blood cells. Interestingly, haemin and haemoglobin induce hyphae

in *C. albicans*, but Haem, free globin and immobilized haemoglobin do not have the same effect (Casanova *et al.,* 1997; Pendrak *et al.,* 2004). The presence of haemoglobin causes the upregulation of the hyphal genes *HWP1* and *ECE1* which explains the effect on the morphology of *C. albicans*. It also increases the expression of cell surfaces adhesins such as fibronectin which may play a role in the switch from commensal to pathogenic infections (Pendrak *et al.,* 2004; Pendrak *et al.,* 2000; Pendrak & Roberts, 2007).

C. albicans can use complement like molecules to bind complement coated red blood cells in order to utilize the iron for growth (Moors *et al.,* 1992) and can secrete a haemolytic factor which lyses erythrocytes to release haemoglobin (Manns *et al.,* 1994; Watanabe *et al.,* 1999). The haemoglobin is then taken up into the cell by Rbt5p and Rbt51p (Weissman, 2004, Weissman *et al.,* 2008). *RBT5* is regulated by iron starvation and is negatively regulated by CaTup1p (Weissman, 2004, Braun *et al.,* 2000). When expressed in *S. cerevisiae RBT51* allows the organism to utilize haemoglobin as an iron source and *RBT5 C. albicans* knockout mutants were unable to utilize haem iron efficiently (Weissman, 2004). Rbt5p has been localized to both the cell surface and the vacuolar compartment. Current information suggests that haemoglobin is internalized by these proteins in an endocytic mechanism into vesicles, which are delivered to the vacuole. The process is thought to be dependent on the endosomal sorting complex required for transport (ESCRT) system (Weissman *et al.,* 2008). The haem is then oxidized to α-Biliverdin by a heam oxygenase, CaHmx1p, the expression of which is induced by the presence of haemoglobin and globin, but not apoglobin (Pendrak *et al.,* 2004). Furthermore, *CaHMX1* expression is higher in iron starvation conditions and when the temperature was switched from 30˚C to 37˚C and it is negatively regulated by Efg1p, but not by CaTup1p. A

Cahmx1Δ/Cahmx1Δ mutant could still take up haemin, but could not use it as a sole iron source (Santos *et al.,* 2003).

1.6: Iron Storage in Fungi:

Due to the toxic nature of iron it has to be stored in a stable form inside living cells to prevent cellular damage (Imlay & Linn, 1988). In animals, plants and bacteria this is usually achieved using iron scavenger molecules called ferritins, which can store up to 4500 iron molecules safely (Chiancone *et al.*, 2004; Schrettl *et al.,* 2004). In fungi, ferritins are only found in the zygomycetes and the systems for storing iron safely are much more varied. Some fungi use siderophores as intracellular iron storage molecules. Ferrichrome is used for intracellular storage in *Ustillago maydis* and *S. pombe* and in *Aspergillus nidulans* and in *Neurospora crassa* ferricrocin is used (Schrettl *et al.*, 2004; Haas, 2003).

Saccaromyces cerevisiae does not produce any siderophores, so instead of using siderophores for storage, it stores iron in the vacuole. ScCcc1p is responsible for the transport of iron into the vacuole in *S. cerevisiae,* it localizes to the vacuolar membrane and it's over expression results in depletion of iron in the cytosol (Li *et al.,* 2001). It is regulated post transcriptionally by Cth1p and Cth2p binding, which destabilizes the mRNA. In low iron conditions *CTH2* expression is activated and Cth2p then destabilizes *ScCCC1* mRNA, preventing the transport of iron into the vacuole (Puig *et al.,* 2005). It is further regulated at the transcriptional level by Yap5p, an iron sensing transcription factor located constitutively in the nucleus which activates transcription of *ScCCC1* in high iron conditions (Li *et al.*, 2008).

The high affinity transport of iron from the vacuole across the vacuolar membrane to the cytosol involves the reduction of ferric iron on the vacuolar membrane by ScFre6p. The reduced iron is then transported across the membrane by ScFth1p, which forms a complex with the ScFet5p mutilcopper oxidase (Urbanowski & Piper, 1999; Singh *et al.,* 2007; Spizzo *et al.,* 1997). Low affinity iron transport from the vacuole to the cytosol is achieved using Smf3p, which is homologous to metal ion transporter Smf1p (Cohen *et al.,* 2000; Portnoy *et al.,* 2000). In *C. albicans* the iron transporter at the cell surface is *CaFTR1* which complexes with a multicopper oxidase (Van Ho *et al.,* 2002). However, other homologs of iron transport proteins and multicopper oxidases have been identified due to homology in the *C. albicans* genome and these may be involved in transporting iron across the vacuolar membrane. A *ScCCC1* homolog is also present in the *C. albicans* genome (Braun *et al.,* 2005).

1.7: High Affinity Copper Uptake in Fungi:

Copper Transport Proteins

S. cerevisiae has three copper transport genes, *ScCTR1-3*; the first copper transport protein in *S. cerevisiae* was identified in a screen for mutants with defective ferrous iron transport (i.e. the multicopper oxidases were unable to function in the absence of copper, thus no ferrous iron uptake could occur). The gene was assumed to be involved in copper transport as the amino terminal region contained eight Met-X_2-Met repeated motifs found in copper transport proteins in some bacterial species (Dancis *et al.,* 1994). The gene was localized, using an epitope tag, to the plasma membrane, where it exists as an oligomer with the

carboxyl terminal inside the cell (Dancis *et al.,* 1994). Another copper transport gene, *ScCTR2,* was identified and deletion of *ScCTR2* resulted in loss of high affinity iron uptake in an *Scctr1Δ/Scctr2Δ* mutant (Portnoy *et al.,* 2001). Furthermore deletion of *ScCTR2* resulted in an accumulation of copper in the vacuole (Rees *et al.,* 2004), even though *ScCTR2* expression is regulated by iron restriction, rather than copper restriction (Rees & Thiele, 2007). Like *ScCTR1, ScCTR2* also has conserved methionine residues essential for its copper transporting function and forms oligomers across a membrane (Rees *et al.,* 2004). Localization of ScCtr2p using epitope tagging and GFP fusion showed it is found in the vacuolar membrane (Portnoy *et al.,* 2001; Rees *et al.,* 2004). *ScCTR3* is found in the plasma membrane as a homotrimer and requires cysteine residues to exit the secretory compartment following assembly (Pena *et al.,* 2000). Deletion of all three copper transport genes results in a respiratory deficiency even when excess copper is added to the medium (Rees *et al.,* 2004).

In *C. albicans CaCTR1* and *CaCTR2* have been identified (Van Ho *et al.,* 2002; Dancis *et al.,* 1994; Marvin *et al.,* 2004; Marvin *et al.,* 2003). The *C. albicans CTR1* gene was identified by its ability to rescue an *S. cerevisiae ctr1Δ* mutant (Marvin *et al.,* 2003) and it is also regulated by copper (Marvin *et al.,* 2004). When the *CaCTR1* deficient mutant was grown on YPD medium, it formed small white 'petite' colonies and after prolonged incubation produced invasive filaments. The lack of a functional *CaCTR1* gene also caused respiratory deficiency on non-fermentable carbon sources and greater susceptibility to oxidative stress. These are phenotypes associated with copper deprivation as copper is required for the enzymes involved in these processes (Marvin *et al.,* 2003).

Three copper transport proteins have been identified so far in *S. pombe*; Ctr4p, Ctr5p and Ctr6p. Ctr4p has 5 Met-X-Met motifs similar to those found in the ScCtr1p, but not in ScCtr3p. However, the carboxyl terminal shows more identity to ScCtr3p; this suggests that the *CTR4* gene is a fusion between *ScCTR1* and *ScCTR3*. Ctr4p is localized to the plasma membrane and is expressed under low copper conditions. Deletion of $Ctr4^{+}$ in *S. pombe* results in loss of iron uptake and mutants were unable to grow on complex carbon sources, as was found for the *ScCTR1* gene (Labbe *et al.,* 1999). However, expression of Ctr4p in an *S. cerevisiae Scctr1Δ/Scctr3Δ* mutant does not rescue the mutant, instead a complex of Ctr4p and Ctr5p is required (Zhou & Thiele, 2001). Ctr6p forms a homotrimer that functions as a vacuolar membrane transporter, it is produced in copper limiting conditions and shows the greatest sequence identity to *ScCTR2*. Deletion of $Ctr6^{+}$ in *S. pombe* did not produce the growth defects seen in the *ctr4Δ* mutant, however the *ctr6Δ* did show a reduction in copper, zinc-super oxide dismutase activity. A *ctr4Δ/ctr6Δ* mutant was devoid of copper, zinc-SOD activity and overexpression of $Ctr6^{+}$ lead to copper sensitivity (Bellemare *et al.*, 2002).

A homolog of the *S. pombe* $Ctr4^{+}$ gene has been identified in *C. neoformans* and the promoter has been utilized as an inducible promoter system in this organism (Ory *et al.*, 2004). Furthermore, GFP reporter experiments have indicated that *C. neoformans CnCTR4* is suppressed with the addition of only 1 μM copper. This regulation was shown to be active *in vivo* in a mouse model, with expression of the GFP reporter under the control of *CnCTR4* promoter in mouse brains during infection, consistent with this being a low copper environment. However, in the Mouse lungs during infection CnCtr4-GFP was not expressed (Waterman *et al.*, 2007).

Copper Chaperone Proteins

In *S. cerevisiae* cells copper loading of the multicopper oxidases involved in iron transport involves a copper chaperone, Atx1p, a copper ATPase, ScCcc2p (Field *et al.*, 2002), and Gef1p, a voltage regulated chloride channel (Van Ho *et al.*, 2002). All these copper binding proteins contain one or more copper binding domains ([M/L][T/S]CXXC). Atx1p and Ccc2p both bind a single copper ion (Xiao *et al.*, 2004) and the C-terminal of Ctr1p, a plasma membrane copper transporter, interacts with Atx1p to provide the copper (Xiao & Wedd, 2002). Copper is transferred from Atx1p to the first N-terminal Atx1p-like domain in ScCcc2p (Huffman & O'Halloran, 2000), during this transfer the proteins physically interact electrostatically with hydrogen bonds stabilizing the complex (Arnesano *et al.*, 2001; Arnesano *et al.*, 2004). The MTCXXC domains are required for this interaction (Pufahl *et al.*, 1997) and Atx1p preferentially interacts with both of the domains in ScCcc2p (van Dongen *et al.*, 2004). This is possible because the Atx1p metal binding loop is flexible (Rosenzweig *et al.*, 1999). ScCcc2p is involved in copper loading of ScFet3p and is found in the Golgi membrane and Atx1p is found free in the cytoplasm. The first N-terminal copper binding motif in ScCcc2p has been characterized as helical and the protein conformation does not appear to change radically upon copper loading (Banci & Presenti, 2000).

Homologs of *ScCCC2* and *ATX1* exist in the genome of *C. albicans* (Van Ho *et al.*, 2002). Deletion of *CaCCC2* abolished high affinity iron transport, but did not affect virulence in the mouse model of systemic infection (Weissman *et al.*, 2002). Virulence may not have been affected by the deletion of *CaCCC2* because there are a number of possible routes of iron uptake including siderophore and haem utilization.

1.8: Regulation of High Affinity Iron Uptake in Fungi:

With the exception of *S. cerevisiae*, most fungi studied to date have iron uptake systems regulated by GATA-type transcription factors. GATA-type transcription factors have one or two zinc finger DNA binding domains (Lowry & Atchley, 2000). Furthermore, fungal GATA-type transcription factors have a cysteine rich region in their centre which is considered a putative iron sensing domain (Haas *et al.*, 1999a). A comparison of these features is shown in figure 1.5. *URBS1* is involved in regulating siderophore production in *Ustilago maydis* (Voisard *et al.*, 1993a). A homolog of *URBS1* has been identified in *Penicillium chrysogenum* that encodes a GATA-type transcription factor (Haas *et al.*, 1997). *SREA* in *Aspergillus nidulans* encodes a protein involved in regulating iron acquisition and siderophore biosynthesis (Haas *et al.*, 1999a, Oberegger *et al.*, 2001a). SRE in *Neurospora crassa* is a GATA-type transcription factor that controls iron transport (Zhou *et al.*, 1998). Fep1p in *S. pombe* regulates high affinity iron uptake and siderophore biosynthesis (Pelletier *et al.*, 2002; Pelletier *et al.*, 2003; Pelletier *et al.*, 2005). A homolog of Fep1p has also been identified and characterized in *Pichia pastoris* (Miele *et al.*, 2007).

S. cerevisiae

In *S. cerevisiae*, the regulation of iron uptake is significantly different from that of the other fungi studied. The iron sensing transcription factor in *S. cerevisiae* is called *ScAFT1* and it regulates transcription of six of the seven ferric reductase genes, *ScCCC2*, *ATX1*, *ScARN1-4*, *ScFET5* and *ScFTH1* (Yamaguchi-Iwai *et al.*, 1996). The amount of ScAft1p present in the cell is relatively constant and although ScAft1p cannot directly bind iron it is thought to sense a signal from the mitochondrial iron-sulphur cluster biosynthesis pathway as an indicator of cellular iron levels (Chen *et al.*, 2004; Rutherford *et al.*, 2005). In low iron

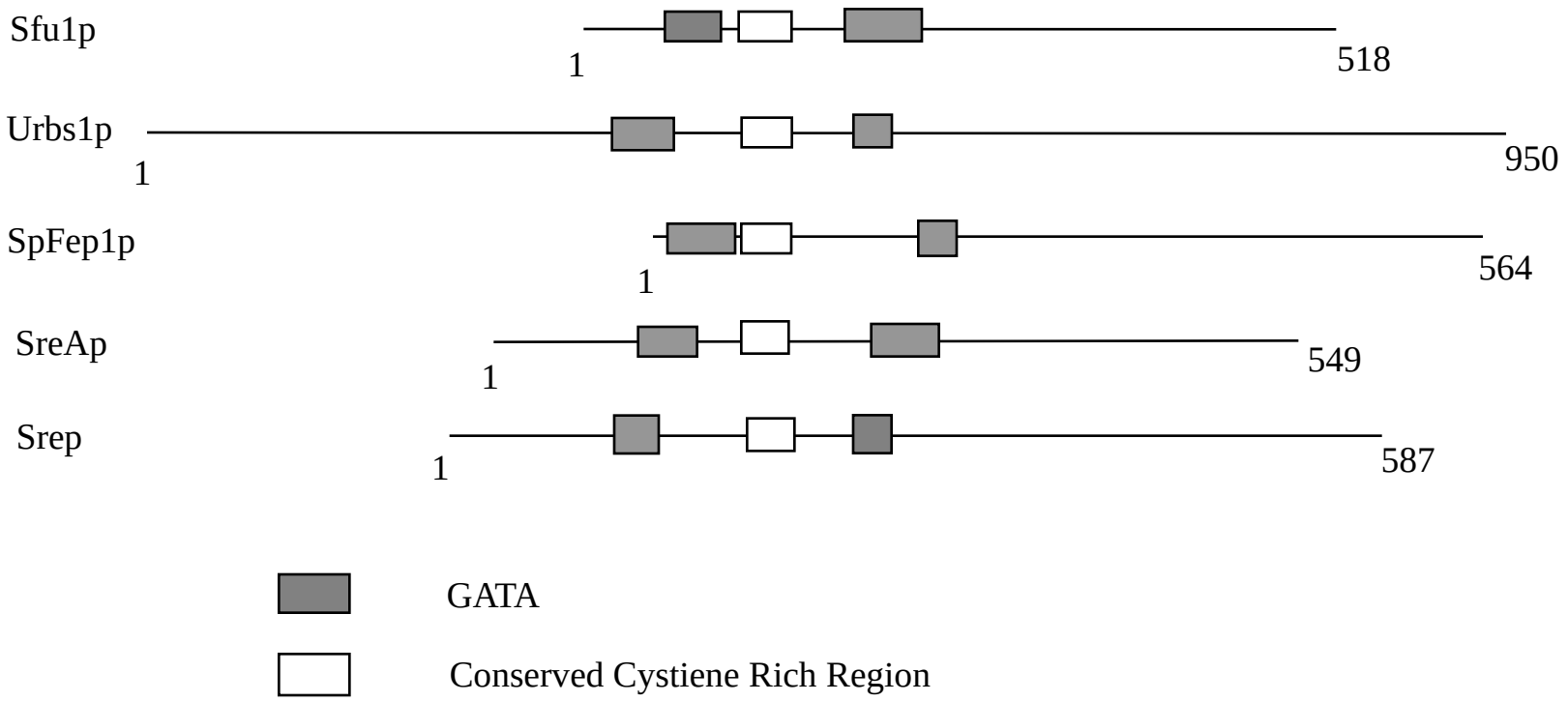


Figure 1.5: Conserved features in the Fungal GATA-type Transcriptional Repressors: In this diagram the conserved cysteine residues are shown as white box sand the GATA DNA binding domains as grey boxes. The black line represents the peptide sequence. Sfu1p is found in the human pathogen *Candida albicans*, Urbs1p is found in the wheat pathogen *Ustillago maytidis*, Fep1p is found in the fission yeast *Schizosaccharomyces pombe*, SreAp is found in the fillamentous fungus *Aspergillus nidulans* and Srep is found in the red bread mold *Neurospora crassa*.

conditions ScAft1p is found in the nucleus of a cell and the nuclear retention of ScAft1p in iron limited conditions ensures transcription of target genes (Yamaguchi-Iwai *et al.,* 2002). In the promoter ScAft1p binds to the consensus sequence (T/C)(G/A)CACCC (Yamaguchi-Iwai *et al.,* 1996). The small, abundant chromatin-associated Nhp6a and Nhp6b proteins and Ssn6p are required for ScAft1p activation of *ScFRE2* and in an *nhp6Δ* mutant transcription of *ScFRE1* was considerably lower (Fragiadakis *et al.,* 2004). Additionally, a paralogue of *ScAFT1*, *ScAFT2* has been identified in *S. cerevisiae* (Blaiseau *et al.,* 2001; Rutherford *et al.,* 2001). An *Scaft1Δ/Scaft2Δ* mutant is unable to grow in low iron conditions and is more sensitive to oxidative stress than *Scaft1Δ* mutants despite the fact that when only *ScAFT2* is knocked out there is no measurable phenotypic difference (Blaiseau *et al.,* 2001). This indicates that *ScAFT2* can regulate the high affinity iron uptake system in *S. cerevisiae* when *ScAFT1* is not present, thus sharing a similar function.

C. albicans

There are two *ScAFT* homologs in *C. albicans*, but they do not appear to have the same function in *C. albicans* as *ScAFT1* has in *S. cerevisiae.* The deletion of *CaAFT1* in *C. albicans* did not produce phenotypes associated with defects in high affinity iron acquisition and although it had a small effect on the expression of *CaFRE10* in response to low iron levels, there was no change in iron replete conditions (Mason, 2006).

Iron responsive regulation has been demonstrated in *C. albicans* in a microarray study. It was found that 526 ORFs were expressed at higher levels in low iron conditions and 626 were expressed at higher levels in high iron conditions. Genes expressed in low iron conditions included components of the high affinity reductive iron uptake system, *HMX1*, which encodes the haem oxygenase, and genes homologous to *S. cerevisiae* genes involved in

transport from the mitochondria to the cytosol. Iron limitation also seems to cause a general stress response with an increase in expression of superoxide dismutase and other stress response genes. Genes expressed in high iron conditions include a number of genes involved in the synthesis of iron containing molecules such as haem (Lan *et al.,* 2004).

As well as proteins directly regulated by the levels of iron, a number of other putative virulence determinants were shown to have increased expression in response to low iron conditions including homologues of Sge1p (a drug export permease in *S. cerevisiae*), adhesins, secreted aspartyl proteases and lipases. Furthermore 11 of the 54 cell surface proteins previously identified as involved in host-pathogen interactions are regulated by iron availability. In addition to this genes involved in the yeast to hyphal transition show increased expression in low iron conditions. The yeast hyphal switch has been implicated in virulence and low iron conditions are a signal that the organism has entered a host (Lan *et al.,* 2004).

SFU1 encodes a GATA type transcriptional repressor identified due to its homology to *URBS1* from *U. maydis*. In a microarray study, Sfu1p has been shown to repress *CaFRE1, CaFRE2, CaFRE5, CaFRE31, CaFET34, CaFET35, CaFTR1,CaFTH1, CaCCC2* and *CaCTR1* in high iron concentrations and induce *CaFTR2* expression in high iron conditions (Lan *et al.,* 2004). Furthermore, in the heterologous *S. pombe* system Sfu1p has been shown to rescue a *fep1Δ* mutant and bind the consensus DNA sequence AGATAA (Pelletier *et al.,* 2007). It was also shown to physically interact with the Tup11 and Tup12 global regulators from *S. pombe*, which are homologous to the *C. albicans* Tup1p, previously shown to be involved in the regulation of *CaFRE10* expression (Pelletier *et al.,* 2007; Knight *et al.,* 2002).

However, Sfu1p is not the only source of iron responsive regulation in *C. albicans*, the *FRP1* gene, encoding a putative ferric reductase was shown to be regulated by iron independently of Sfu1p. The iron responsive regulation of *FRP1* was mediated by achieved by the recruitment of Hap43 to the CBF complex, which binds the CCAAT motif, in iron starvation conditions (Baek *et al.,* 2008). In eukaryotes 3 Hap proteins form heterotrimer with DNA binding capacity at the CCAAT motif, then the addition of a final subunit to the complex (e.g. Hap43p) allows activation of gene expression (Mantovani, 1999). This data indicates that *C. albicans* has both an activator (the CBF complex) and a repressor (Sfu1p) acting to increase expression of genes in low iron conditions.

Ustilago maydis.

The GATA-type transcription factor Urbs1p is encoded by *URBS1* and was identified due to its ability to rescue *U. maydis* mutants with constitutive siderophore production (Voisard, *et al.,* 1993b). Electrophoretic gel mobility shift analysis (EMSA) was used to show that Urbs1p interacts directly with DNA and mutation analysis of a siderophore production (*SID1*) promoter – β-glucuronidase (GUS) fusion identified the consensus binding sequence as G/TGATAA. The *SID1* promoter contained two of these consensus sequences and mutation of one of these sequences lead to a partial deregulation of GUS in the promoter fusion, and deletion of both lead to constitutive expression of GUS (An *et al.,* 1997). Urbs1p has two zinc finger DNA binding domains and mutation analysis of these domains has shown that the C-terminal region is required for *SID1* repression (An *et al.,* 1997).

Aspergillus sp.

The iron sensing GATA-type transcription factor in *A. nidulans* is encoded by *SREA*, which represses the iron uptake and siderophore biosynthesis systems (Haas *et al.,* 1999b;

Oberegger *et al.*, 2002a; Oberegger *et al.*, 2002b). Deletion of *SREA* leads to increase uptake of ^{59}Fe, increased TAFC and fusigen production, deregulation of siderophore bound iron uptake, increased sensitivity to oxidative stress and cellular accumulation of ferricrocin (Haas *et al.*, 1999b; Oberegger *et al.*, 2001a). SreAp is responsible for the regulation of *SIDABC*, three siderophore biosynthesis genes, *MIRABC*, three sidrerophore transporter genes, *SODAB*, two superoxide dismutase genes, *AMCA*, a mitochondrial orthonine carrier and *ATRH*, an ABC transporter (Oberegger *et al.*, 2002a; Haas *et al.*, 2003). However, siderophore biosynthesis and transcription of the gene encoding the ferric reductase *FREA* are still metalloregulated in a *sreAΔ* mutant, indicating that other regulators are involved (Oberegger *et al.*, 2002a; Oberegger *et al.*, 2001b). It has also been demonstrated that in a mutant deficient in *SREA*, transcription of genes encoding proteins which contain iron cofactors; *ACOA*, *LYSF*, and *CYCA*, were higher compared to the wildtype. Furthermore, in both the *sreaΔ* mutant and the wildtype transcription of these three genes was increased in response to iron replete conditions, again suggesting the role of further regulators of these genes besides SreAp (Oberegger *et al.*, 2002b). Expression of *SREA* is increased in low iron conditions. DNA binding of SreAp was shown by *in vitro* gel shift assays to require a single GATA site (Haas *et al.*, 1999b; Oberegger *et al.*, 2001b).

One of the genes with increased transcription in *sreaΔ* is *HAPX*, which is also upregulated in response to low iron in both the wildtype and *sreaΔ* mutant. When *HAPX* is deleted growth in iron limited conditions is impaired and when *HAPX* is knocked out in conjunction with *SREA* the double mutation is lethal. The Hap proteins form a complex that binds the consensus sequence CCAAT. When *HAPC*, a gene encoding a CCAAT- Binding subunit, or *HAPX* in *A. nidulans* is knocked out a number of iron regulated genes are deregulated including *SREA* itself and some genes encoding proteins which contain iron sulfer clusters

and heme subunits - *ACOA*, *LYSF* and *CYCA*. However *SIDA* and *MIRC* are not deregulated when *HAPC* a gene encoding a CCAAT- Binding subunit, or *HAPX* in *A. nidulans* is deleted and, but *SIDA* and *MIRC* are deregulated in a *sreaΔ* mutant. HapXp has been shown to physically interact with other Hap proteins *in vitro* and *in vivo* only under iron limited conditions and the complex binds the CCAAT boxes of *SREA* and *LYSF* (Hortschansky *et al.,* 2007). The putative ortholog of HapX in *C. albicans* was shown to be iron regulated by *SFU1* and repression of aerobic respiration by a Hap complex has been demonstrated (Lan *et al.,* 2004; Hortschansky *et al.,* 2007; Johnson *et al.,* 2005). Analysis of gene expression at different iron concentrations and promoter analysis of iron regulated genes in *Aspergillus fumigatus* suggests a similar *SREA* mediated system of regulation in response to iron availability (Power, *et al.,* 2006).

Neurospora crassa

The GATA-type transcription factor *SRE* has been shown to repress siderophore biosynthesis in the red bread mold *N. crassa*. When *SRE* is knocked out siderophore biosynthesis is increased, but increased transcription in response to low iron conditions is still present (Zhou *et al.,* 1998; Zhou & Marzluf, 1999). It was shown by Gel Electrophoresis Mobility Shift Assays (EMSA) that Srep binds to DNA at GATA sites and mutation of either one of the zinc finger domains weakened binding. Mutation of both abolished binding all together. However, when these mutations were studied *in vivo*, all the mutants showed levels of siderophore biosynthesis comparable to the *sreΔ* mutant (Zhou & Marzluf, 1999). The space between the GATA elements has also been implicated in binding efficiency. Srep binds more efficiently to promoters with 25 bp between the two GATA elements, than to those with 10 bp between the GATA elements. The four cysteine residues found between the two zinc finger elements in Srep have also been shown to have a role in DNA binding affinity.

Mutations in these residues lead to lower affinity DNA binding (Harrison, 2002). Unlike *SREA* in *A. nidulans*, *SRE* is constitutively transcribed (Zhou *et al.*, 1998).

Schizosaccharomyces pombe

In *S. pombe*, Fep1p is the iron sensing transcription factor and it binds to the consensus sequence 5'-(T/A)GATA(A/T)-3.' The Fep1 protein acts as a transcriptional repressor, preventing expression of genes in iron replete conditions (Pelletier *et al.*, 2002; Pelletier *et al.*, 2003). The Fep1 protein is located in the nucleus in both iron replete and iron starved conditions and contains two zinc finger motifs, ZF1 at the N-terminal and ZF2 at the C-terminal. Mutations in the zinc finger binding domains have shown that both ZF1 and ZF2 are necessary for iron sensitive regulation of *Fio1*$^{+}$ *in vivo*. EMSA studies went on to reveal that ZF1 is necessary for DNA binding, and ZF2 enhances that binding ~5-fold *in vitro*. The Fep1 protein also contains a conserved cysteine rich region at position 68-94 which when mutated resulted in loss of *Fio1*$^{+}$ iron sensitive regulation (Pelletier *et al.*, 2005).

The Fep1 protein can only function in the presence of its corepressor Tup11p. Furthermore, the C-terminal of Fep1p and the WD40 repeats of Tup11p (which form a β-propeller structure) have been shown to interact physically (Pelletier *et al.*, 2002; Znaidi *et al.*, 2004). The C-terminal region of Fep1p also dimerizes in baker's yeast in a two-hybrid assay and there is evidence that this also occurs *in vivo* in *S. pombe* using His-tagged Fep1 proteins. Mutations in the putative α-helix found in the C-terminal indicate that the two Fep1 proteins interact by forming a coiled-coil structure (Pelletier *et al.*, 2005).

Similar to the system in *A. nidulans*, *S. pombe* also has a protein complex that binds a CCAAT motif. This protein complex contains Php2p, Php3p and Php5p. Repression of

Pcl1^{+}, a gene involved in iron storage, in iron limited conditions was lost when the -205 to -201 CCAAT motif was mutated. Inactivation of the *Php2*$^{+}$ and *Php4*$^{+}$ genes also resulted in the loss of iron sensitive regulation of *Pcl1*$^{+}$. The genes *Php2*$^{+}$, *Php3*$^{+}$ and *Php5*$^{+}$ are not regulated in response to iron availability; however, *Php4*$^{+}$ transcription was shown to be upregulated by Fep1p in response to low iron conditions (Mercier *et al.,* 2006).

Pichia pastoris

Iron responsive regulation in *P. pastoris* was demonstrated by *LacZ* promotor fusion experiments to be dependent on the AGATAA consensus sequence being present in the promoter of genes regulated by iron levels. As a result a homolog of *S. pombe FEP1* was identified using degenerate primers and designated *PpFEP1*. The product of the gene contained two zinc finger motifs and the conserved cystiene residue that is distinctive to the fungal GATA-type transcriptional repressors. A *Ppfep1Δ* mutant demonstrated deregulation of iron regulated genes and increase of ferric reductase activity. The *PpFEP1* gene itself was not regulated in response to iron, which is also the case for *URBS1* and *S. pombe FEP1*. Again, like the *S. pombe* Fep1p, PpFep1p was shown by EMSA only to bind DNA in the presence of iron (Miele *et al.,* 2007).

1.9: Regulation of High Affinity Copper Uptake in Fungi

S. cerevisiae

S. cerevisiae contains a copper sensing transcription factor called ScMac1p that regulates a number of genes involved in copper and iron acquisition (Van Ho *et al.,* 2002; Marvin *et al.,* 2004). ScMac1p regulated genes are transcribed when copper concentrations are below 1μM and not above this level (Zhu *et al.,* 1998). Copper binding to ScMac1p induces an

intramolecular interaction that inactivates ScMac1p (Jensen *et al.,* 1998). Binding of copper to ScMac1p occurs at the consensus site Cys-X-Cys-X_4-Cys-X-Cys-X_2-Cys-X_2-His. Two of these motifs bind 8 Cu^+ ions (Jensen & Winge, 1998; Keller *et al.,* 2000). In the absence of copper the ScMac1p is activated and binds to DNA at Copper Responsive Elements (CuREs), which have the consensus sequence TTTGCTC (Labbe *et al.,* 1997). In addition, ScMac1p has also been implicated in post transcriptional regulation of ScCtr1p by endocytosis and degradation when copper concentrations exceed 10μM (Ooi *et al.,* 1996; Yonkovich *et al.,* 2002).

The ferric reductase genes *ScFRE1* and *ScFRE7* are regulated by ScMac1p, *ScFRE1* (but not *ScFRE7*) is co-regulated by ScAft1p (Van Ho *et al.,* 2002). Although both *ScCTR1* and *ScCTR3* are both transcriptionally regulated by ScMac1p, ScCtr3p is not post transcriptionally regulated by ScMac1p (Pena *et al.,* 2000). The genes which are transcriptionally regulated by ScMac1p all contain CuREs in their promoters (Labbe *et al.,* 1997; Jensen *et al.,* 1998). The *ScMAC1* gene is transcribed constitutively, but at copper concentrations exceeding 10μM the ScMac1p is degraded to prevent transcription of genes that would take up toxic amounts of copper into the cell (Yonkovich *et al.,* 2002; Zhu *et al.,* 1998).

C. albicans

Our laboratory has identified a homolog of *ScMAC1* in *C. albicans* that regulates *CaCTR1* in response to low copper conditions (Marvin *et al.,* 2004). This regulation of *CaCTR1* has been shown to be dependent on three putative CuREs found upstream of the gene at -397,-275 and -273 of the start codon. Mutation of all three of the CuRE elements in the *CaCTR1* promoter was found to abolish copper sensitive regulation of the *lacZ* reporter. Mutation of only the -

397 bp upstream CuRE resulted in a decrease of expression of the *lacZ* reporter, but up regulation in response to low copper was still present. When the -275 bp upstream CuRE was mutated there was a 40% decrease in the *lacZ* reporter expression in copper replete conditions, but there was no significant difference to wildtype in low copper conditions. Mutation of the -237 bp upstream CuRE had no significant effect on expression of the *lacZ* reporter. Furthermore, in a *Camac1Δ/Camac1Δ* mutant copper sensitive regulation of the wildtype *CaCTR1* promoter was not observed. All this supports the theory that CaMac1p is responsible for copper sensitive regulation of *CaCTR1* via the CuRE promoter sequences (Woodacre *et al.,* 2008).

CuRE elements were also found in the promoters of a number of other genes, including the putative ferric reductase *CaFRE7* (Woodacre *et al.,* 2008; Levitin & Whiteway, 2007). The *CaFRE7* promoter contains 2 CuREs and mutation of both of these CuREs resulted in a loss of copper sensitive regulation, mutation of the -177 CuRE resulted in an increase in *lacZ* reporter expression in low copper conditions, but a decrease in expression in copper replete conditions. Mutation of the -132 CuRE resulted in a decrease of reporter expression copper replete conditions, but no significant difference from wildtype expression in low copper conditions. Copper sensitive expression of the reporter under the native *CaFRE7* promoter was not detected in a *Camac1Δ/Camac1Δ* mutant. Again, this supports the theory that CaMac1p in responsible for copper sensitive regulation of genes via the CuRE promoter sequences (Woodacre *et al.,* 2008; Levitin & Whiteway, 2007). However in yeast form *CaFRE7* is also repressed by Tup1p in response to the immune protein prostaglandin E and therefore shows increases expression in response to prostaglandin E in hyphae (Levitin & Whiteway, 2007).

In *S. cerevisiae* the *ScMAC1* gene is constitutively expressed, but using a *lacZ* reporter it has been shown that the *C. albicans CaMAC1* gene is activated in response to low copper conditions and that this copper sensitive regulation is lost in a *Camac1Δ/Camac1Δ* mutant. The copper regulation is also lost with the mutation of the single CuRE found in the *CaMAC1* promoter. This indicates that *CaMAC1* is autoregulated in response to copper (Woodacre *et al.,* 2008).

Other Fungi

Copper sensitive gene regulation in *S. pombe* is achieved by the copper sensing transcription factor Cuf1p, which has been shown to regulate components of both the iron and copper uptake systems (Labbe *et al.,* 1999). While Cuf1p is orthologous to ScMac1p, the protein sequence is similar both to ScMac1p and ScAce1p. The ScAce1protein is involved in regulating the expression of metallothionein genes. In fact a chimeric Cuf1p-ScAce1p can rescue a *Cuf1Δ* mutant (Labbe *et al.,* 1999; Beaudoin *et al.,* 2003; Jungmann *et al.,* 1993). Cuf1p functions using a Crm1-nuclear export system in which Cuf1p is located in the nucleus in low copper conditions, but during the shift to high copper conditions it is transported to the cytoplasm (Beaudoin & Labbe, 2006; Beaudoin & Labbe, 2007).

A homolog of the *S. pombe Cuf1*$^{+}$ has been identified in *C. neoformans.* The *C. neoformans* Cuf1p contains a putative copper binding motif ^{349}C-X-C-X_3-C-X-C-X_2-C-X_2-H (Ory *et al.,* 2004; Waterman *et al.,* 2007). When *Cuf1*$^{+}$ was knocked out of *C. neoformans* growth was deficient in low copper concentrations and the virulence of the organism was reduced in a mouse model, which is consistent with its regulation of the copper uptake gene *Ctr4*$^{+}$ (Waterman *et al.,* 2007).

1.10: Background to Project:

The essential nature and toxicity of iron and copper has lead to the evolution of complex homeostatic control of the levels of these metals within the cells and filaments of all fungi. Parts of these systems have been characterized in many different fungi, but no one system is fully understood. Consequently, this study aims to further characterize the high affinity iron and copper uptake systems of the pathogenic fungus *Candida albicans*. In our laboratory a knockout mutant of the major ferric reductase in *C. albicans*, *CaFRE10* was constructed and tested for cell surface cupric reductase activity. In a wildtype *C. albicans* strain it was demonstrated that cell surface cupric reductase activity increased in response to low iron and low copper conditions. It was found that in the *Cafre10Δ/Cafre10Δ* mutant the iron responsive increase in cell surface cupric reductase activity was not observed, but the copper responsive increase in activity was still detected (Mason, 2006). Furthermore, it has previously been demonstrated that the expression of the putative ferric/cupric reductase *CaFRE7* was increased in low copper conditions (Woodacre *et al.,* 2008; Levitin & Whiteway, 2007). This led to the question, is *CaFRE7* responsible for the copper responsive cell surface cupric reductase activity? A previous microarray study (Lan *et al.*, 2004) had demonstrated that some components of the high affinity iron uptake system in *C. albicans*, including *CaFRE10*, were regulated by Sfu1p, an iron responsive transcriptional repressor; but no functional analysis of this regulation had been undertaken. This led to the question, does *sfu1Δ/sfu1Δ* show phenotypic changes consistent with the role of Sfu1p as an iron sensitive repressor of transcription. All previous work on the high affinity iron and copper uptake systems had been performed in the yeast form of *C. albicans* and this led to the question, is the high affinity iron uptake system the same in hyphae as it is in yeast? From this background information the aims of this project were:

- To characterize the role of *CaFRE7* in cell surface iron and copper reduction.
- To further characterize the role of Sfu1p in iron responsive regulation of the high affinity iron and copper uptake systems in *C. albicans*.
- To investigate the possibility that high affinity iron uptake may be different in hyphae compared to yeast.

Chapter 2: Materials and Methods

2.1: Strain Information:

The *Candida albicans* strains used in this study are shown in table 2.1 and the *E. coli* stains used in this study are shown in table 2.2. The name of the strain is given along with the genotype and a reference where appropriate.

Strain	**Genotype**	**Source/Reference**
SC5314	Isolated from patient with disseminated candidosis.	Gillum *et al.* 1984
CAI-4	Δ*ura3::λimm434/*Δ*ura3::λimm434*	Fonzi & Irwin, 1993
CAF-2	*ura3Δ::λimm434/URA3*	Fonzi & Irwin, 1993
DAY185	As CAI4 but *HIS1::his1::hisG/his1::hisG; ARG4::URA3::arg4::hisG/arg4::hisG*	Davis *et al.* 2000
BWP17	Δ*ura3::λimm434/*Δ*ura3::λimm434; arg4::hisG/arg4::hisG; his1::hisG/his1::hisG*	Wilson *et al.* 1999
CNA6	*ura3Δ::λimm434/ura3Δ::λimm434 his1::hisG/his1/hisG sfu1Δ::URA3/sfu1Δ::HIS1*	Lan *et al.* 2000
MEM-m2	CAI4 but *arg4::hisG/arg4::hisG; his1Δ::hisG/his1Δ::hisG mac1Δ::URA3/mac1Δ::ARG4; his1::hisG/his1::hisG::HIS1*	Marvin *et al.* 2004
MEM-c3	CAI4 but *arg4::hisG/arg4::hisG; his1Δ::hisG/his1Δ::hisG ctr1Δ::URA3/ctr1Δ::ARG4; his1::hisG/his1::hisG::HIS1*	Marvin *et al.* 2003

RM10.5	As CAI4 but *fre10Δ::hisG/fre10Δ::hisGURA3hisG*	Mason, 2006
MEM-m2	As BWP17 but *mac1Δ::URA3/mac1Δ::ARG4; his1::hisG/his1::hisG::HIS1*	Marvin *et al.*, 2004
MEM-c3	As BWP17 but *ctr1Δ::URA3/ctr1Δ::ARG4; his1::hisG/his1::hisG::HIS1*	Marvin *et al.*, 2003
AWP1	BWP17 with *RPS10*::placpoly/*RSP10*	Woodacre *et al.* 2008
AWM1	BWP17 with *RPS10*::pAWM1/*RSP10*	Woodacre *et al.* 2008
RJ1.1	As CAI4 but *FRE7/fre7Δ::hisGURA3hisG*	This Study
RJ1.2	As CAI4 but *FRE7/fre7Δ::hisGURA3hisG*	This Study
RJ1.3	As CAI4 but *FRE7/fre7Δ::hisG*	This Study
RJ1.4	As CAI4 but *fre7Δ::hisG/fre7Δ::hisGURA3hisG*	This Study
RJ1.5	As CAI4 but *fre7Δ::hisG/fre7Δ::hisGURA3hisG*	This Study
RJ1.6	As CAI4 but *fre7Δ::hisG/fre7Δ::hisG*	This Study
RJ1.7	As CAI4 but *fre7Δ::hisG/fre7Δ::hisG*	This Study
RJ1.8	As CAI4 but *fre7Δ::hisG/fre7Δ::hisG/CaRPS10::URA3/CaRPS10*	This Study
RJ1.9	As CAI4 but *fre7Δ::hisG/fre7Δ::hisG/CaRPS10::URA3/CaRPS10*	This Study
RJ1.10	As CAI4 but *fre7Δ::hisG/fre7Δ::hisG/RPS10::URA3::FRE7/CaRPS10*	This Study
RJ2.1	As RM10.5 but *FRE7/fre7Δ::hisGURA3hisG*	This Study

RJ2.3	As RM10.5 but *FRE7/fre7Δ::hisG*	This Study
RJ2.4	As RM10.5 but *fre7Δ::hisG/fre7Δ::hisGURA3hisG*	This Study
RJ2.5	As RM10.5 but *fre7Δ::hisG/fre7Δ::hisGURA3hisG*	This Study
RJ2.6	As RM10.5 but *fre7Δ::hisG/fre7Δ::hisG*	This Study
RJ2.7	As RM10.5 but *fre7Δ::hisG/fre7Δ::hisG*	This Study
RJ2.8	As RM10.5 but *fre7Δ::hisG/fre7Δ::hisG/CaRPS10::URA3/CaRPS10*	This Study
RJ2.9	As RM10.5 but *fre7Δ::hisG/fre7Δ::hisGCa/RPS10::URA3/CaRPS10*	This Study
RJ2.10	As RM10.5 but *fre7Δ::hisG/fre7Δ::hisG/CaRPS10::URA3::FRE7/CaRPS10*	This Study
RJ3	BWP17 with *CaRPS10*::placpoly*SFU1*promoter/*CaRSP10*	This Study
RJ4	MEM-m2 with *CaRPS10*::placpoly*SFU1*promoter/*RSP10*	This Study
RJ5	CNA6 with *CaRPS10*::placpoly*SFU1*promoter/*CaRSP10*	This Study
RJ6	CNA6 with *CaRPS10*::pAWM1/*CaRSP10*	This Study

Table 2.1: *Candida albicans* strains used in this study

Strain	**Genotype**	**Source/Reference**
DH5α	*Φ80lacZΔM15; recA1; endA1; gyrA96; thi-1; hsdR17($r_k^-m_k^+$); supE44; relA1; deoI Δ(lacZYA-argF)U169*	Hanahan, 1983

Table 2.2: *E. coli* strains used in this study

2.2: Plasmid Information:

The plasmids used in this study are shown in table 2.3. The name of the plasmid is given along with the genotype and a reference where appropriate. All plasmids were maintained in *E. coli* DH5α and recovered as described in section 2.4. Maps of the plasmids created in this study and the plasmids that they were derived from can be seen in figures 2.1-3.

Plasmid	**Genotype**	**Source/Reference**
pMB-7	*ori*; *lacz*α^{+}; *amp*^{R}; *hisGCaURA3hisG*	Fonzi & Irwin, 1993
pMB-FRE7	*ori*; *lacz*α^{+}; *amp*^{R}; *hisGCaURA3hisG* with *CaFRE7* disruption cassette.	This Study
CIp10	*Ori; amp*^{R}*;CaRPS10; CaURA3*	Murad *et al.* 2000
CIp10*CaFRE7*	CIp10 with *CaFRE7* ORF	This study
placpoly	*Ori; amp*^{R}*; CaRPS10; CaURA3; lacZ*	Uhl & Johnson, 2001 Brown *et al.*, unpublished
placpoly *SFU1* promoter	placpoly with 959bp *SFU1* promoter	This study
pAWM1	placpoly with 683bp *CaMAC1* promoter	Woodacre *et al.* 2008

Table 2.3: Plasmids used in this study

2.3: Media and Growth Conditions:

E. coli was grown on Luria-Bertani medium (1 % Bacto-tryptone, Oxoid; 0.5 % bacto-yeast extract, Oxoid; 0.5 % Sodium chloride, pH 7.2) at 37ºC. Liquid cultures were aerated by shaking at 200 rpm. When solid media was required 2 % agar was added. Spectrophotometry at OD 600 nm was used to observe the cell titre of liquid culture.

C. albicans was grown in yeast extract peptone dextrose (YPD) medium (1 % yeast extract, Oxoid; 2 % Bacto-peptone, Oxoid; 2 % glucose). When auxotrophic selection was required synthetic dextrose (SD) medium (0.67 % yeast nitrogen base, bioline; 2 % glucose) was used with the addition of appropriate amino acid supplements as described in Table 2.4 (Sherman *et al.* 1986). Growth in differing iron concentrations was achieved using minimal defined (MD) medium (10 % salt and trace solution, see table 2.5; 0.1 % vitamin solution, see table 2.6; 7 mM calcium chloride; 20 mM sodium citrate; pH 4.2; 2 % glucose) with supplements as described in table 2.4 (Sherman *et al.* 1986; Wickerham, 1951; Eide *et al.* 1992). When solid media was required 2 % agar was added to SD and YPD and 4 % agar to MD. Liquid media was aerated by shaking at 200 rpm and all growth was carried out at 30°C for yeast. Hyphae were induced in liquid media by the addition of 20 % serum and growth at 37°C, aerated by shaking at 200 rpm.

2.4: Isolation of Nucleic Acids:

Rapid Isolation of Yeast Chromosomal DNA.

A 10 ml yeast culture was grown overnight in YPD and in the morning centrifuged for 5 minutes at 3000 rpm, the supernatant was discarded and the cells resuspended in 0.5 ml

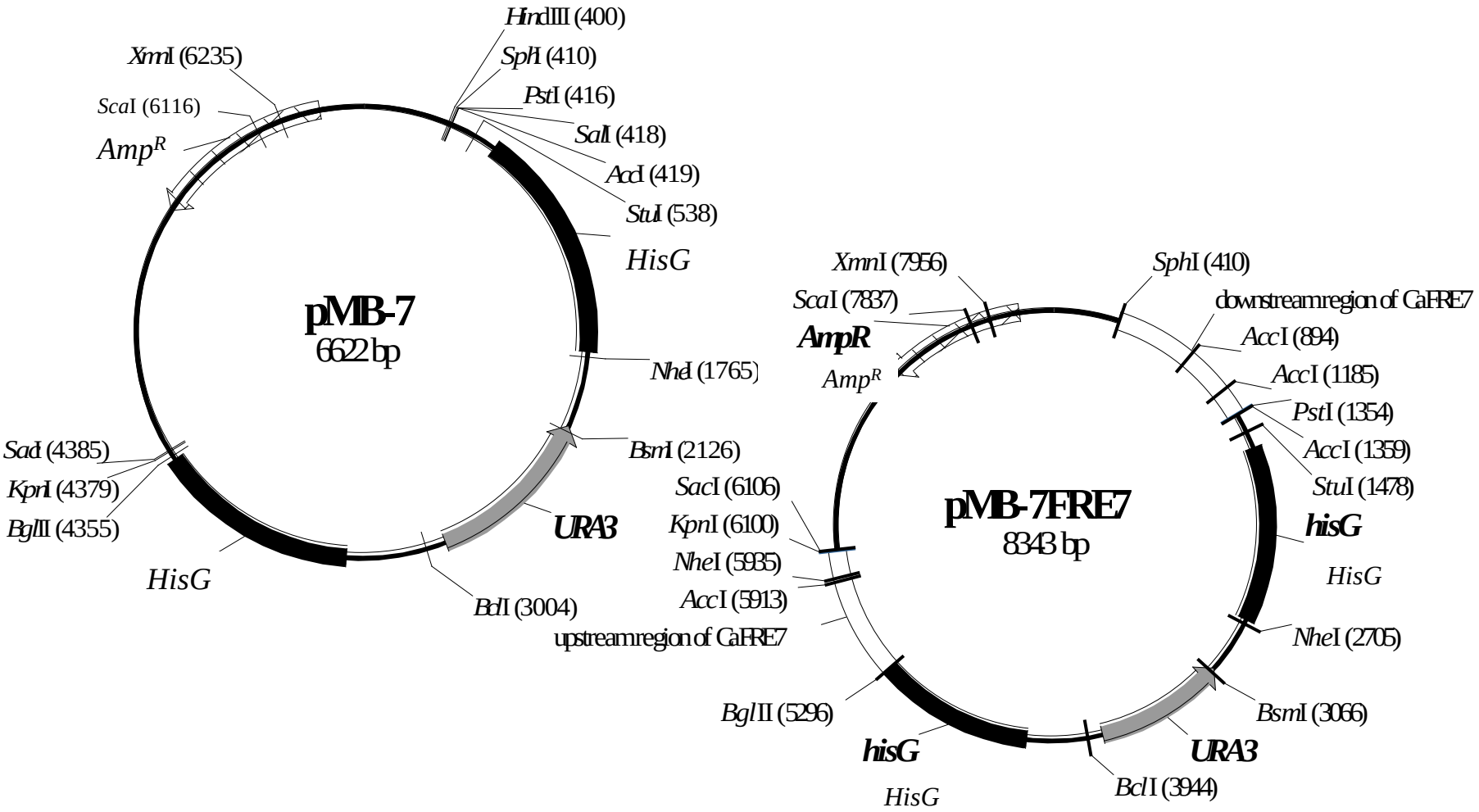
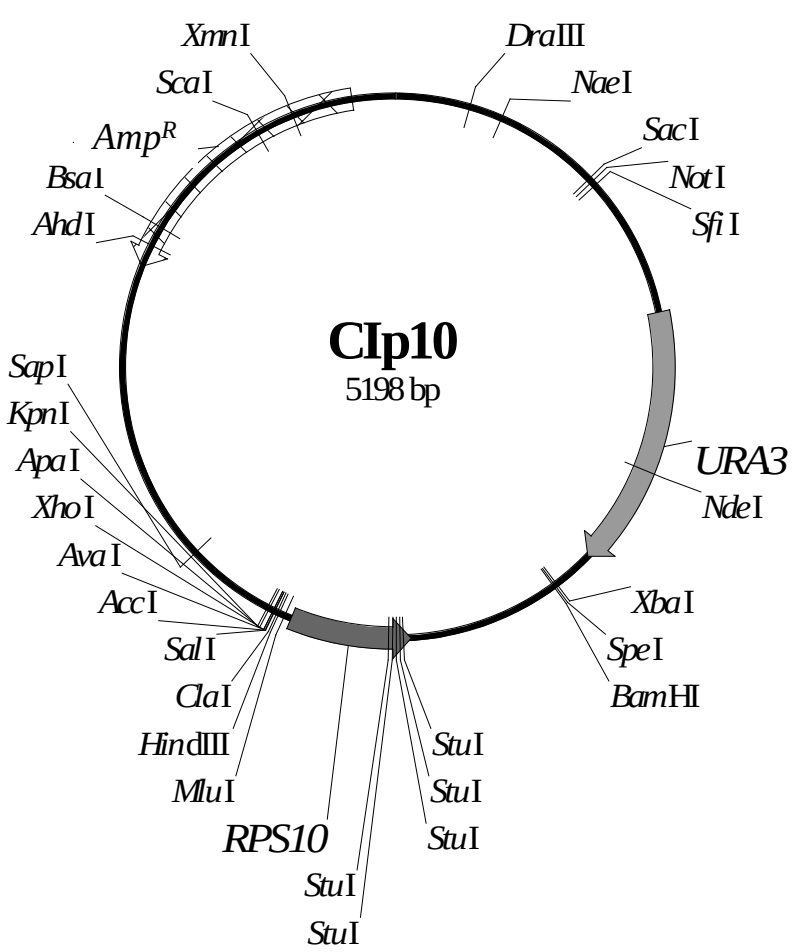


Figure 2.1: 'URA' Blaster Plasmids: pMB-7 contains an *Amp*^{R} gene for selection when cloning in *E. coli* and two *HisG* repeats flanking the *CaURA3* gene, this comprises the 'URA'-blaster cassette. In pMB-7*FRE7* an upstream region of *CaFRE7* has been cloned in the multicloning site one side of the cassette and a downstream region has been cloned into the multicloning site the other side of the cassette.



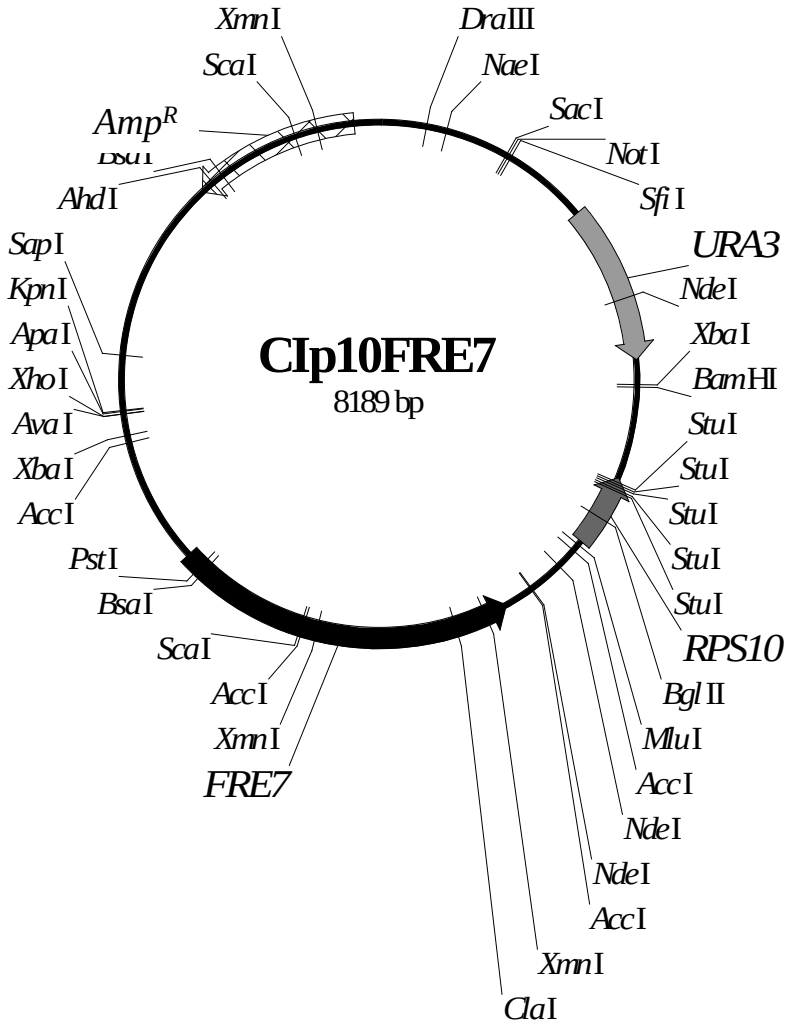


Figure 2.2: Reintroduction of *CaURA3* and *CaFRE7* Compliment Plasmids: CIp10 contains an *Amp*^{R} gene for selection when cloning in *E. coli* and the *CaURA3* gene, it also contains a region of homology to *RPS10* for integration into this locus of the *C. albicans* genome. In CIp10*FRE7* the whole of *CaFRE7* and its promoter have been cloned into CIp10 to reintegrate *CaFRE7* at a different locus to compliment *CaFRE7* deletions.

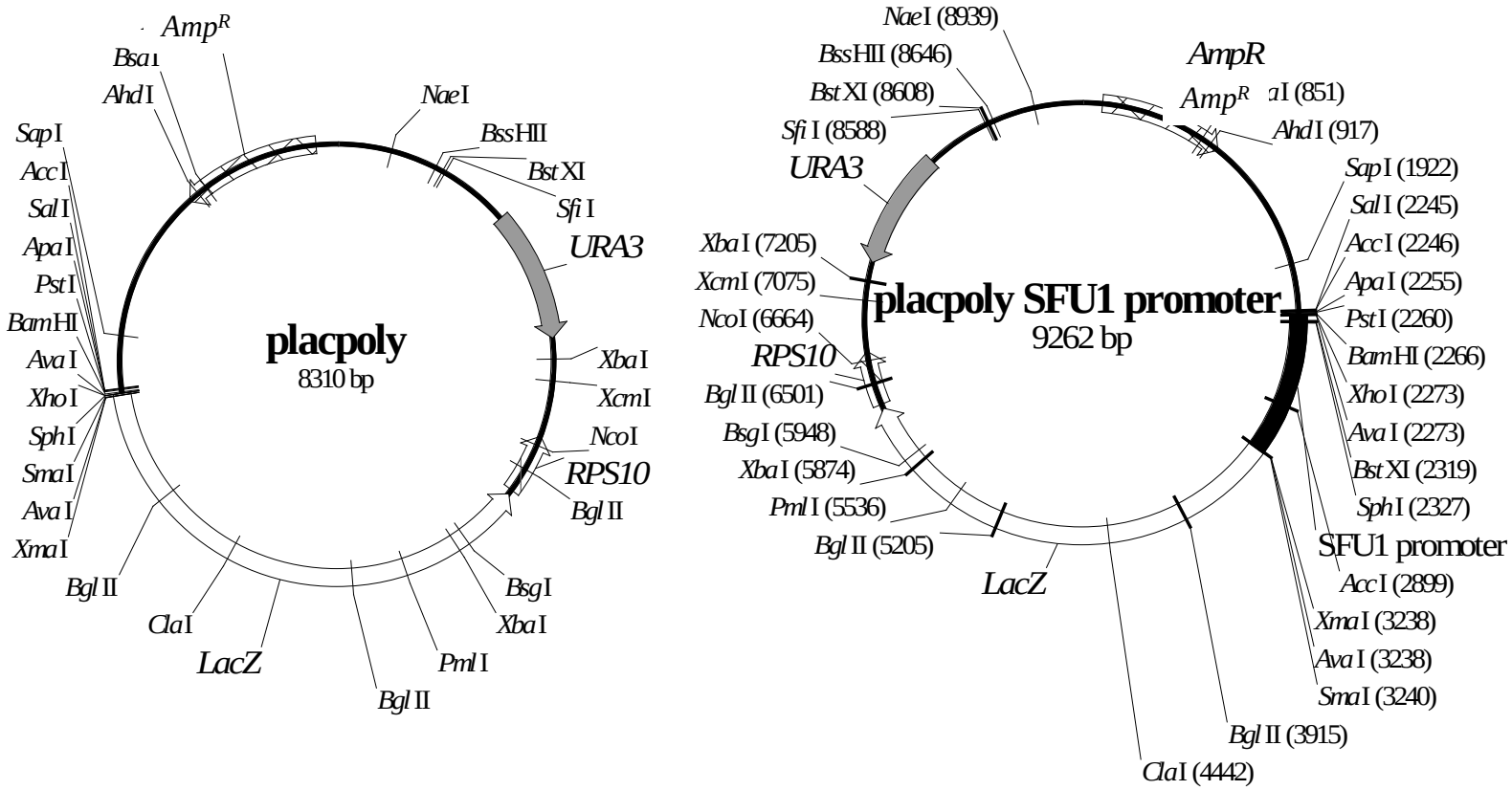


Figure 2.3: β-Galactosidase Reporter Plasmids: placpoly contains an *Amp*^{R} gene for selection when cloning in *E. coli* and a *CaURA3* gene for selection in *C. albicans*, it also contains a region of homology to *RPS10* for integration into this locus of the *C. albicans* genome. *C. albicans* has an alternative codon usage and recognises CTG as serine, so the *lacZ* of *Streptococcous thermophilus* is used because it was found to have a low CTG content and so fewer CTG codons had to be mutated to make the *lacZ* optimal for use in *C. albicans* (Ule & Johnson, 2001). In placpoly *SFU1* promoter, ~1 kb of the *SFU1* promoter has been cloned into placpoly upstream of the *lacZ* gene to create a reporter construct.

Supplement	Concentration in medium
arginine	20 µg/ml^{-1}
histidine	20 µg/ml^{-1}
uridine	50 µg/ml^{-1}
5-Flouroorotic Acid (5-FOA)	1 µg/ml^{-1}

Table 2.4: Amino acid and base supplements

Supplement	Concentration in medium
ammonium sulphate	7.57 mM
potassium dihydrogen orthophosphate	5.02 mM
di-potassium hydrogen orthophosphate	0.92 mM
magnesium sulphate	2.03 mM
sodium chloride	1.71 mM
boric acid	162 nM
potassium iodide	60 nM
zinc sulphate	244 nM
di-potassium hydrogen orthophosphate	0.92 mM
magnesium sulphate	2.03 mM

Table 2.5: Salt and trace solution

Supplement	Concentration in medium
d-biotin	8.19 nM
thiamine hydrochloride	1.19 µM
pyridoxine hydrochloride	1.95 µM
myo-inositol	11 µM
d-pantothenic acid calcium salt	0.84 µM

Table 2.6: Vitamin solution

distilled water. The cells were then centrifuged again at 13000 rpm. The supernatant was discarded and the pellet was disrupted by brief vortexing. Cells were resuspended in 200 µl breaking buffer (2 % v/v TritonX-100, 1 % v/v SDS, 100 mM NaCl) and vortexed for 7 minutes in phenol/chloroform/Isoamyl Alcohol (IAA) with glass beads. 200 µl TE buffer (10mM Tris-Cl, 1mM EDTA) was added and again the sample was vortexed briefly followed by spinning at 13000 rpm for 5 minutes. The aqueous layer was removed to a clean tube and 1 ml 100 % ethanol was added. The tubes were incubated overnight at -20°C to precipitate the nucleic acids. The sample was then centrifuged at 13000 rpm for 25 minutes, the supernatant discarded and the pellet resuspended in 0.4 ml TE buffer. 3 µl of 10 mg/ml DNase free RNase was then added and the sample was incubated at 37°C for 1 hour. 40 µl 3M Sodium Acetate and 1 ml 100 % ethanol was then added and the samples were incubated at -20°C overnight. The sample was then centrifuged for 25 minutes at 13000 rpm and the DNA resuspended in distilled water. The concentration was determined by spectrophotometery and the samples stored at -20°C.

Isolation of plasmid DNA from *E. coli*.

All plasmids were extracted using a QIAprep Spin Miniprep Kit (Qiagen) or using a E.Z.N.A.® Plasmid Mini kit (Omega) following the manufacturer's instructions.

***C. albicans* RNA Extraction (adapted from Schmitt *et al.* 1990).**

Cells were grown in 50 ml cultures to log phase. The cells were harvested by centrifugation at 3000 rpm for 5 minutes and resuspended in 400 µl AE buffer (50 mM Sodium Acetate pH 5.3, 10 mM EDTA). To disrupt the cells 40 µl of 10 % SDS was added and the cells were vortexed for 30 seconds, 400 µl of phenol was then added and

a further vortex for 30 seconds. The cells were then incubated at 65°C for 4 minutes, and then rapidly frozen in a dry ice ethanol bath for 3 minutes; this was repeated 3-4 times with a final incubation at 65°C for 4 minutes. Cell debris was then removed by centrifugation and the proteinacious matter was removed from the aqueous phase by phenol/chloroform/IAA precipitation. The RNA was then precipitated by adding ethanol and incubating overnight at -20°C. The RNA was pelleted by centrifugation for 15 minutes at 13000 rpm, washed with 80 % ethanol, allowed to air dry and resuspended in 100 µl DEPC treated water. The concentration of the RNA was determined by Spectrophotometery at 260 nm.

2.5: Manipulation of Nucleic Acids:

Restriction digestion of DNA.

All enzymes used were supplied by New England Biolabs Ltd and digests were performed using the buffers supplied with the enzymes.

Ligation of DNA.

Following vector digestion phosphate groups were removed from the sticky ends of the molecule to prevent the vector re-ligating using Antarctic phosphotase (New England Biolabs Ltd) as per the manufacturer's instructions. The phosphotase was inactivated by incubation at 65°C for 30 minutes.

Ligations were typically performed with a insert:vector ratio of 3:1 (Dugaiczyk, *et al.* 1975). The vector and insert were diluted in a total volume of 20 µl distilled H_2O and denatured at 65°C for 5 minutes then cooled on ice for 5 minutes. Reactions were then

carried out using T4 DNA ligase from New England Biolabs Ltd using the buffer supplied following the manufacturer's instructions. The reactions were incubated overnight at 16°C.

Polymerase Chain Reaction (PCR).

PCR reactions were made up to a total volume of 20-50 µl with distilled H_2O and contained 50-100 ng template DNA, 1 pmol forward primer, 1 pmol reverse primer, 11.1x buffer (450 mM Tris-HCl, pH 8.8; 110 mM ammonium sulphate; 45 mM magnesium chloride; 67 mM β-mercaptoethanol; 44 µM EDTA, pH 8.0; 10 mM dATP; 10 mM dGTP; 10 mM dTTP; 10 mM dCTP; 1.13 $mg.ml^{-1}$ BSA) and a DNA polymerase. Normally *Taq* DNA polymerase (ABgene or KAPA biosystems) was used, however when a high fidelity polymerase was required Bio-X-Act (Bioline) was used instead. Primers used were purchased from Sigma, Invitrogen or the University of Leicester's Protein and Nucleic Acid Chemistry Laboratory (PNACL) and the sequences are shown in table 2.7. The temperature cycles of each enzyme pair is given in table 2.8. When purification of the PCR product was required it was either purified by agarose gel electrophoresis followed by extraction of DNA from the gel (see below) or a QIAquick PCR Purification Kit (Qiagen) was used according to the manufacturer's instructions.

Agarose gels for DNA separation.

LE agarose (Amersham) was dissolved in 1xTris Acetate EDTA (TAE) buffer and 0.5 $\mu g.ml^{-1}$ Ethidium bromide was added to the gel prior to it setting. Loading buffer (15 % (w/v) Ficoll type 400; 0.06 % (w/v) bromophenol blue, 0.06 % (w/v) xylene cyanol FF, 30 mM EDTA) was added to DNA samples at a 1/6 dilution. The gels were then

Primer Name	Sequence 5’ 3’	Target Site (relative to start codon)
CaACT1 probe F	GGTAGACCAAGACATCAAGG	*CaACT1* +61 to +80
CaACT1 probe R	GAACCACCAATCCAGACAGAG	*CaACT1* +979 to +966
CaFRE5 probe F	TCCTTGTATTATGGAGCAGG	*CaFRE5* +469 to +488
CaFRE5 probe R	ATAAATGCATGACCACCGGG	*CaFRE5* +1379 to +1360
CaFRE10 probe F	ATAACGGTAAAGGTATTGGC	*CaFRE10* +985 to +1005
CaFRE10 probe R	GTTTGAGCAGGTTGTTGAGCC	*CaFRE10* +1529 to +1508
CaFRE7 probe F	GCCTACATGAAGTACAAGC	*CaFRE7* +61 to +79
CaFRE7 probe R	GGACCACGTGAATGACTG	*CaFRE7* +607 to +590
CaFTR1 probe F	TACTTTGACAGAAACACCAA	*CaFTR1* -42 to -22
CaFTR1 Probe R	TTGGTTTCGAAATACCAAAT	*CaFTR1* +680 to +700
*CaFRE7KOKpn*I	GATCGGTACCCCAAATGCCAAATAAGCCTC	*CaFRE7* -829 to -810
*CaFRE7KOBgl*II	CTAGAGATCTGACAGTTATGAGTCGCTTAC	*CaFRE7* -57 to -38
*CaFRE7KOPst*I	GATCCTGCAGCTGAGTCTTTTGGA	*CaFRE7* +1742 to

	TGGTAA	+1761
*CaFRE7KOSph*I	CTAGGCATGCTTGGTAATTTGCCATTCTCG	*CaFRE7* +2658 to +2677
*CaFRE7CXho*I	GATCCTCGAGGATTTCTTGCATTGTTTCGG	*CaFRE7* -796 to -777
*CaFRE7CMlu*I	GATCACGCGTACTGTCATTTTCGACAGTTG	*CaFRE7* +2035 to +2055
CaFRE7 -1315 F	GAACTACTCAATCCACTGGC	*CaFRE7* -1315 to -1296
RP10F	CCAGCTCTCACAGATACTC GAGTATCTGTGAGAGCTGG	*RPS10* -136 to -117
URA3R	GGTGATGGATTAGGACAAC	*CaURA3* +640 to +658
Orf19.7076 F	GCTTCTGTCGCCATAACTAG	Orf19.7076 +556 to +575
Orf19.7076 R	CGTTCAAGAGGTATGGCTAC	Orf19.7076 -12 to +8
*SFU1 Xho*I F	CTCGAGGTTAAAATTCTCATACTTTTATG	*SFU1* -3 to -26
*SFU1 Xma*I R	CCCGGGTATATTAAATGATTGTTATAGCG	*SFU1* -939 to -962
LacZ F	CGAGGCTTCAAATTCTGAAC	LacZ +2081 to +2101

Table 2.7: Primers used in this study: Incorporated restriction enzyme sites are underlined.

Primers	Initial Denaturing	Denaturing	Annealing	Extension	Final Extension	Cycles
CaFRE7KOKpnI *CaFRE7KOBglII*	95°C 5 minutes	95°C 1 minute	56.3°C 45 seconds	72°C 1 minutes	72°C 5 minutes	30
CaFRE7KOPstI *CaFRE7KOSphI*	93°C 5 minutes	93°C 30 seconds	55°C 30 seconds	72°C 1 minutes	72°C 5 minutes	30
*CaFRE7CXho*I *CaFRE7CMlu*I	95°C 4 minutes	95°C 30 seconds	51.2°C 30 seconds	70°C 3 minutes	72°C 5 minutes	35
CaFRE7 -1315 F *URA3* R	94°C 5 minutes	94°C 1 minute	55°C 30 seconds	72°C 3 minutes	72°C 5 minutes	30
RP10F *URA3R*	94°C 5 minutes	94°C 1 minute	55°C 30 seconds	72°C 1.5 minutes	72°C 5 minutes	30
*SFU1 Xho*I F *SFU1 Xma*I R	95°C 4 minutes	95°C 30 seconds	59°C 30 seconds	70°C 1 minutes	72°C 5 minutes	30
CaACT1F *CaACT1R*	94°C 5 minutes	94°C 1 minute	55°C 30 seconds	72°C 1.5 minutes	72°C 5 minutes	30
CaFRE5F *CaFRE5R*	94°C 5 minutes	94°C 1 minute	55°C 30 seconds	72°C 1 minutes	72°C 5 minutes	30
CaFRE7F *CaFRE7R*	94°C 5 minutes	94°C 1 minute	55°C 30 seconds	72°C 1.15 minutes	72°C 5 minutes	30

CaFRE10F *CaFRE10R*	94°C 5 minutes	94°C 1 minute	55°C 30 seconds	72°C 1 minutes	72°C 5 minutes	30
CaFTR1 F *CaFTR1* R	95°C 5 minutes	95°C 30 seconds	48.1°C 30 seconds	72°C 1 minutes	72°C 10 minutes	30
Orf19.7076 F Orf19.7076 R	95°C 5 minutes	95°C 1 minute	48.6°C 1 minute	72°C 1 minutes	72°C 10 minutes	35

Table 2.8: PCR cycles. The primers for a PCR reaction have differing annealing temperatures based on their sequence. The extension time in a PCR reaction is dependent on the size of the sequence being amplified. The temperature and duration of each step of the PCR cycle is given. The cycles refer to how many times the denaturing, annealing and extension steps were repeated.

placed in 1x TAE running buffer and samples were then loaded onto the gels. 60-100 volts was passed through the buffer for 30-90 minutes.

Recovery of DNA from gels when required was achieved by excision of the band using a scalpel blade and DNA was subsequently extracted using either a QIAquick Gel Extraction Kit (Qiagen) or a Zymoclean™ Gel DNA Recovery Kit (Zymo Research) as per the manufacturer's instructions.

Denaturing gels for RNA separation.

Gels containing 1.5 % LE agarose (Amersham), 1 % MOPS and 0.8 % formaldehyde were prepared. The RNA samples were denatured in 10 µl deionised formamide, 3.5 µl formaldehyde (37 % w/v) and 2 µl 10xMOPS by heating at 65ºC for 10 minutes. 1 µl 1 mg/ml ethidium bromide and 3 µl loading buffer (15 % (w/v) Ficoll type 400; 0.06 % (w/v) bromophenol blue, 0.06 % (w/v) xylene cyanol FF, 30 mM EDTA) was added to each sample and the samples were loaded onto the gel. Gels were run at 90-100 volts for 2-3 hours.

DNA sequencing.

DNA sequencing was achieved using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Reactions contained 4 µl BigDye (previously diluted 1/8 in sequencing buffer, Applied Biosystems) and 2 pmol primer. 10-500 ng template DNA was used depending on the template size and source. The reactions were made up to 10 µl with distilled water. Samples were then incubated at 30 cycles of 94°C for 0.3 minutes, 96°C for 0.1 minutes, 50°C for 0.05 minutes and 60°C for 0.4 minutes. Performa® Gel Filtration Cartridges (Edge Biosystems) were used to purify the

reactions and these were analysed by an Applied Biosystems 3730 sequencer at the University of Leicester's PNACL.

2.6: Transfer of Nucleic Acids from Gels to Nylon Membrane:

Northern Transfer of RNA.

The RNA was transferred onto a nylon membrane (Hybond-N+, Amersham) using a gradient of SSC. This was achieved by placing the gel on 3MM paper soaked in 20x SSC and resting in a tank of 20x SSC to act as a wick. The nylon membrane was then soaked in 6x SSC and placed over the gel. 2 sheets of gel sized 3MM soaked in 6x SSC were then placed over the nylon membrane. A stack of paper towels was then placed on top of the 3MM and weighted down to ensure even coverage. This was left overnight to allow the RNA to be transferred to the membrane. After transfer the nylon membrane was dried on a piece of 3MM and the RNA was crosslinked to the filter using a UV crosslinker (Amersham life sciences) at 70 x 10^3 μjoules/cm^2.

Southern Transfer of DNA (Southern, 1975; Wahl *et al.* 1979).

5-10 μg DNA was digested with the appropriate restriction enzyme and run on an agarose gel for 4-5 hours at 60 volts. The gel was then washed for 20 minutes in depurinating solution (0.25 M hydrochloric acid), 20 minutes in denaturing solution (0.5 M sodium hydroxide; 1 M sodium chloride) and finally 20 minutes in neutralising solution (0.5 M Tris-HCl, pH 7.4), all with constant agitation. The gel was rinsed with distilled water and the DNA was transferred onto a nylon membrane (Hybond-N+, Amersham) using a gradient of SSC as described for the Northern blot. After transfer

the nylon membrane was dried on a piece of 3MM and the DNA was crosslinked to the filter using a UV crosslinker (Amersham life sciences) at 70 x 10^3 µjoules/cm^2.

2.7: Radioactive Labelling, Filter Hybridisation and Probe Detection (adapted from Feinberg & Vogelstein, 1984):

[α-32P]CTP and unlabelled ATP, TTP and GTP were used in a reaction using the Klenow fragment of DNA polymerase I with random hexamer primers to label probe DNA. Probes were constructed by PCR from the wildtype genome or by digestion of a plasmid containing the appropriate DNA followed by subsequent PCR or gel purification. Unlabelled probe DNA was diluted to 30 ng in 16 µl distilled water, boiled at 95-100ºC for 5 minutes, and then cooled on ice for 5 minutes. The DNA was then mixed with 5 µl oligolabelling buffer (240 mM Tris-HCl pH 8.0, 75 mM 2-mercaptoethanol, 0.1 mM dATP, 0.1 mM dGTP, 0.1 mM dTTP, 1 M HEPES pH 6.6, 0.1 mg/ml hexadeoxyribonucleotides 2.1 OD units/ml), 1 µl purified 200 µg/µl BSA (New England Biolabs) and 1µl Klenow fragment of DNA *pol I* (New England Biolabs). 2.5 µl of [α-32P]CTP was then added and the reaction was incubated at 37ºC for 1-2 hours. The probe was then purified using a Sephadex™ G-50 column (Amersham Biosciences), boiled on a heating block for 10 minutes, and then cooled on ice for ten minutes. The radiolabelled probe was then added to a hybridisation chamber containing the blot prehybridised in Church Gilberts Buffer (0.5M Na_2HPO_4/NaH_2PO_4 pH 7.4, 7 % SDS, 1mM EDTA). The probe was allowed to hybridise to the blot overnight. Blots were rinsed in 3xSSC with 0.1 % SDS. Blots were then washed in three 30 minute washes at 65ºC followed by 1-2 hour wash at 65ºC in the same buffer. Blots were wrapped in cling film and exposed for 1 hour to two weeks at -80ºC or at

room temperature depending on the level of radioactivity of the blot Blots were then stripped of probe using two 15-20 minute washes in boiling 0.1 % SDS and reprobed using the control probe for the highly constitutively expressed gene *CaACT1*.

2.8: Transformation of DNA to Cells:

***C. albicans* lithium acetate transformations (adapted from Wilson *et al.* 1999).**

Cells were grown overnight in 5 ml YPD with uridine and this culture was used to inoculate a 50 ml culture of YPD to an optical density of 0.168. The cells were then grown to log phase (approximate optical density 0.8) and centrifuged for 5 mins at 4000 rpm at room temperature. Cells were washed in 10 ml sterile distilled water and resuspended in 0.5 ml TeLiAc (0.1M lithium acetate; 1xTris). 10 mg/ml ssDNA was incubated at 65°C for 10 minutes, then cooled on ice for 10mins. 5 µl of ssDNA was then added to an 1.5 ml tube with 1-5 µg of transforming DNA. 0.1 ml of cells was then added to the tube and it was incubated at room temperature for 30 minutes. 0.7 ml of PLATE mix (0.1M lithium acetate; 1xTris; 40 % polyethylene glycol MW 3350) was added to each tube and the tubes were incubated overnight at room temperature. Cells were then heat shocked at 44°C for 15 minutes and then collected by centrifugation at 3000 rpm at room temperature for 3 minutes. The pellet was suspended in 0.2 ml sterile distilled water and 0.1 ml of the suspension was plated out onto each of two selective plates. The plates were then grown for 3-5 days at 30°C.

E. coli calcium chloride transformations (Mandel & Higa, 1970).

Cells were grown in 5 ml LB broth overnight and diluted 1/100 in warm LB broth to a volume of 50 ml and grown to an OD of 0.4-0.6. The culture was then put on ice for

10 minutes followed by pelleting the cells in a centrifuge at 4000 rpm at 4°C for 5 minutes. The supernatant was discarded and cells washed in 25 ml ice cold 100 mM $CaCl_2$. Cells were then pelleted once more at 4000 rpm at 4°C for 5 minutes in a centrifuge. Again, the supernatant was discarded and cells washed in 12.5 ml ice cold 100 mM $CaCl_2$. The cells were then incubated on ice for 30-60 minutes, followed by a final pelleting of the cells at 4000 rpm at 4°C for 5 minutes in a centrifuge. Cells were then resuspended in 0.625 ml ice cold 100 mM $CaCl_2$ and transformed immediately or stored at -80°C in 50 % glycerol.

2.9: Ferric and Cupric Reductase Assays:

Cells were grown to mid exponential phase, harvested, washed twice in ice-cold distilled water and resuspended in ice cold assay buffer (5 % Glucose, 50 mM sodium citrate pH 6.5). Cells were then incubated for a further 10 minutes at 30°C prior to the addition of reductase buffer (5 % Glucose, 50 mM sodium citrate pH 6.5, 1 mM $FeCl_3$/$CuCl_2$, 1 mM BPS/BCS). Cells were incubated for a further 5 minutes, and the OD of the solution determined by spectrophotometry at OD 520 nm for ferric reductase assays and OD 480 nm for cupric reductase assays. Calibration curves of known amounts of $FeCl_2$/CuCl were constructed to ascertain the amount (mM) of ferrous/cuprous ions produced by reduction at the cell surface.

2.10: Radioactive Iron Uptake Assays:

Cells were grown to mid log phase in YPD then harvested by centrifugation and resuspended in assay buffer (5 % glucose, 10mM sodium citrate pH 6.5) to $1x10^7$

cells/ml. 1 μM $^{55}FeCl_3$ was added and the cells were incubated at 0°C and 30°C for 30-90 minutes. Cells were then harvested by vacuum manifold onto glass filters (Whatman GF/C 25mm) and washed three times with EDTA, pH 6.5 and three times with water. Filters were air dried and 3ml scintillation fluid was added, the β emissions were then counted in a scintillation counter.

2.11: β-galactosidase Assays (Rupp, 2002):

Cells were grown up in 5ml cultures to an approximate concentration of $1x10^7$ cells/ml. They were then harvested by centrifugation at 4000 rpm for 5 minutes, washed twice in 1 ml distilled H_2O and resuspended in 1 ml Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM potassium chloride, 0.1 mM magnesium chloride, 50 mM β-mercaptoethanol). The optical density (OD) at 600nm of 200 μl of the culture in Z-buffer was measured in assays with yeast or a wet weight of 200 μl culture measured in assays with hyphae (and converted to g/l of culture). 60 μl chloroform and 40 μl 0.1 % SDS was added to the remaining 800 μl of culture and this was vortexed for 10 seconds to permeabalise the cells. The cells along with a Z buffer only control were then incubated for 30°C for 5 minutes. 200 μl ONPG (4mg/ml^{-1} in Z buffer) was then added to start the reaction and the time was recorded. Samples were incubated for 30 minutes and then the reaction was stopped by the addition of 400 μl 1M sodium carbonate and the time recorded again. Samples were centrifuged at 13000 rpm for 5 minutes and the absorbance of 1 ml supernatant was measured at OD_{420} and OD_{550}. Activity was calculated using this equation:

Activity = OD_{420} – (reaction volume x OD_{550}) / (OD_{600} or g/lx culture volume x time)

Reaction volume = 1.5 ml

Culture volume = 0.8 ml

The OD_{550} measurement is for the light scattered by cell debris and this is corrected for in the equation. OD_{600} is the cell concentration taken at the beginning of the experiment for yeast or a g/l weight for hyphae and OD_{420} is the yellow colour produced by the degradation of ONPG (Rupp, 2002).

Chapter 3: The Role of *CaFRE7* in High Affinity Iron Uptake.

3.1: Introduction:

Ferric and cupric reductase proteins reduce iron and copper to their more soluble ferrous and cuprous forms, which can then be transported into the cell (Van Ho *et al.* 2002). In *Candida albicans* the major cell surface ferric and cupric reductase is CaFre10p which is responsible for 70% of cell surface ferric reductase activity (Knight *et al.* 2005; Mason, 2006). The expression of *CaFRE10* is regulated in response to the concentration of iron in the growth medium. However, the expression of *CaFRE10* is not regulated in response to the levels of copper in the growth medium (Knight *et al.* 2005; Mason, 2006). Cell surface cupric reductase activity in *C. albicans* has been shown to increase in response to both low iron and low copper conditions (Mason, 2006). Deletion of *CaFRE10* resulted in an approximately 50% decrease in cell surface cupric reductase activity. Furthermore, the increase in cupric reductase activity in response to low iron conditions was not observed in a *Cafre10Δ/Cafre10Δ* mutant, but the cupric reductase activity was still increased in response to low copper conditions (Mason, 2006).

In the model organism *Saccharomyces cerevisiae*, *ScFRE7* is regulated by the copper content of the growth medium, but not by the iron content of the growth medium (Bakel *et al.* 2005; Georgatsou & Alexandraki, 1999; Martins *et al.*1998). Overexpression of *ScFRE7* can rescue an Sc*fre1Δ/Scfre2Δ* strain, indicating a role in cell surface ferric reductase activity (Georgatsou & Alexandraki, 1999; Martins *et al.*1998; Labbe *et al.* 1997;

Rees & Thiele, 2007). In *C. albicans* a homolog of *ScFRE7* exists (*CaFRE7*) and has been shown to be activated at the transcriptional level in response to low copper conditions by CaMac1p (Levitin & Whiteway, 2007; Woodacre *et al.* 2008). To investigate the possibility that *CaFRE7* encodes a cell surface ferric and cupric reductase that is regulated by copper, two *Cafre7Δ/Cafre7Δ* mutants were constructed; one in the wild-type CAI-4 background and another in the *Cafre10Δ/Cafre10Δ* mutant background. To make the mutants, a 'URA' blaster gene disruption strategy was employed (Fonzi & Irwin, 1993).

The 'URA' blaster gene disruption method (figure 3.1) was developed by Fonzi and Irwin (1993), the flanking regions of the gene of interest are amplified by PCR and cloned into plasmid pMB7 (see materials and methods table 2.3) either side of *hisG* repeats which flank the *URA3* gene. The completed cassette can then be excised from pMB-7 and transformed into a uridine auxotrophic mutant of *C. albicans.* Recombinants are selected using synthetic dextrose (SD) media without any amino acid supplements. The culture is then streaked onto SD media containing uridine and 5-Fluoroorotic Acid (5-FOA).

The 5-FOA selects for *ura*$^{-}$ strains created by recombination between the *hisG* repeats either side of the *URA3* gene in the disruption cassette. In the presence of *URA3* in the pyrimidine pathway 5-FOA is converted to fluoroorotidine monophosphate, which is followed by decarboxlation to form 5- fluoroorotidine monophosphate. The inhibition of the growth is thought to be due to the production of fluorodexoyuridine from the 5-FOA synthetic substrate, which inhibits thymidylate synthetase (Boeke *et al.* 1984; Rode & Les, 1996). A second cassette is then transformed into the mutant with a single allele disrupted. The transformants produced from this second round of transformations have to be carefully

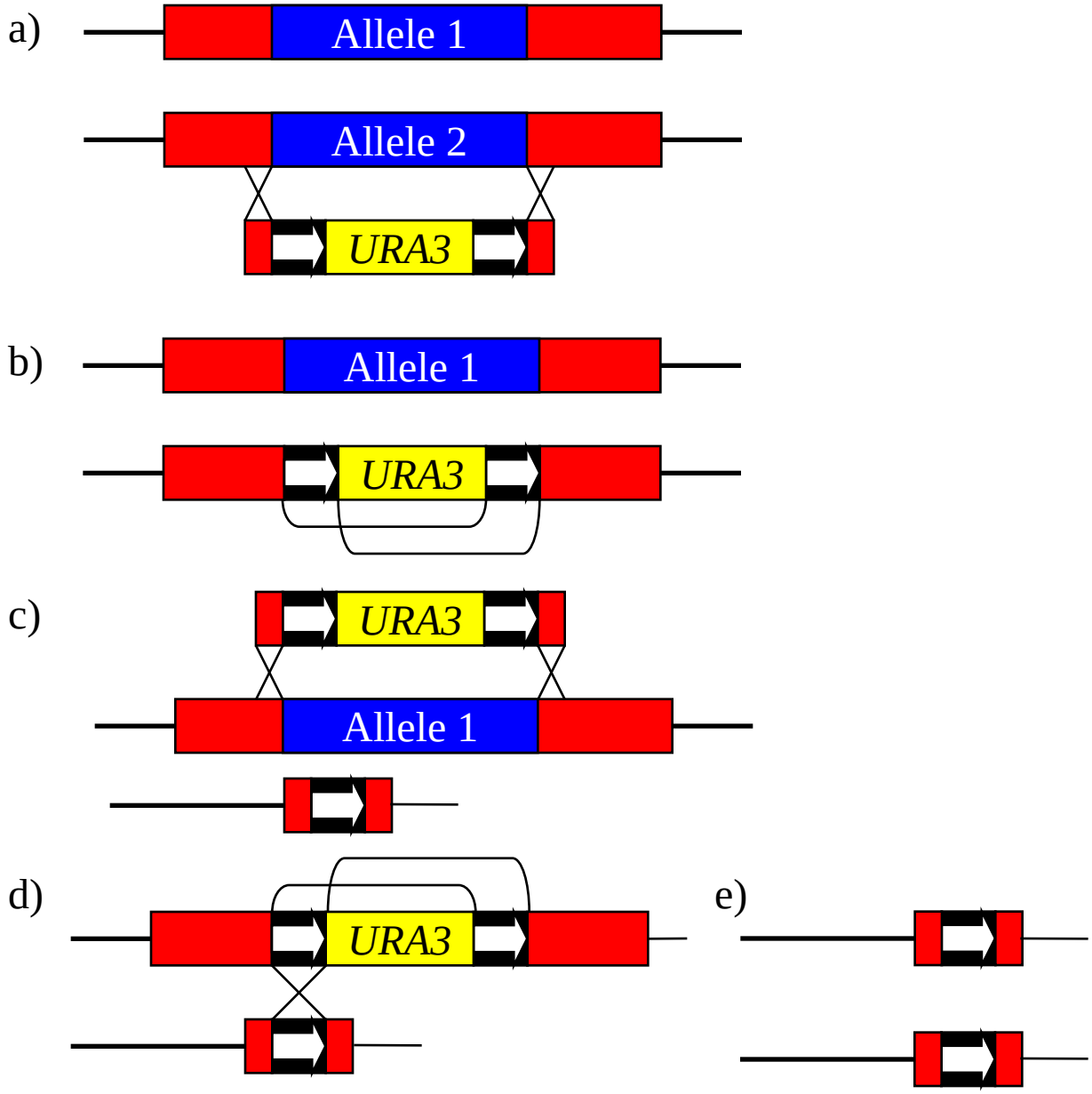


Figure 3.1: 'URA' Blasting to Knock Out Genes in *C. albicans*: a) Areas adjacent to the gene of interest are shown in red, these combine with areas of homology cloned into the 'URA' blaster cassette. Due to homologous recombination the 'URA' blaster cassette replaces one of the alleles. b) The *HisG* repeats, shown as white arrows, adjacent to the *URA3* marker gene recombine to excise *URA3*. c) a second transformation knocks out the second allele. d) The *HisG* repeats recombine to excise *URA3*. e) Both alleles of the gene are knocked out.

screened as the cassette will often recombine into the *hisG* repeat still present in the disrupted allele. Confirmation of the mutant genotype is achieved using Southern blotting.

3.2: Construction of the *CaFRE7* knockout and the *CaFRE10* and *CaFRE7* double knockout mutants:

Construction of a *CaFRE7* 'URA' Blaster Cassette

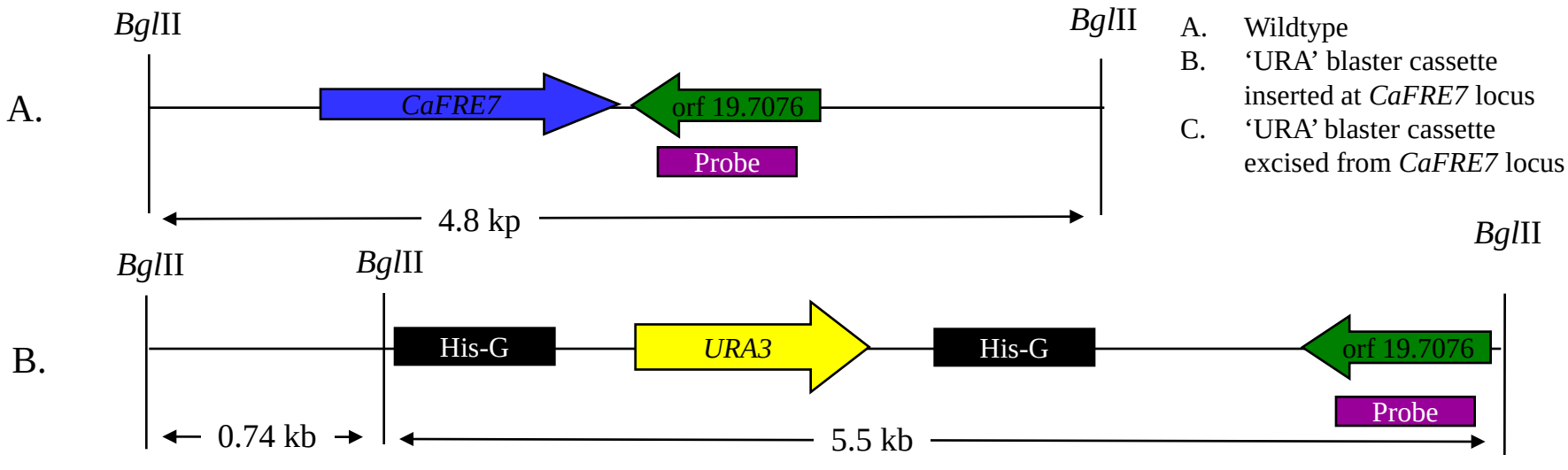
To construct the *CaFRE7* 'URA' blaster cassette, primers *CaFRE7KOKpn*I and *CaFRE7KOBgl*II were designed with *Bgl*II and *Kpn*I sites at either end to amplify the -829 to -38 sequence relative to the start codon of *CaFRE7*. All primers in this section can be found in Materials and Methods, table 2.7 and all PCR cycles can be found in Materials and Methods table 2.8. The PCR product produced by these primers was ligated into a pGEMT®-Easy Vector (Promega) as per the manufacturers instructions and transformed into the *E. coli* DH5α strain. The plasmid recovered was then digested with *Bgl*II and *Kpn*I and the excised PCR product was gel extracted. The pMB-7 plasmid (see Materials and Methods table 2.3) was then digested with *Bgl*II and *Kpn*I and gel extracted and dephosphorylated. The upstream region of *CaFRE7* was then ligated into the digested pMB-7 plasmid and transformed into the *E. coli* DH5α strain. A similar procedure was used to amplify the +1742 to +2677 sequence relative to the start codon of *CaFRE7* with primers *CaFRE7KOPst*I and *CaFRE7KOSph*I, incorporating *Sph*I and *Pst*I sites. The PCR product was cloned into pMB-7 already containing the -829 to -38 region to create pMB-FRE7. The *CaFRE7* 'URA' blaster cassette was excised from pMB-FRE7 by digestion with *Sph*I and *Kpn*I followed by gel extraction of the cassette.

Construction of CIp10*CaFRE7* Plasmid for Complementation of *CaFRE7* Deletion

To confirm that any phenotype detected in the *CaFRE7* deletion mutants was as a result of the absence of *CaFRE7* a complementation of the mutation was necessary. To complement the mutation, the *CaFRE7* gene including its promoter was inserted into the CIp10 integration vector (Materials and Methods table 2.2). The CIp10 plasmid contains a section of the *RPS10* locus and a copy of the *URA3* gene. When linearized with *Stu*I and transformed into *C. albicans* CIp10 integrates at the *RPS10* locus. A region of DNA containing *CaFRE7* and its promoter was amplified up using *CaFRE7CXho*I and *CaFRE7CMlu*I primers and Bio-X-Act DNA polymerase in a PCR reaction. The primers contain sites for *Xho*I and *Mlu*I enzymes, which in the PCR are introduced to either end of the sequence to be inserted into CIp10, the PCR product was then PCR purified. The purified PCR product and CIp10 were both digested with *Xho*I and *Mlu*I, run on an agarose gel and the appropriate bands gel extracted. The digested CIp10 was then dephosphorylated and the purified digestion of the PCR product was ligated into the dephosphorylated vector. Five of the resultant colonies produced were grown up in luria broth supplemented with ampicillin and the plasmids isolated. All five of the colonies were confirmed by restriction digest to contain an insert the same size as the PCR product.

Construction of a *CaFRE7* Knockout Mutant.

To disrupt a single allele of *CaFRE7* the *CaFRE7* 'URA' Blaster cassette was transformed into CAI-4 (see Materials and Methods table 2.1) using the lithium acetate method. From two separate transformations, only two transformants were produced, but both of these were confirmed as having a single allele of *CaFRE7* disrupted by Southern blot, these strains were named RJ1.1 (figure 3.2) and RJ1.2 (data not shown). Both strains were plated



A. Wildtype
B. 'URA' blaster cassette inserted at *CaFRE7* locus
C. 'URA' blaster cassette excised from *CaFRE7* locus

1. CAF-2
2. CAI-4
3. SC5314
4. RM10.5
5. RJ2.3
6. RJ2.4
7. RJ2.5
8. RJ2.6
9. RJ2.7
10. RJ2.8
11. RJ2.9
12. RJ2.10
13. RJ1.1
14. RJ1.3
15. RJ1.4
16. RJ1.5
17. RJ1.6
18. RJ1.7
19. RJ1.8
20. RJ1.9
21. RJ1.10

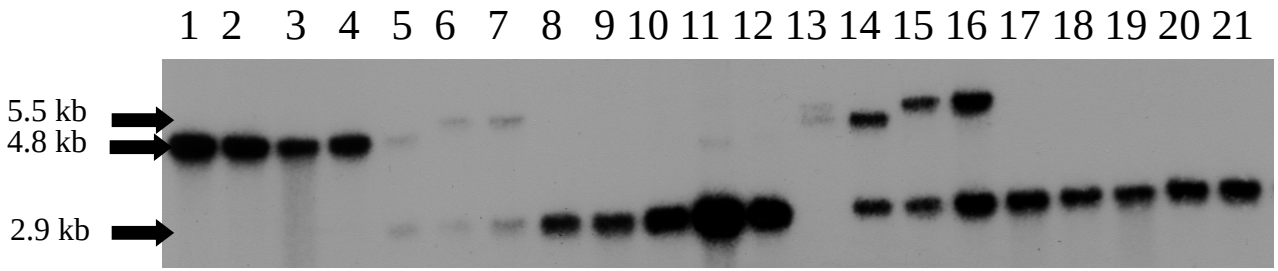
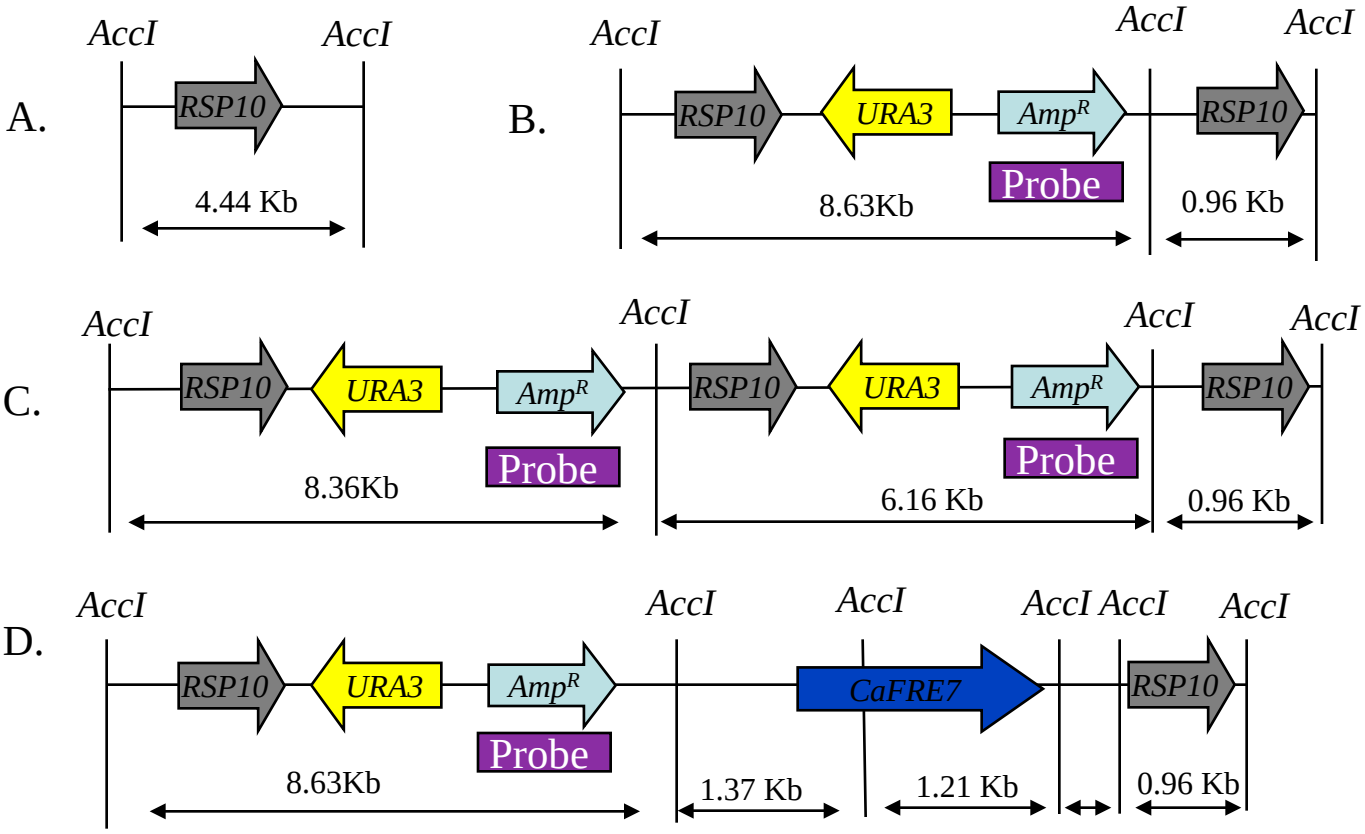
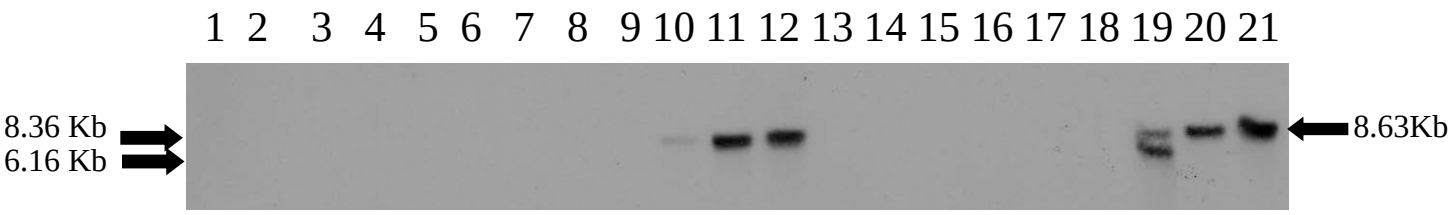


Figure 3.2: Southern Blot Confirmation of *CaFRE7* Knockout Mutants: Strains were grown overnight in 10 ml YPD supplemented with uridine and the DNA was extracted by the rapid yeast chromosomal DNA method described in Materials and Methods. The DNA was quantified by separation on an agarose gel and 5-10μg DNA was digested with *Bgl*II and run on a 1% agarose gel for 5 hours at 70 volts. The gel was southern blotted overnight and the DNA was crosslinked to the membrane as described in Materials and Methods. A probe was constructed by PCR using the orf19.7076 F and 19.7076 R primers. The PCR product was then purified by gel extraction. The probe was radiolableled and hybridised with the blot overnight in Church Gilberts buffer, then washed in 3xSSC;0.1% SDS and exposed to X-ray film at -80˚C for 2-3 days as described in the materials and methods section. CAF-2 , CAI-4 and SC5314 are wildtype strains, RM10.5 is *fre10Δ::hisG/fre10Δ::hisGURA3hisG,* RJ1.1 is *FRE7/fre7Δ::hisGURA3hisG* RJ1.3 is *FRE7/fre7Δ::hisG* RJ1.4 and RJ1.5 are *fre7Δ::hisG/fre7Δ::hisGURA3hisG* RJ1.6 and RJ1.7 are *fre7Δ::hisG/fre7Δ::hisG* RJ1.8 and RJ1.9 are *fre7Δ::hisG/fre7Δ::hisG/CaRPS10::URA3/CaRPS10* RJ1.10 is *fre7Δ::hisG/fre7Δ::hisG/RPS10::URA3::FRE7/CaRPS10.* RJ2.3 is *fre10Δ::hisG/fre10Δ::FRE7/fre7Δ::hisG,*RJ2.4 and RJ2.5 are *fre10Δ::hisG/fre10Δ::fre7Δ::hisG/fre7Δ::hisGURA3hisG,*RJ2.6 and RJ2.7 are *fre10Δ::hisG/fre10Δ::fre7Δ::hisG/fre7Δ::hisG ,* RJ2.8 and RJ2.9 are *fre10Δ::hisG/fre10Δ::fre7Δ::hisG/fre7Δ::hisG/CaRPS10::URA3/CaRPS10* and RJ2.10 is *fre10Δ::hisG/fre10Δ::fre7Δ::hisG/fre7Δ::hisG/CaRPS10::URA3::FRE7/CaRPS10.*





A. Wildtype *RPS10* locus
B. One copy of CIp10 inserted into *RPS10*
C. Two copies of CIp10 inserted into *RPS10*
D. CIp10*CaFRE7* inserted into *RPS10*

1. CAF-2
2. CAI-4
3. SC5314
4. RM10.5
5. RJ2.3
6. RJ2.4
7. RJ2.5
8. RJ2.6
9. RJ2.7
10. RJ2.8
11. RJ2.9
12. RJ2.10
13. RJ1.1
14. RJ1.3
15. RJ1.4
16. RJ1.5
17. RJ1.6
18. RJ1.7
19. RJ1.8
20. RJ1.9
21. RJ1.10

Figure 3.3: Southern Blot Confirmation of reintegration of *URA3* and *CaFRE7* Complement into *CaFRE7* Knockout Mutants: Strains were grown overnight in 10 ml YPD supplemented with uridine and the DNA was extracted by the rapid yeast chromosomal DNA method described in Materials and Methods. The DNA was quantified by separation on an agarose gel and 5-10µg DNA was digested with *Acc*I and run on a 1% agarose gel for 5 hours at 70 volts. The gel was southern blotted overnight and the DNA was crosslinked to the membrane as described in Materials and Methods. A probe was constructed by digestion of CIp10 (see Materials and Methods table 2.3) with *Acl*1. The band containing a fragment of the Amp^R gene was purified by gel extraction. The probe was then radiolablled and hybridised with the blot overnight in Church Gilberts buffer, then washed in 3xSSC;0.1% SDS and exposed to X-ray film at -80˚C for 2-3 days as described in Materials and Methods. CAF-2 , CAI-4 and SC5314 are wildtype strains, RM10.5 is *fre10Δ::hisG/fre10Δ::hisGURA3hisG,* RJ1.1 is *FRE7/fre7Δ::hisGURA3hisG* RJ1.3 is *FRE7/fre7Δ::hisG* RJ1.4 and RJ1.5 are *fre7Δ::hisG/fre7Δ::hisGURA3hisG* RJ1.6 and RJ1.7 are *fre7Δ::hisG/fre7Δ::hisG* RJ1.8 and RJ1.9 are *fre7Δ::hisG/fre7Δ::hisG/CaRPS10::URA3/CaRPS10* RJ1.10 is *fre7Δ::hisG/fre7Δ::hisG/RPS10::URA3::FRE7/CaRPS10.* RJ2.3 is *fre10Δ::hisG/fre10Δ::FRE7/fre7Δ::hisG,*RJ2.4 and RJ2.5 are *fre10Δ::hisG/fre10Δ::fre7Δ::hisG/fre7Δ::hisGURA3hisG,*RJ2.6 and RJ2.7 are *fre10Δ::hisG/fre10Δ::fre7Δ::hisG/fre7Δ::hisG ,* RJ2.8 and RJ2.9 are *fre10Δ::hisG/fre10Δ::fre7Δ::hisG/fre7Δ::hisG/CaRPS10::URA3/CaRPS10* and RJ2.10 is *fre10Δ::hisG/fre10Δ::fre7Δ::hisG/fre7Δ::hisG/CaRPS10::URA3::FRE7/CaRPS10.*

on SD plates supplemented with uridine and 5-FOA to select for colonies which had lost the URA Blaster cassette. A colony derived from RJ1.1 (*Cafre7Δ/CaFRE7*) was selected and plated onto YPD supplemented with uridine and was named RJ1.3 (figure 3.2). The URA blaster cassette was then transformed into the RJ1.3 (*Cafre7Δ/CaFRE7/ura*$^-$) strain. The second round of transformations produced a large number of colonies because of the tendency for the *CaFRE7* 'URA' blaster cassette to integrate into the remaining *HisG* found at the locus of the previously disrupted allele of *CaFRE7*. Eighty of the colonies produced were plated onto YPD and screened using colony PCR with the *CaFRE7F* and *CaFRE7R* primers to amplify *CaFRE7* if it was still present in the strain. Of the eighty colonies, six were found to be negative for *CaFRE7* by PCR and five were shown by southern blot to contain a disruption of both *CaFRE7* alleles. Two of these five strains (named RJ1.4 and RJ1.5) were plated onto 5-FOA and derivatives each lacking *URA3* were selected and named RJ1.6 and RJ1.7.

Plasmid CIp10 was linearized by digestion with *Stu*I and transformed into RJ1.6 (*Cafre7Δ/Cafre7Δ/ura*$^-$) and RJ1.7 (*Cafre7Δ/Cafre7Δ/ura*$^-$). Transformation of CIp10 into RJ1.6 (*Cafre7Δ/Cafre7Δ/ura*$^-$) yielded three transformants which all contained *URA3* at the *RPS10* locus; one of these was selected for further study and called RJ1.8 (figure 3.3). Transformation of CIp10 into RJ1.7 (*Cafre7Δ/Cafre7Δ/ura*$^-$) produced only one transformant; but this had *URA3* integrated at the *RPS10* locus and was named RJ1.9. RJ1.8 (*Cafre7Δ/Cafre7Δ*) has a double insertion of CIp10 at the *RPS10* locus, so RJ1.9 (*Cafre7Δ/Cafre7Δ*) was predominantly used for further study, although no phenotypic differences were observed between the two strains. Plasmid CIp10*CaFRE7* was linearized by digestion with *Stu*I and transformed into RJ1.7. Transformation of CIp10*CaFRE7* into

RJ1.7 (*Cafre7Δ/Cafre7Δ/ura*$^-$) produced two transformants, but only one of these had *URA3* and *CaFRE7* integrated at the *RPS10* locus, this was named RJ1.10. Confirmation of the presence of *CaFRE7* in RJ1.10 (*Cafre7Δ/Cafre7Δ/CaFRE7*) was achieved by PCR with *CaFRE7F* and *CaFRE7R* primers (data not shown).

Construction of a *Cafre10ΔΔ/Cafre7ΔΔ* Knockout Mutant

The strain RM10.5 (*Cafre10Δ/Cafre10Δ*) was streaked onto SD supplemented with uridine and 5-FOA and, once grown, a colony was selected lacking *URA3* and used for further study (data not shown). The purified 'URA'-blaster cassette was then transformed into RM10.5 *ura*$^-$ using the lithium acetate method described in the materials and methods chapter. Of the numerous colonies produced, six were selected and their DNA extracted and a single allele disruption of *CaFRE7* was confirmed in one of these transformants via PCR using the *CaFRE7* -1315 F and *URA3* R primers (data not shown). The strain was named RJ2.1 and plated onto SD supplemented with uridine and 5-FOA to excise the *CaURA3* gene from the *CaFRE7* locus. Of the colonies produced, one was selected for further study and named RJ2.3, confirmation of the absence of *URA3* at the *CaFRE7* locus was achieved by Southern blot (figure 3.2). The *CaFRE7* 'URA' blaster cassette was then transformed into RJ2.3 (*Cafre10ΔΔ/Cafre7Δ/ura*$^-$). A total of 45 colonies were produced from two transformations and these were screened for the presence of *CaFRE7* by colony PCR with the *CaFRE7F* and *CaFRE7R* primers. The PCR showed three of the transformants were negative for *CaFRE7*, but southern blot identified that only two of these had homozygous deletion of *CaFRE7*. These two strains were named RJ2.4 and RJ2.5 (figure 3.2).

Strains RJ2.4 (*Cafre10ΔΔ/Cafre7ΔΔ*) and RJ2.5 (*Cafre10ΔΔ/Cafre7ΔΔ*) were plated onto SD supplemented with uridine and 5-FOA and one derivative of each strain lacking *CaURA3* was selected for further study. The derivative of RJ2.4 was called RJ2.6 and the derivative of RJ2.5 was called RJ2.7. To reintroduce *CaURA3*, CIp10 was transformed into RJ2.6 (*Cafre10ΔΔ/Cafre7ΔΔ/ura*$^-$) and RJ2.7 (*Cafre10ΔΔ/Cafre7ΔΔ/ura*$^-$); only a single transformant was produced for RJ2.6, but this was confirmed by southern blot to contain *CaURA3* at the *RPS10* locus and named RJ2.8 (Figure 3.3). Transformation of CIp10 into RJ2.7 (*Cafre10ΔΔ/Cafre7ΔΔ/ura*$^-$) yielded 4 transformants, screening with colony PCR using the *RP10F URA3R* primers showed only two of these contained *CaURA3* at the *RPS10* locus (data not shown). One was selected for further study and named RJ2.9; this was confirmed to have *CaURA3* at the *RPS10* locus by southern blot (figure 3.3). To complement the *CaFRE7* mutation the CIp10*CaFRE7* plasmid was transformed into RJ2.6 (*Cafre10ΔΔ/Cafre7ΔΔ/ura*$^-$), this yielded three transformants, two of which were shown by screening with colony PCR using the *RP10F* and *URA3R* primers to contain CIp10*CaFRE7* at the *RPS10* locus, one of these was selected for further study and named RJ2.10 and confirmed as containing CIp10*CaFRE7* at the *RPS10* locus by southern blot (figure 3.3). Confirmation of the presence of *CaFRE7* in RJ2.10 (*Cafre10ΔΔ/Cafre7ΔΔ/CaFRE7*) was achieved by PCR with *CaFRE7F* and *CaFRE7R* primers (data not shown).

3.3: *CaFRE7* Plays a Role in Cell surface Ferric Reductase Activity

It has previously been demonstrated that *CaFRE10* is responsible for 70% of cell surface ferric reductase activity in the yeast form of *C. albicans* and that in a *Cafre10Δ/Cafre10Δ* mutant the increase in ferric reductase activity in response to low iron conditions is not

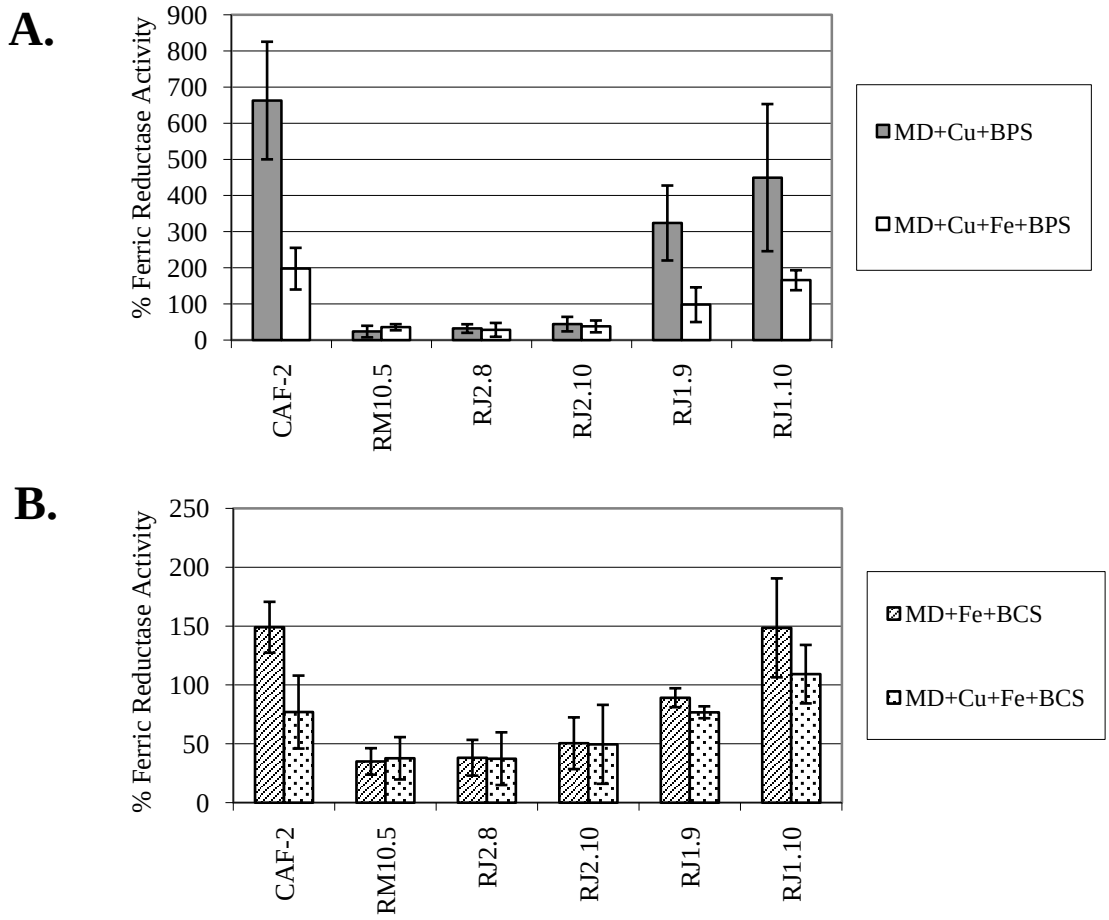


Figure 3.4: Cell Surface Ferric Reductase Activity in *CaFRE7* Knockout Strains: Strains were grown in MD with the addition of 100μM $CuCl_2$, 100μM BPS, 100μM $FeCl_3$, and 100μM BCS as indicated for 5-6 hours to mid-exponential phase at 30°C with shaking (200 rpm). The cell surface cupric reductase activity was then determined as described in Materials and Methods section 2.9. The data is expressed as a % of the ferric reductase activity of CAF-2 grown in MD supplemented with 100μM $CuCl_2$ and 100μM $FeCl_3$. The mean values of three reductase assays are shown and the error bars represent standard deviation. A shows iron responsive ferric reductase activity and B shows copper responsive ferric reductase activity. CAF-2 is the wildtype strain, RM10.5 is *fre10Δ/fre10Δ*, RJ1.9 is *fre7Δ/fre7Δ* RJ1.10 is *fre7Δ/fre7Δ/FRE7,* RJ2.8 is *fre10ΔΔ/fre7ΔΔ* and RJ2.10 is *fre10ΔΔ/fre7ΔΔ/FRE7.*

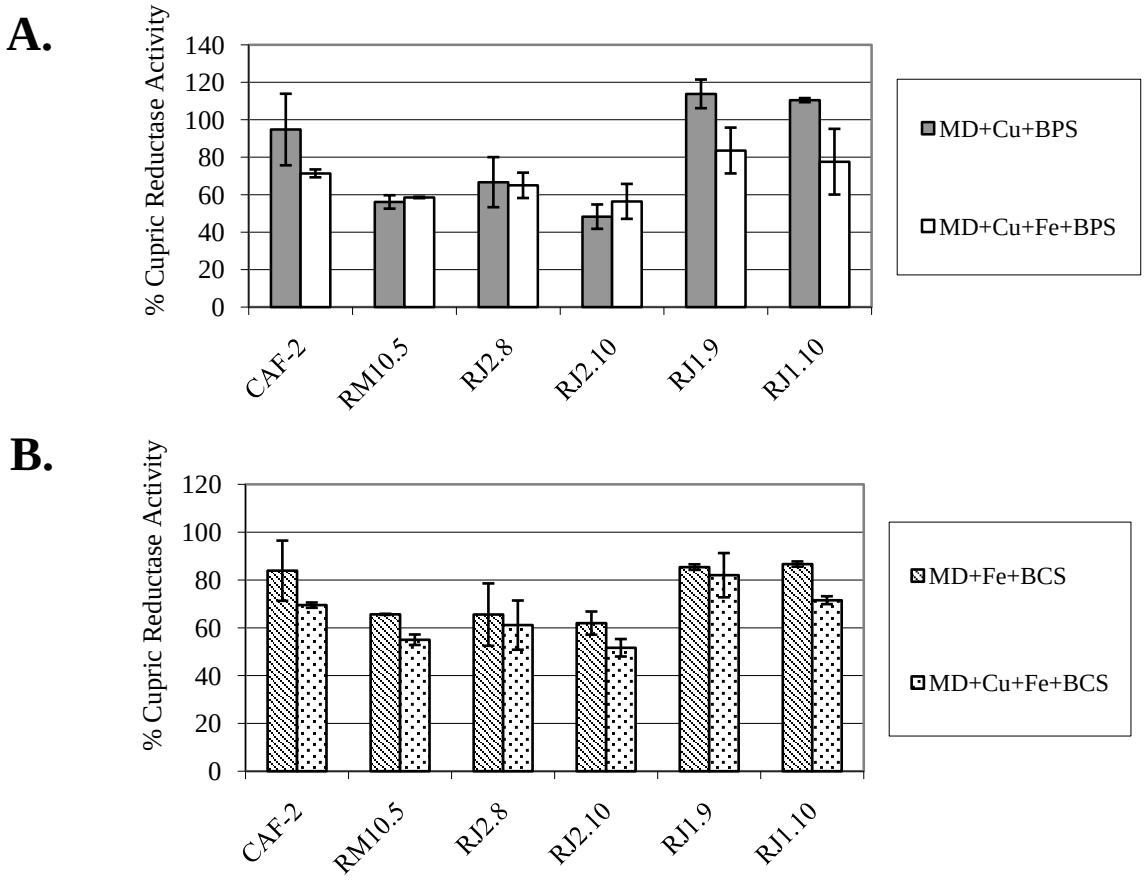


Figure 3.5 : Cell Surface Cupric Reductase Activity in *CaFRE7* Knockout Strains: Strains were grown in MD with the addition of 100µM $CuCl_2$, 100µM BPS, 100µM $FeCl_3$, and 100µM BCS as indicated for 5-6 hours to mid-exponential phase at 30°C with shaking (200 rpm). The cell surface cupric reductase activity was then determined as described in Materials and Methods section 2.9. The data is expressed as a % of the ferric reductase activity of CAF-2 grown in MD supplemented with 100µM $CuCl_2$ and 100µM $FeCl_3$. The mean values of two reductase assays are shown and the error bars represent standard deviation. A shows iron responsive cupric reductase activity and B shows copper responsive cupric reductase activity. CAF-2 is the wildtype strain, RM10.5 is *fre10Δ/fre10Δ,* RJ1.9 is *fre7Δ/fre7Δ* RJ1.10 is *fre7Δ/fre7Δ/FRE7,* RJ2.8 is *fre10ΔΔ/fre7ΔΔ* and RJ2.10 is *fre10ΔΔ/fre7ΔΔ/FRE7.*

detected (Knight *et al.* 2005, Mason, 2006). In figure 3.4 A it can be seen that in the RM10.5 (*Cafre10Δ/Cafre10Δ*) mutant iron-responsive ferric reductase activity is not detected, and that it is also absent in RJ2.8 and RJ2.10, both of which are derived from RM10.5 and are therefore *Cafre10Δ/Cafre10Δ* mutants. In RJ1.9 (*Cafre7Δ/Cafre7Δ*) the iron responsive increase in ferric reductase activity is still present, however, overall activity is approximately 50% lower than CAF-2 in iron replete conditions and this is significant in a Tukey's one way ANOVA (p=0.001). This is partially restored in the complimented strain RJ1.10 (*Cafre7Δ/Cafre7Δ/CaFRE7*), although the difference is not significant, possibly because the gene is only present in a single copy at a different locus. The ferric reductase activity in the wildtype CAF-2 strain increases by an approximately 2 fold in response to low copper as can be seen in figure 3.4 B, which is significant in a Tukey's one way ANOVA (P=0.009). This copper responsive increase in cell surface ferric reductase activity is lost in mutants containing deletions of *CaFRE10* (RM10.5, RJ2.8 and RJ2.10) and in mutants containing deletions in *CaFRE7* (RJ1.9). The *Cafre7Δ/Cafre7Δ* phenotype is partially rescued in the complemented mutant RJ1.10 (*Cafre7Δ/Cafre7Δ/CaFRE7*), although this is not significant, again possibly due to the difference in copy number and locus of the *FRE7* gene compared to wildtype. This indicates that whilst *CaFRE10* is the major ferric reductase *CaFRE7* may also play a role in cell surface ferric reductase activity. The results for RJ2.8 were replicated in RJ2.9 and the results for RJ1.9 were replicated in RJ1.8 (data not shown).

3.4: Investigation of the role of *CaFRE7* Cell Surface Cupric Reductase Activity

It has previously been shown in our laboratory that cell surface cupric reductase activity was increased in response to both low iron and low copper conditions in a wildtype

C. albicans strain. Furthermore it was shown that *CaFRE10* was responsible for the increase in cupric reductase activity in response to low iron (Mason, 2006). You can see in figure 3.5 A that in RM10.5 (*Cafre10Δ/Cafre10Δ*) and the *CaFRE7* double mutants derived from it (RJ2.8 and RJ2.10) that the iron responsive increase in cupric reductase activity is not observed. The iron responsive increase in cupric reductase activity is still present in strains containing only a deletion in *CaFRE7* (RJ1.9) and this is significant in a Tukey's one way ANOVA (P=0.003). Even though *CaFRE10* is clearly responsible for the iron responsive cell surface cupric reductase activity, figure 3.5 B shows that the increase in cell surface cupric reductase activity in response to low copper conditions is still significant in a Tukey's one way ANOVA (P<0.001) in a *Cafre10Δ/Cafre10Δ* mutant (RM10.5). the role of *CaFRE7* in cupric reductase activity is less clear, with overall activity not significantly different from wildtype. However, this may be as a result of the upregulation of one of the other ferric reductases in the absence of *CaFRE7* to compensate. In a mutant lacking both *CaFRE10* and *CaFRE7* (RJ2.8) no iron or copper sensitive regulation of cupric reductase activity can be detected. A mean of two repeats is shown in figure 3.5, however the experiments were repeated at least 3 times with RJ1.8 and RJ1.9 (both *Cafre7Δ/Cafre7Δ*), RJ1.10 (*Cafre7Δ/Cafre7Δ/CaFRE7*), RJ2.8 and RJ2.9 (*Cafre10ΔΔ/Cafre7ΔΔ*) and RJ2.10 (*Cafre10ΔΔ/Cafre7ΔΔ/CaFRE7*). Although the absolute values varied, the pattern observed was always the same for each genotype.

3.5: Deletion of *CaFRE7* has no Effect on Growth in Low Iron or Low Copper Conditions:

To determine whether the deletion of *CaFRE7* has any effect on growth in low iron and low

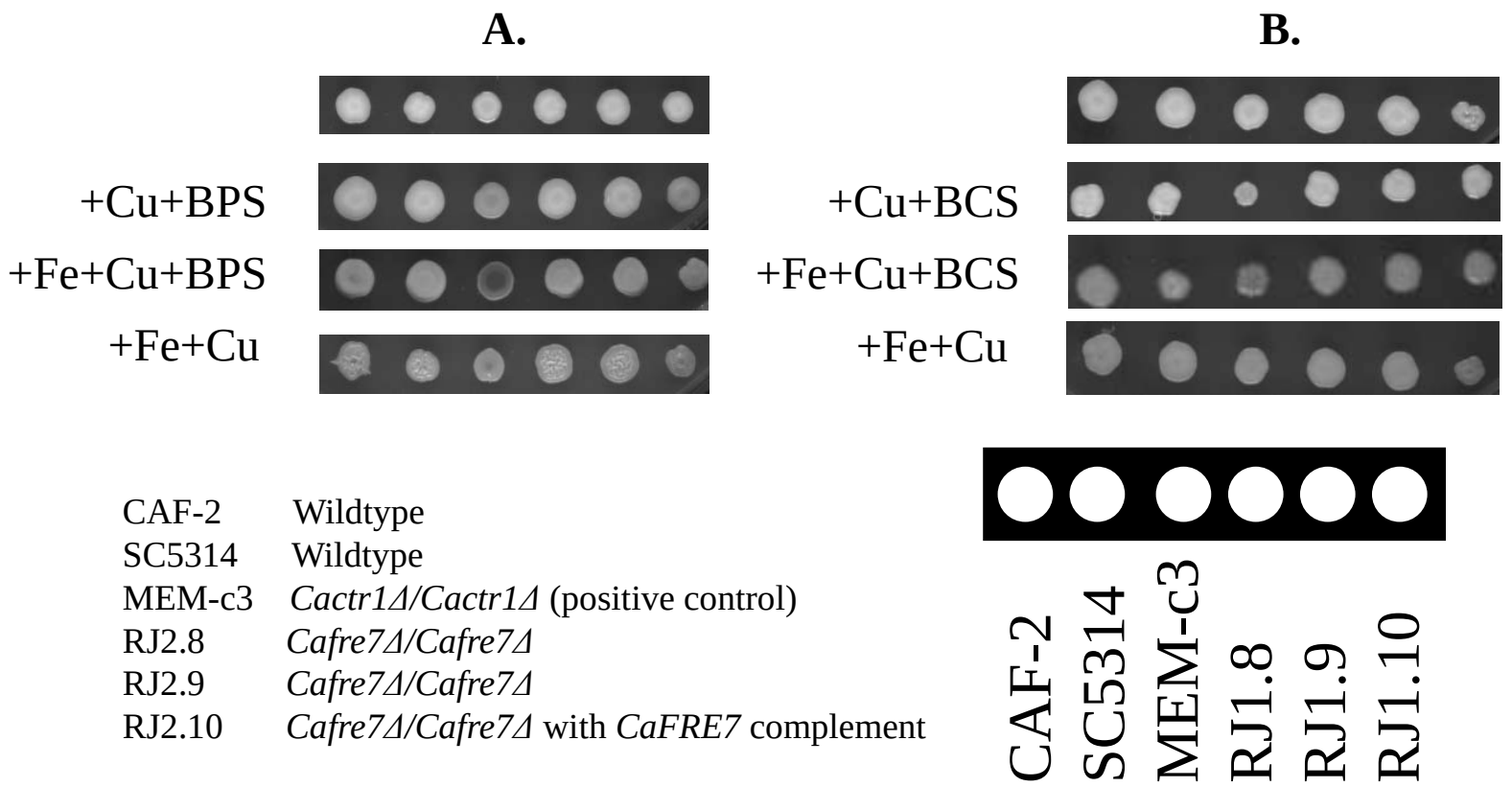
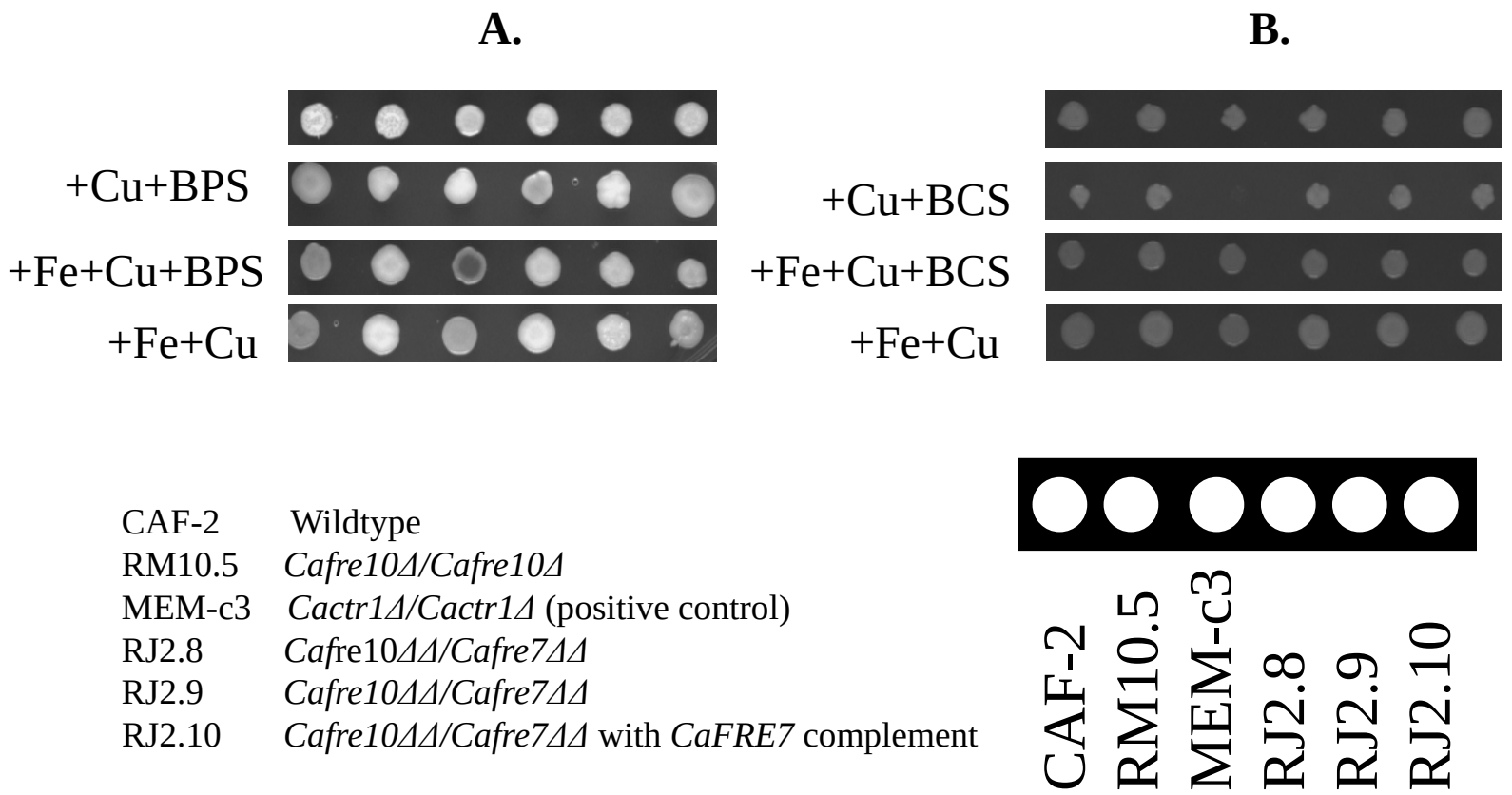


Figure 3.6: Tests for Growth Deficiency in *Cafre7Δ /Cafre7Δ* Strains in Response to Low Iron and Low Copper Conditions:

Cells were grown to mid-log phase in MD with the addition of 100 μM BPS, then serially diluted and spotted onto plates using a 48 pin replicator and grown at 30˚C for 3 days. A.100 μM BPS, 100 μM $FeCl_3$ or 100 μM $CuCl_2$ were added to MD plates as indicated to test for growth in low iron conditions. B. 50 μM BPS, 50 μM $FeCl_3$ or 50 μM $CuCl_2$ were added to MD plates as indicated to test for growth in low copper conditions. MEM-c3 is a positive control known to grow slower in the absence of copper. An example of each strain is shown.



CAF-2	Wildtype
RM10.5	*Cafre10Δ/Cafre10Δ*
MEM-c3	*Cactr1Δ/Cactr1Δ* (positive control)
RJ2.8	*Caf*re10*ΔΔ/Cafre7ΔΔ*
RJ2.9	*Cafre10ΔΔ/Cafre7ΔΔ*
RJ2.10	*Cafre10ΔΔ/Cafre7ΔΔ* with *CaFRE7* complement

Figure 3.7: Tests for Growth Deficiency in *Cafre10ΔΔ/Cafre7ΔΔ* Strains in Response to Low Iron and Low Copper Conditions: Cells were grown to mid-log phase in MD with the addition of 100 μM BPS, then serially diluted and spotted onto plates using a 48 pin replicator and grown at 30˚C for 3 days. A.100 μM BPS, 100 μM $FeCl_3$ or 100 μM $CuCl_2$ were added to MD plates as indicated to test for growth in low iron conditions. B. 50 μM BPS, 50 μM $FeCl_3$ or 50 μM $CuCl_2$ were added to MD plates as indicated to test for growth in low copper conditions. MEM-c3 is a positive control known be growth deficient in the absence of copper. An example of each strain is shown.

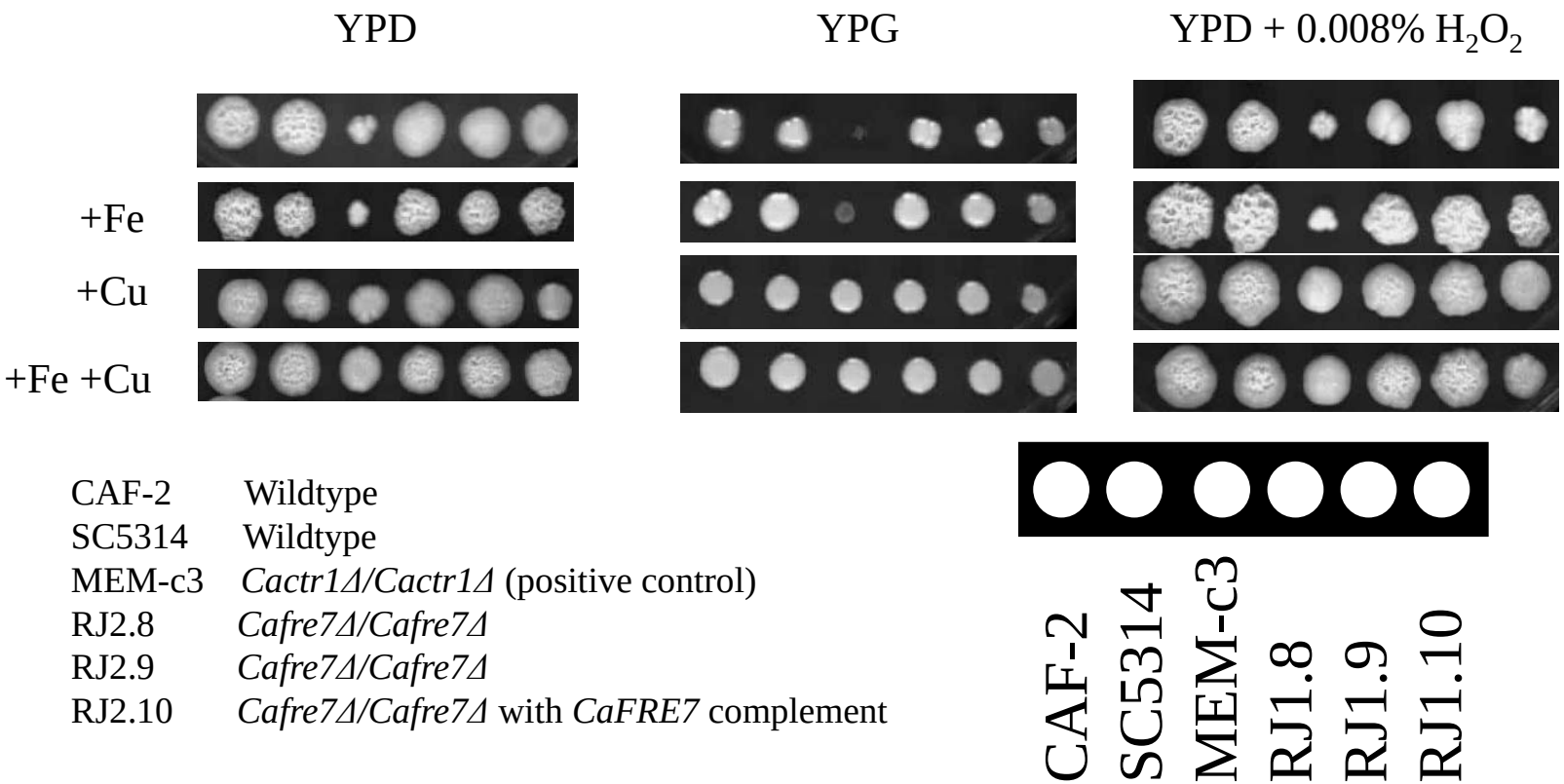


Figure 3.8: Tests for Sensitivity to Oxidative Stress and Respiratory Deficiency in *Cafre7Δ/Cafre7Δ* Strains:

Cells were grown to mid-log phase in MD with the addition of 100 μM BPS, then serially diluted and spotted onto plates using a 48 pin replicator and grown at 30˚C for 4 days. Sensitivity to oxidative stress was tested for by addition of 0.008% H_2O_2 to YPD medium. YPG contains glycerol as the sole carbon source to test for respiratory deficiency and YPD contains glucose as the sole carbon source for comparison. 100 μM $FeCl_3$ or 100 μM $CuCl_2$ were added as indicated. MEM-c3 is a positive control known to be sensitive to oxidative stress and is respiratory deficient without the addition of excess copper. An example of each strain is shown.

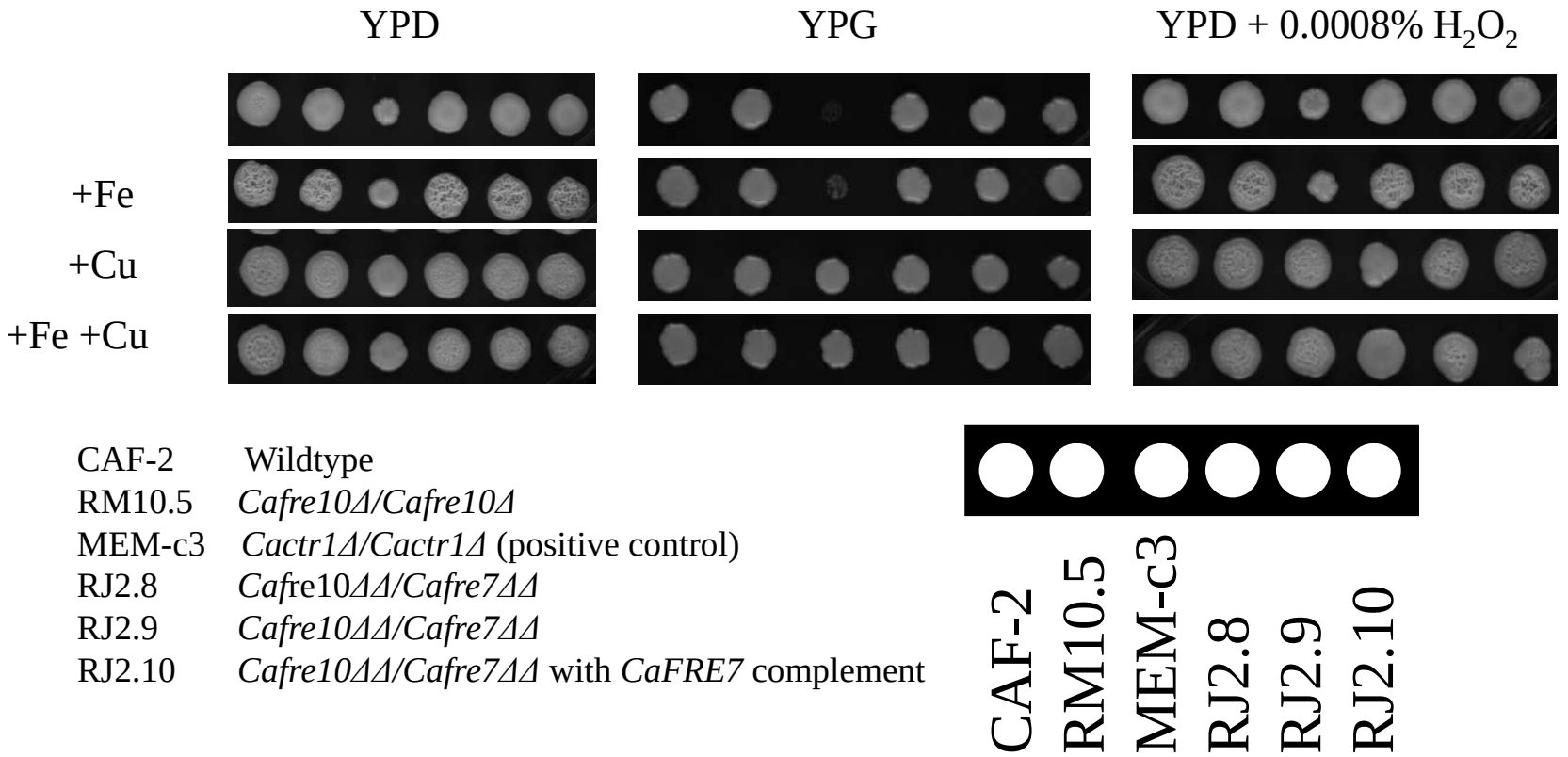


Figure 3.9: Tests for Sensitivity to Oxidative Stress and Respiratory Deficiency in *Cafre10ΔΔ/Cafre7ΔΔ* Mutants:

Cells were grown to mid-log phase in MD with the addition of 100 μM BPS, then serially diluted and spotted onto plates using a 48 pin replicator and grown at 30˚C for 4 days. Sensitivity to oxidative stress was tested for by addition of 0.008% H_2O_2 to YPD medium. YPG contains glycerol as the sole carbon source to test for respiratory deficiency and YPD contains glucose as the sole carbon source for comparison. 100 μM $FeCl_3$ or 100 μM $CuCl_2$ were added as indicated. MEM-c3 is a positive control known to be sensitive to oxidative stress and respiratory deficient without the addition of excess copper. An example of each strain is shown.

copper the RJ1.8-10 and RJ2.8-10 mutants were grown on plates containing minimal media with the supplementation of iron and copper chelators and iron and copper to create low iron and low copper conditions. Strains were grown overnight in MD, then this culture was used to inoculate a 10ml MD culture supplemented with 100 µM BPS. Cells were washed and the number of cells counted. Serial dilutions of $1x10^6$ cells down to$1.6x10^4$ were made of each strain and plated onto MD plates supplemented with iron and copper chelators and iron and copper to create low iron and low copper conditions. This was repeated at least three times for each condition. An example of one spot of each strain is shown in figures 3.6 and 3.7. None of the mutants constructed showed any deficiencies in growth in low iron or low copper conditions.

3.6: Deletion of *CaFRE7* has no Effect on Respiration and Sensitivity to Oxidative Stress:

Deficiencies in high affinity iron and copper uptake have been shown previously to result in an increased sensitivity to oxidative stress and an inability to utilise complex carbon sources (Casas *et al.* 1997; Blaiseau *et al.* 2001; De Freitas *et al.* 2000; Jungmann *et al.*1993). Strains were grown overnight in YPD, then this culture was used to inoculate a 10ml YPD culture. Cells were washed and the number of cells counted. Serial dilutions of $1x10^6$ cells down to $1.6x10^4$ were made of each strain and plated onto YPD containing glycerol as a sole carbon source to test for respiratory competency and with glucose as the carbon source supplemented with 0.008% hydrogen peroxide to test for sensitivity to oxidative stress. This was repeated at least three times for each condition. An example of one spot of each strain is shown in figure 3.8 and 3.9. None of the *CaFRE7* deletion mutants showed any respiratory deficiency or any increased sensitivity to oxidative stress.

3.7: Discussion:

Ferric reductases in the model organism *S. cerevisiae* are regulated by both iron and copper (Bakel *et al.* 2005; Georgatsou & Alexandraki, 1999; Martins *et al.*1998). However, regulation of the cell surface ferric reductases in *S. cerevisiae* differs significantly from that in *C. albicans.* In *C. albicans* the major ferric reductase at the cell surface, CaFre10p is regulated by iron, but not copper and there is an supplementary ferric reductase in the form of CaFre7p which is transcriptioanally regulated by copper (Woodacre *et al.,* 2008). In *S. cerevisiae* the major cell surface ferric reductase, *ScFRE1*, is regulated by levels of both iron and copper in the growth medium (Bakel *et al.* 2005; Georgatsou & Alexandraki, 1999; Martins *et al.*1998).

CaFRE7 is regulated by copper (Levitin & Whiteway, 2007; Woodacre *et al.* 2008) and when it is deleted the copper responsive cell surface ferric reductase activity is not observed. However, the increase in activity in low copper conditions is not seen in the mutants containing deletions of only *CaFRE10*, which is surprising since *CaFRE10* is not regulated by copper and the copper responsive ferric reductase activity is mediated by *CaFRE7*. It may be that the increase in activity in response to copper that is attributable to *CaFRE7* is not detectable by the ferric reductase assay when applied to a *Cafre10Δ/Cafre10Δ* mutant because the levels of activity are so greatly reduced in the absence of *CaFRE10*.

Another surprising result is that cell surface cupric reductase activity is not reduced in a *CaFRE7* knockout mutant. This may be due to an increase in the expression of another

cupric reductase in the absence of *CaFRE7* in order to respond to copper deficiency. This is supported by previous cupric reductase assays performed in a mutant of the gene encoding the copper sensing transcription factor CaMac1p, a similar increase in cupric reductase activity in high copper conditions is seen (Mason, 2006). Since CaMac1p regulates *CaFRE7* it is possible that it is the absence of *CaFRE7* expression in both strains is the cause of the increase in cupric reductase activity in high copper conditions.

CaFre10p is responsible for an increase in cell surface ferric reductase activity in response to low iron conditions (Knight *et al.* 2005; Mason, 2006). The data presented in this chapter indicates that the increase in ferric reductase activity seen in response to low copper conditions is attributable to CaFre7p. Therefore CaFre7p has a significant role in reduction of iron at the cell surface.

CaFre7p has a role in cell surface ferric reduction, but all the experiments performed were at pH4.2 and evidence from previous studies have indicated that expression of ferric reductases is also regulated by pH (Bensen *et al.*, 2004; Baek *et al.,* 2008). These changes in expression result in differences in cell surface ferric reductase activity with CaFre10p being less important for reductive uptake of iron from ferric sources at pH 6.2 compared to pH 4.4. Further studies identifying which ferric reductases contribute to cell surface activity at different pH and possibly other environmental factors would be a good direction in which to take this research. These studies indicate that cell surface ferric reductase activity is not simply regulation by iron and copper of *CaFRE10* and *CaFRE7*, but also includes regulation by other environmental factors. The diverse set of factors regulating the

ferric reductase genes may be an adaptation to the different conditions found in the different regions of the host body.

Chapter 4: The role of Sfu1p in Regulating the Expression of the High Affinity Iron Uptake System in *C. albicans*.

4.1: Introduction:

In Chapter 1 the importance of iron homeostasis was discussed. Iron is an essential element and is required as a cofactor for a number of proteins. However, as a result of Fenton Chemistry, toxic free radicals are produced from iron that damages the cell. As a result of this any organism needs to tightly regulate how much iron it takes up, so that it can take up enough to fulfil the requirements of the cell, but not so much that it causes cellular damage (Crichton & Pierre, 2001).

The iron sensing transcription factor in *S. cerevisiae*, ScAft1p, regulates transcription of six of the seven ferric reductase genes as well as *ScCCC2*, *ATX1*, *ScARN1-4*, *ScFET5* and *ScFTH1* (Yamaguchi-Iwai *et al.*, 1996). In low iron conditions ScAft1p is found in the nucleus of the cell, this nuclear retention of ScAft1p in iron limited conditions (in high iron conditions it is found in the cytoplasm) ensures transcription of target genes (Yamaguchi-Iwai *et al.*, 2002). In the promoter of a gene regulated by ScAft1p, the ScAft1p binds to the consensus sequence (T/C)(G/A)CACCC (Yamaguchi-Iwai *et al.*, 1996). Two *ScAFT1* homologs have been identified due to sequence homology in *Candida albicans*, but they do not appear to have the same function as ScAft1p has in *S. cerevisiae*. The deletion of *CaAFT1* in *C. albicans* did not produce phenotypes associated with defects in high affinity iron acquisition and although it had a small effect on the expression of *CaFRE10* in response to low iron levels, there was no change in iron replete conditions (Mason, 2006). It is clear from this that although the

high affinity iron uptake system in *C. albicans* may function in a manner similar to that of *S. cerevisiae*; it is not regulated in the same way.

URBS1 encodes a GATA type transcriptional repressor involved in regulating siderophore production in *Ustillago maydis*. Production of siderophores is another means of gaining iron in iron limited conditions (Voisard *et al.,* 1993). Other GATA type transcription factors in fungi include SreAp in *Aspergillus nidulans*, which is also involved in regulating iron acquisition and siderophore biosynthesis (Oberegger *et al.,* 2001; Haas *et al.,* 1999a) and Srep in *Neurospora crassa*, which controls iron transport (Zhou *et al.,* 1998). All GATA type transcription factors contain one or two zinc finger DNA binding domains (Lowry & Atchley, 2000). Furthermore, all GATA-type transcription factors found in fungi have a cysteine rich region in their centre which is considered a putative iron sensing domain (Haas *et al.,* 1999a). *SFU1* encodes a GATA-type transcriptional repressor in *C. albicans* which was identified due to its homology to the *URBS1* gene from *U. maydis* (Lan *et al.,* 2004). Sfu1p has been demonstrated in a microarray study to decrease expression of *CaFRE1*, *CaFRE2*, *CaFRE5*, *CaFRE10*, *CaFRE31*, *CaFET34*, *CaFET35* and *CaFTH1* in response to high iron conditions (Lan *et al.,* 2004). However, the effect of Sfu1p on expression of the major ferric iron permease (*CaFTR1*) was not shown. Nor were the iron specific phenotypic effects of the absence of the *SFU1* gene, although it showed no growth deficiency in iron limited conditions (Lan *et al.,* 2004). A further study in which *SFU1* was shown to rescue a *Schizosaccharomyces pombe fep1Δ* mutant, $Fep1^{+}$ encodes an iron binding GATA-type transcriptional repressor. This provides some evidence that *SFU1* is functional (Pelletier *et al.,* 2007). However, this study was in a heterologous system and does not offer direct functional evidence of the role of *SFU1* in *C. albicans.*

The aim of this part of the study was to investigate the phenotype of a *sfu1Δ/sfu1Δ* mutant to provide functional evidence for its role as a repressor of high affinity iron uptake in *C. albicans*.

4.2: Expression of Ferric Reductases is altered in *sfu1Δ/sfu1Δ*:

It has previously been demonstrated in our laboratory that, in the yeast form of *C. albicans*, expression of *CaFRE10* and *CaFRE5* is increased in response to low iron conditions and expression of *CaFRE7* is increased in response to low copper conditions (Mason, 2006). In the CNA6 (*sfu1Δ/sfu1Δ*) mutant *CaFRE10* and *CaFRE5* (figure 4.1) expression is much higher than in the wildtype SC5314 (figure 4.1). In the absence of *SFU1* the metalloregulation of *CaFRE10* and *CaFRE5* is not detected and it appears to be expressed at the same level over all conditions (figure 4.1). This indicates that Sfu1p is involved in the iron sensitive regulation of ferric reductase genes. The expression of *CaFRE7* is unaltered in the CNA6 (*sfu1Δ/sfu1Δ*) mutant (figure 4.1), indicating that Sfu1p is not involved in copper sensitive regulation of the ferric reductases. To see if the changes in expression of *CaFRE10* in the CNA6 (*sfu1Δ/sfu1Δ*) mutant lead to any changes in the cell surface ferric reductase activity at the cell surface, the ferric reductase activity of the CNA6 (*sfu1Δ/sfu1Δ*) was investigated.

4.3: Cell surface ferric reductase activity is elevated in *sfu1Δ/sfu1Δ*:

In our laboratory and others, it has been previously demonstrated that cell surface ferric reductase activity in wild type *C. albicans* is higher in response to low iron conditions (Mason, 2006; Knight *et al.*, 2005). Data presented here shows that in the CNA6

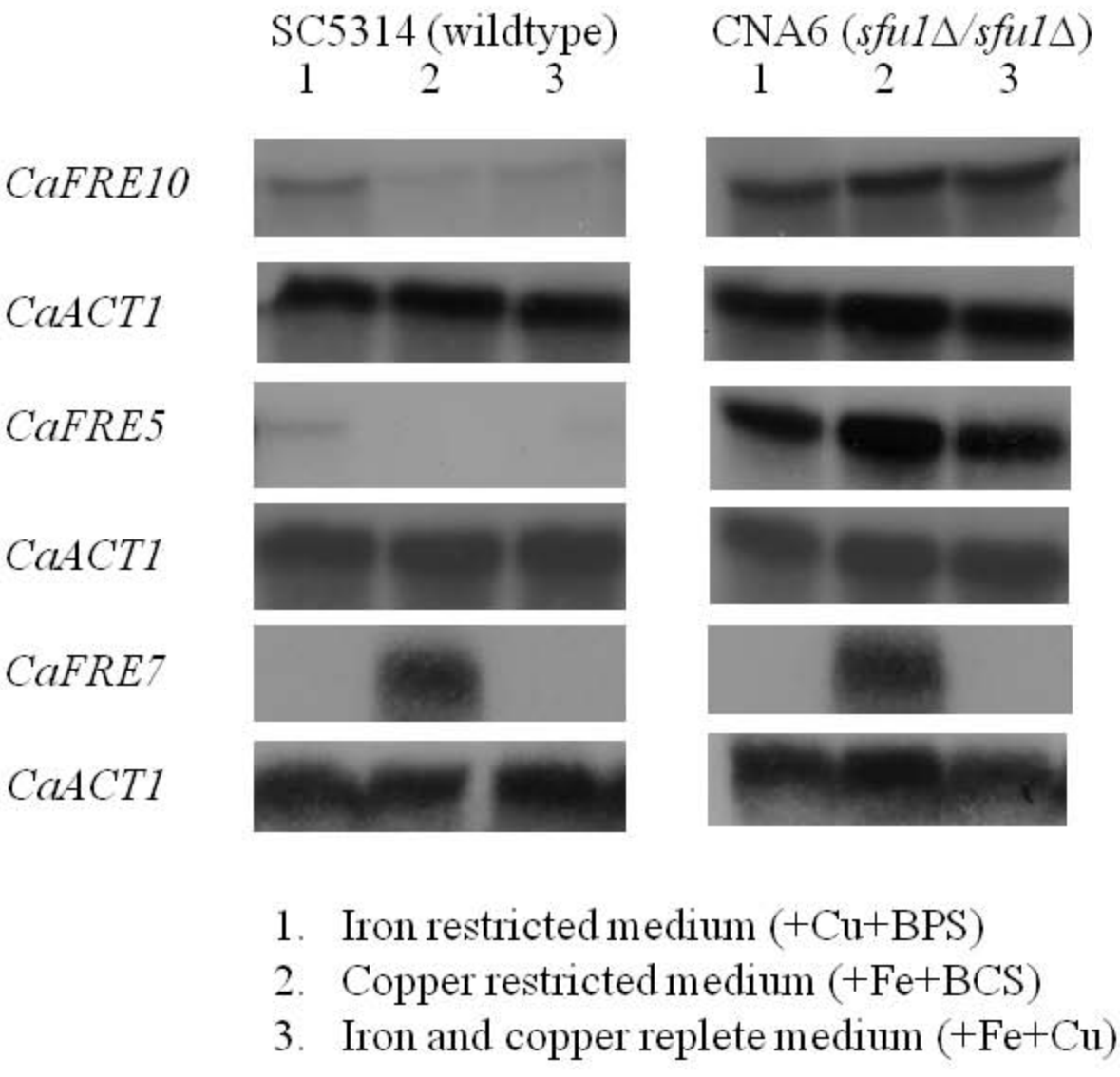


Figure 4.1: Expression of Ferric Reductases is Altered in the CNA6 *sfu1Δ/sfu1Δ* Knockout Mutant: Strains were grown in MD with the addition of 100 μM $CuCl_2$, 100 μM BPS, 100 μM $FeCl_3$, and 100 μM BCS as indicated for 5-6 hours to mid-exponential phase at 30°C with shaking (200 rpm). Cells were harvested and RNA extracted and run on a formaldehyde gel as described in Materials and Methods section 2.5. The RNA was transferred to a nylon membrane and the northern blot was probed with a probe constructed by PCR using either the *CaFRE10F* and *CaFRE10R* primers, the *CaFRE5F* and *CaFRE5R* primers and the *CaFRE7F* and *CaFRE7R* primers. The blots were stripped and reprobed with a probe generated by PCR using the *CaACT1F* and *CaACT1R* primers as a loading control.

(*sfu1Δ/sfu1Δ*) mutant this iron responsive regulation of cell surface ferric reductase activity is not detected (figure 4.2 A). The ferric reductase activity in the CNA6 (*sfu1Δ/sfu1Δ*) is also higher overall than that seen in the wildtype CAF-2 strain. In wildtype CAF-2 strain there is an approximately 2 fold increase of cell surface ferric reductase activity in response to low copper conditions (figure 2.4 B), which is significant in a Tukey's one way ANOVA (P=0.002). This increase is still present, but it is not significant in the CNA6 (*sfu1Δ/sfu1Δ*) mutant possibly because expression is much higher than in wildtype. *CaFRE10* is responsible for ~70% of cell surface ferric reductase activity in *C. albicans* (Mason, 2006, Knight *et al.*, 2005). Given that the expression of *CaFRE10* is so much higher in the CNA6 (*sfu1Δ/sfu1Δ*) mutant, it is not surprising that there is a corresponding increase in cell surface ferric reductase activity in this mutant.

4.4: Cell Surface Cupric Reductase Activity is Increased in *sfu1Δ/sfu1Δ*:

In the wildtype CAF-2 strain, cupric reductase activity is higher when the cells are grown in the iron-restricted or copper-restricted conditions. It was previously found to be approximately 2 fold higher when iron-restricted (Mason, 2006), however, here it is shown to be closer to 1.5 fold higher (figure 4.3 A). In the CNA6 *sfu1Δ/sfu1Δ* mutant there is still a significant increase (Tukey's one way ANOVA) in cupric reductase activity in response to low iron (P=0.001) and low copper (P=0.003) conditions, but the overall cupric reductase activity is significantly higher than is found in the CAF-2 wildtype strain (figure 4.3). This indicates that although Sfu1p represses cupric reductase activity, it is not responsible for the iron or copper responsive regulation of the cupric reductases in *C. albicans*.

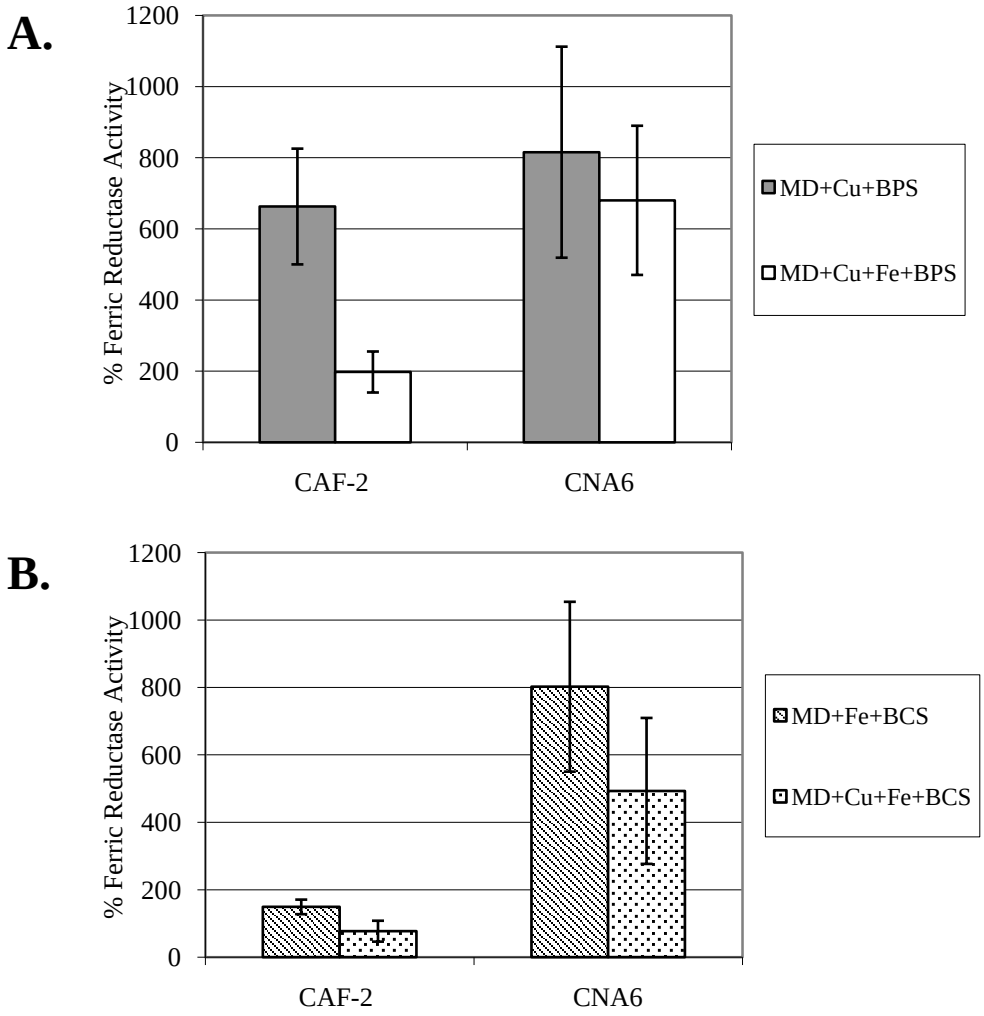


Figure 4.2: Cell Surface Ferric Reductase Activity in CNA6:

Strains were grown in MD with the addition of 100 μM $CuCl_2$, 100 μM BPS, 100 μM $FeCl_3$, and 100 μM BCS as indicated for 5-6 hours to mid-exponential phase at 30°C with shaking (200 rpm). The cell surface ferric reductase activity was then determined as described in Materials and Methods section 2.9. The data is expressed as a % of CAF-2 cell surface ferric reductase activity when grown in MD supplemented with 100 μM $CuCl_2$ and 100 μM $FeCl_3$. The mean values of three reductase assays are shown and the error bars represent standard deviation. A shows iron responsive ferric reductase activity and B shows copper responsive ferric reductase activity.

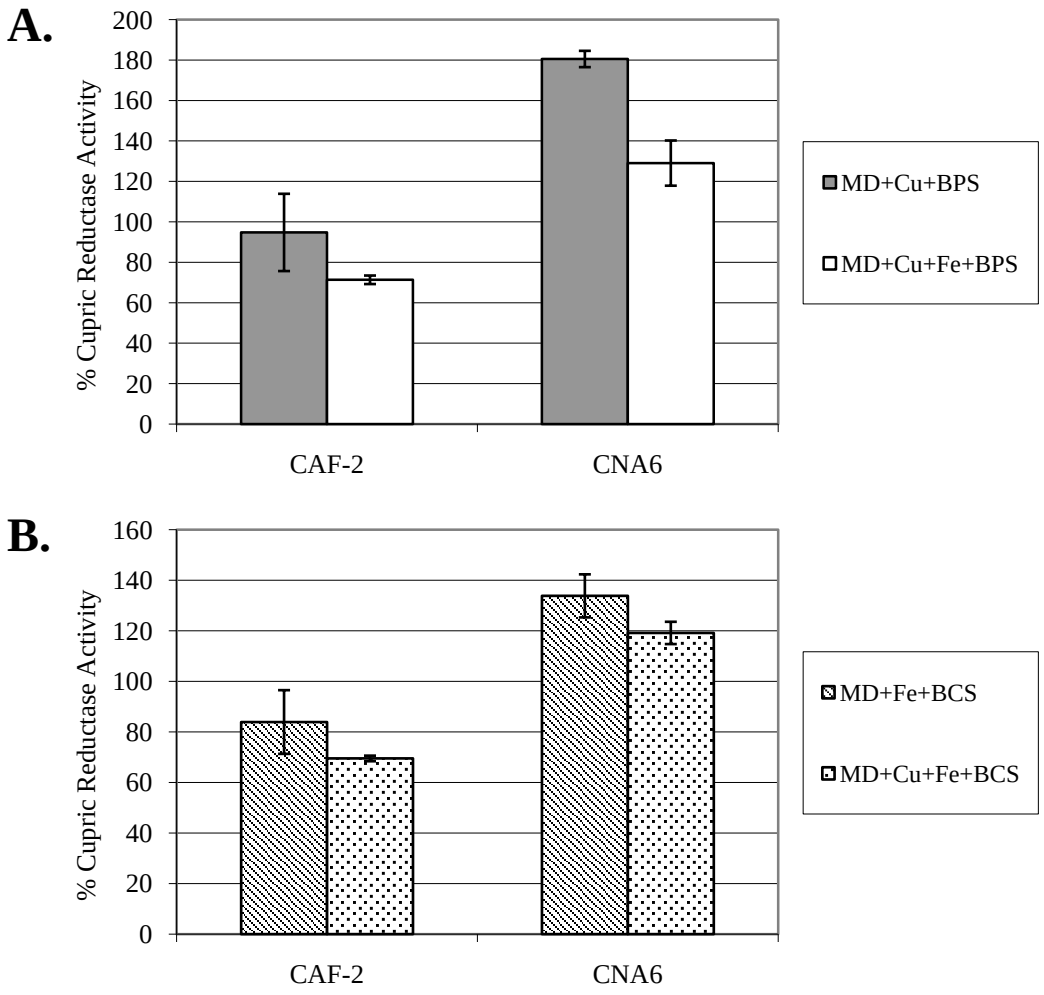


Figure 4.3: Cell Surface Cupric Reductase Activity in CNA6:

Strains were grown in MD with the addition of 100 μM $CuCl_2$, 100 μM BPS, 100 μM $FeCl_3$, and 100 μM BCS as indicated for 5-6 hours to mid-exponential phase at 30°C with shaking (200 rpm). The cell surface cupric reductase activity was then determined as described in Materials and Methods section 2.9. The data is expressed as a % of CAF-2 cell surface ferric reductase activity when grown in MD supplemented with 100 μM $CuCl_2$ and 100 μM $FeCl_3$. The mean values of two reductase assays are shown and the error bars represent standard deviation. A shows iron responsive cupric reductase activity and B shows copper responsive cupric reductase activity.

4.5: Iron Uptake is increased in *sfu1Δ/sfu1Δ*:

Ferric reductase activity is a measure of how much ferric iron is reduced at the cell surface, but does not indicate how much is actually taken up into the cell itself. Radioactive iron uptake assays determine the amount of iron taken up into the cell. The CNA6 (*sfu1Δ/sfu1Δ*) mutant has a rate of iron uptake approximately 5 fold higher than that of wildtype, indicating that it may be involved in the repression of the iron permease genes (figure 4.4 A). The transcription of *CaFTR1*, which encodes the major iron permease (Ramanan & Wang, 2000), is higher in the CNA6 (*sfu1Δ/sfu1Δ*) mutant (figure 4.4 B), indicating that it is repressed by Sfu1p in an iron dependent manner.

4.6: Construction of a β-galactosidase *SFU1* promoter reporter construct and its transformation into *C. albicans* strains:

Previous northern blot analysis had shown that transcription of *SFU1* was increased in response to high copper and high iron conditions (Woodacre *et al.*, 2008). Furthermore, *SFU1* has also been shown to be repressed in some way by CaMac1p, with *SFU1* expression being 3 fold higher in a *Camac1Δ/Camac1Δ* mutant; this is supported by the presence of a CuRE (CaMac1p binding site) in the *SFU1* promoter. CaMac1p has been shown to be an activator of transcription in low copper conditions; the data from the *SFU1* northern blot suggested the possibility of some novel form of regulation by CaMac1p (Woodacre *et al.*, 2008). To further investigate this regulation a more sensitive method of quantifying gene expression was required and to this end a *SFU1* β-galactosidase reporter was constructed. Given the way in which iron and copper uptake

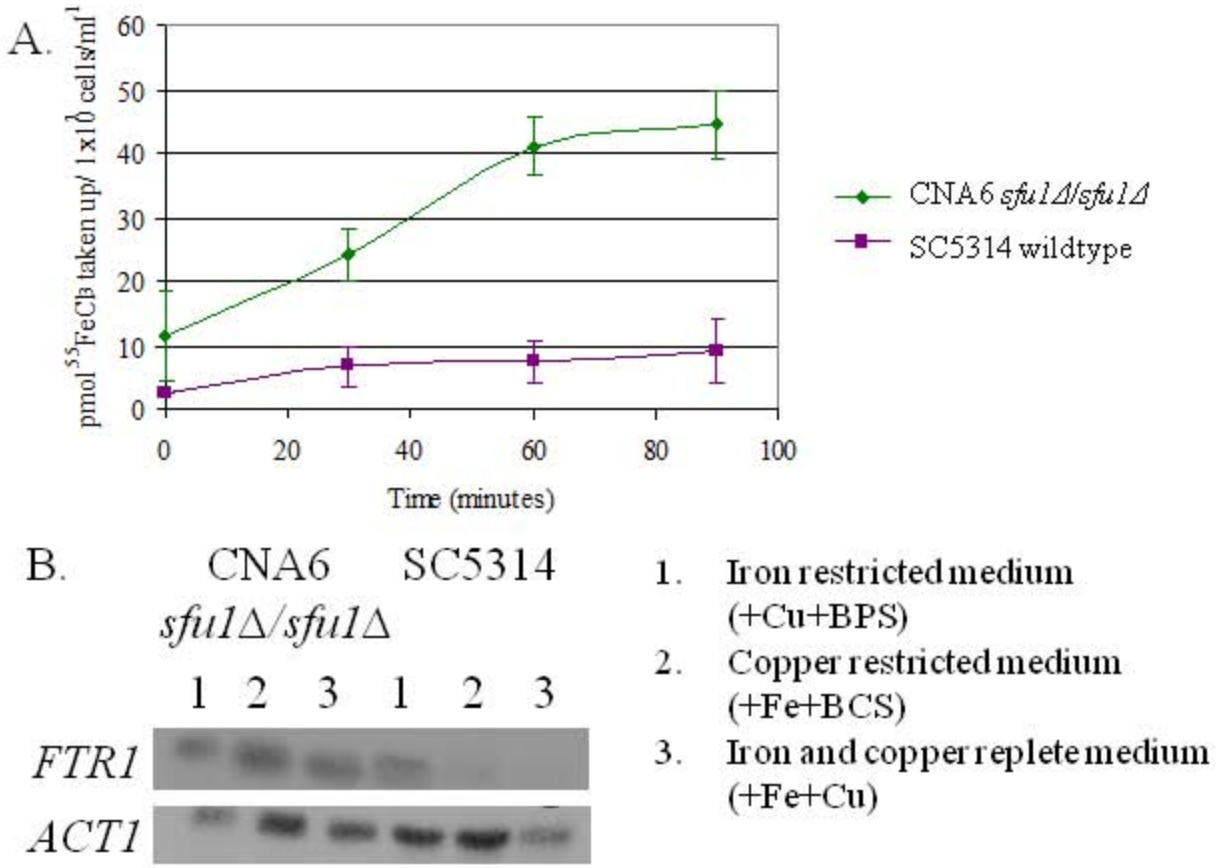


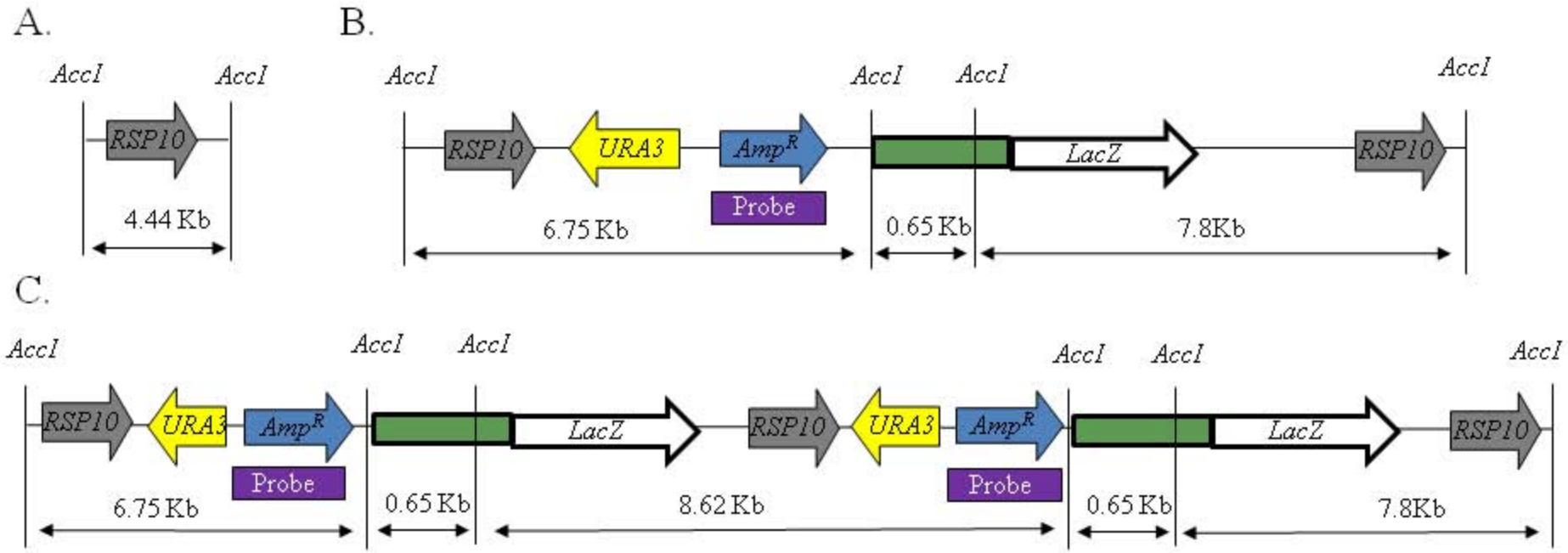
Figure 4.4: Radioactive Iron Uptake and Iron Permease Expression:

A: Cells were grown for 5-7 hours in YPD to log phase then assayed as described in Materials and Methods section 2.10. The data are shown as iron uptake over a period of 90 minutes, the mean of three repeats is shown and the error bars represent standard deviations. B: Strains were grown in MD with the addition of 100 μM $CuCl_2$, 100 μM BPS, 100 μM $FeCl_3$, and 100μM BCS as indicated for 5-6 hours to mid-exponential phase at 30°C with shaking (200 rpm). Cells were harvested, RNA was extracted and run on a formaldehyde gel as described in Materials and Methods section 2.5. The RNA was transferred to a nylon membrane and the northern blot was probed with a probe created by PCR using the *CaFTR1F* and *CaFTR1R* primers. The blots were stripped and re-probed with a probe generated by PCR using the *CaACT1F* and *CaACT1R* primers as a loading control.

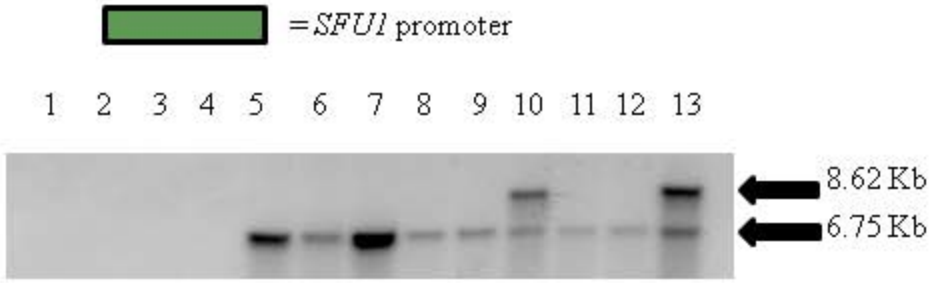
in *C. albicans* are intimately linked and the regulation of *SFU1* by CaMac1p, the regulation of *CaMAC1* by Sfu1p was also investigated.

The *SFU1* β-galactosidase reporter was constructed using placpoly which contains the *lacZ* gene, a *URA3* selectable marker and a region of homology to *RPS10* locus in *C. albicans*. The promoter of interest can be cloned in front of *LacZ* and the construct will integrate into the *C. albicans* genome at the *RPS10* locus. The *SFU1* promoter construct was transformed into the wildtype BWP17 strain (see Materials and Methods table 2.1). It was also transformed into MEM-m2 (*Camac1Δ/Camac1Δ*) to further investigate the regulation of *SFU1* by CaMac1p and into CNA6 (*sfu1Δ/sfu1Δ*) to see if *SFU1* is autoregulated in any way. To see if *CaMAC1* is regulated in any way by Sfu1p a *CaMAC1* promoter β-galactosidase reporter construct (pAWM1; Woodacre *et al.*, 2008), was transformed into CNA6 (*sfu1Δ/sfu1Δ*).

To construct the *SFU1* reporter, the region of the *SFU1* promoter at -3 bp to -962 bp upstream of the start codon, which contains the CaMac1p binding motif, was amplified in a PCR reaction using primers *SFU1 Xho*I F and *SFU1 Xma*I R (Materials and Methods table 2.7) and a proof reading DNA polymerase. The primers contain sites for the *Xho*I and *Xma*I enzymes which are introduced either side of the region of interest. The DNA in the PCR was run on an agarose gel and purified by gel extraction. The purified product was ligated into a pGEMT®-Easy Vector (Promega) following the manufacturer's instructions and transformed into the *E. coli* DH5α strain. The pGEMT®-Easy plasmid with the *SFU1* promoter insert and the placpoly plasmid (table 2.3) were both digested with *Xho*I and *Xma*I enzymes and gel extracted. The digested placpoly was then dephosphorylated and the purified digestion of the *SFU1* promoter



A. Wildtype *RPS10* locus
B. One copy of plapoly *SFU1* promoter inserted into *RPS10*
C. Two copies plapoly *SFU1* promoter inserted into *RPS10*



1. SC5314
2. BWP17
3. CNA6 (*sfu1Δ/sfu1Δ*)
4. MEMm-2 (*Camac1Δ/Camac1Δ*)
5. BWP17 placpoly SFU1 promoter plasmid 1 (RJ3)
6. BWP17 placpoly SFU1 promoter plasmid 2
7. MEM-m2 placpoly SFU1 promoter plasmid 1 (RJ5)
8. MEM-m2 placpoly SFU1 promoter plasmid 2
9. MEM-m2 placpoly SFU1 promoter plasmid 3
10. CNA6 placpoly SFU1 promoter plasmid 1
11. CNA6 placpoly SFU1 promoter plasmid 2 (RJ4)
12. CNA6 pAWM1 1 (RJ6)
13. CNA6 pAWM1 2

Figure 4.5: Southern Blot Confirmation of Integration of Plasmids at the *RPS10* Locus: Strains were grown overnight in 10 ml YPD supplemented with uridine and the DNA was extracted by the rapid yeast chromosomal DNA method described in Materials and Methods. The DNA was quantified by separation on an agarose gel and 5-10μg DNA was digested with *Acc*I and run on a 1% agarose gel for 5 hours at 60 volts. The gel was Southern blotted overnight and the DNA was crosslinked to the membrane as described in Materials and Methods. A probe was constructed by digestion of CIp10 (see materials and methods table 2.3) with *Acl*1. The band containing the Amp^R gene was purified by gel extraction. The probe was then radiolablled and hybridised with the blot overnight in Church Gilberts buffer, then washed in 3xSSC;0.1% SDS and exposed to X-ray film at -80˚C overnight as described in Materials and Methods. The diagram shows the integration of placpoly containing the *SFU1* promoter (placpoly *SFU1* promoter). In lanes 12 and 13 are transformants with integrations of placpoly with the *CaMAC1* promoter (pAMW1). The smaller band is the same size for both placpoly *SFU1* promoter and pAMW1 as the section of DNA does not contain the promoter region inserted into placpoly. However the larger band seen in a double insertion of pAMW1 is 8.99 Kb. Although only 683 bp *CaMAC1* promoter is included, compared with 959 bp *SFU1* promoter, the *SFU1* promoter contains an *Acc*I site that shortens the fragment.

was ligated into the plasmid. The promoter sequence in the plasmid was confirmed in a sequencing reaction using the *lacZ* F primer (Materials and Methods, table 2.7).

The placpoly *SFU1* promoter plasmid was transformed, using the lithium acetate method, into BWP17, CNA6 (*sfu1Δ/sfu1Δ*) and MEM-m2 (*Camac1Δ/Camac1Δ*). The transformation into BWP17 yielded 15 transformants; DNA was extracted (Materials and Methods, section 2.4) from 8 of the transformants and screened for an insert using the PCR with the *RP10F* and *CaURA3R* primers. Two of these were found to have inserts at the *RPS10* locus. Transformation of placpoly *SFU1* promoter plasmid into MEM-m2 resulted in three transformants, all of which were found to have inserts at the *RPS10* locus using PCR with the *RP10F* and *CaURA3R* primers. Transformation of placpoly *SFU1* promoter plasmid into CNA6 resulted in two transformants, both of which were found to have inserts at the *RPS10* locus using PCR with the *RP10F* and *CaURA3R* primers. Transformation of pAWM1 plasmid into CNA6 resulted in two transformants, both of which were found to have inserts at the *RPS10* locus using PCR with the *RP10F* and *CaURA3R* primers. The positive transformants were then tested for double insertions using Southern blotting (figure 4.5). Both of the placpoly *SFU1* promoter BWP17 transformants were found to have only a single insertion in the *RPS10* locus. The first of these on figure 4.5 was selected for further study and called RJ3. One of the two placpoly *SFU1* promoter CNA6 transformants was found to have a single insertion in the *RPS10* locus, this was named RJ4. All three of the placpoly *SFU1* promoter MEM-m2 transformants were found to have a single insertion in the *RPS10* locus. The first of these on figure 4.5 was selected for further study and called RJ5. One of the two pAWM1 CNA6 transformants was found to have a single insertion in the *RPS10* locus, this was named RJ6.

4.7: Investigation of the regulation of *SFU1* by CaMac1p and Sfu1p using a β-galactosidase reporter construct:

Expression of *SFU1* had previously been shown in our laboratory by northern blot analysis to be increased in response to high iron conditions compared to low iron conditions (Woodacre *et al.,* 2008). As was shown in the northern blots, in the wildtype (RJ3) *SFU1* expression is significantly increased (Student's t-test at 5% level) in high iron conditions compared to low iron conditions (figure 4.6 A). This iron sensitive regulation is significant in the *Camac1Δ/Camac1Δ* strain, but overall expression of *SFU1* is much higher. The expression of *SFU1* is also much higher in the *sfu1Δ/sfu1Δ* strain, RJ5, and the iron sensitive regulation is not observed.

Previous northern blot analysis has shown that expression of *SFU1* was also increased in response to high copper conditions compared to low copper conditions and increased in the MEM-m2 (*Camac1Δ/Camac1Δ*) mutant (Woodacre *et al.,* 2008). Contrary to the northern blot analysis, expression of *SFU1* was seen here to be significantly increased in low copper conditions (Student's t-test at 5% level) compared to copper replete conditions in the wildtype strain (RJ3, figure 4.6 B). The copper sensitive expression is lost in both the *Camac1Δ/Camac1Δ* strain (RJ4) and the *sfu1Δ/sfu1Δ* strain (RJ5) and expression of *SFU1* in both these mutants is higher than in the wildtype.

4.8: Investigation of the regulation of *CaMAC1* by Sfu1p using a β-galactosidase reporter construct:

It has previously been demonstrated in our laboratory that expression of *CaMAC1* was

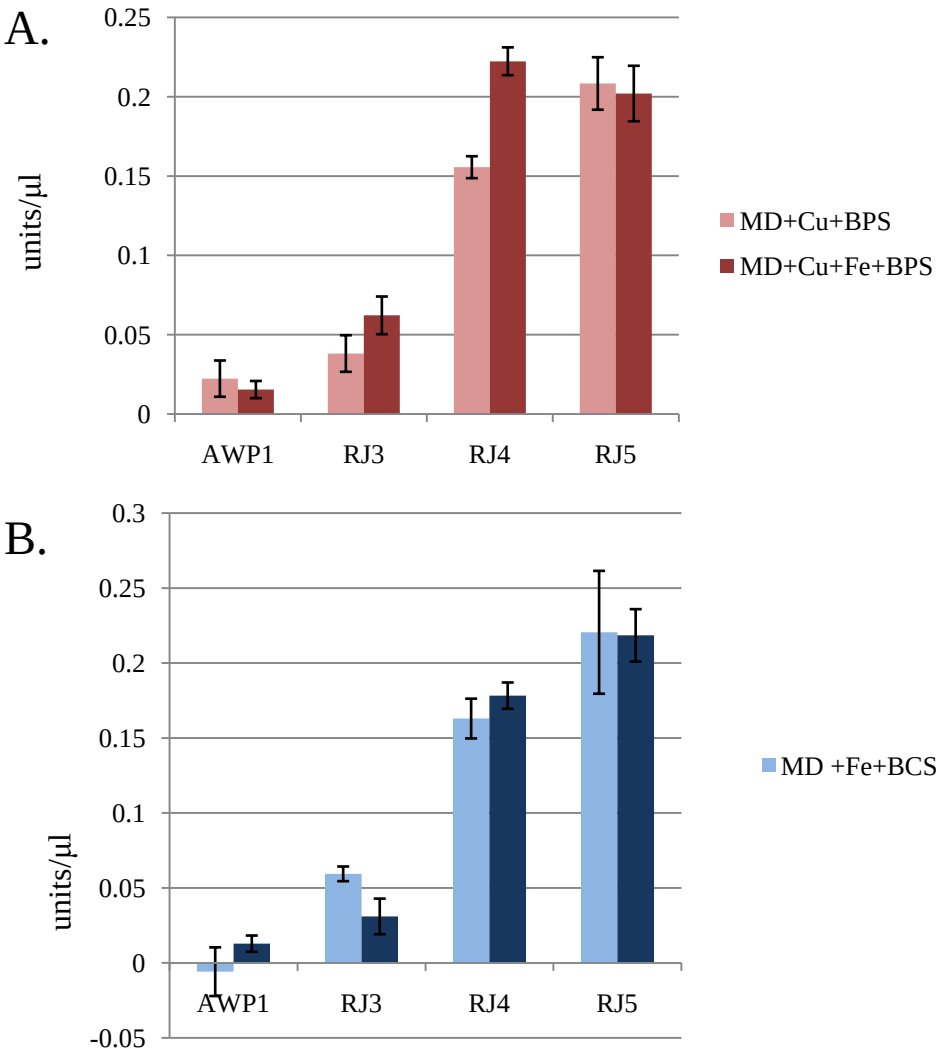


Figure 4.6: Expression *SFU1* Reporter Construct in RJ3 wildtype, RJ4 *Camac1Δ/Camac1Δ* and RJ5 *sfu1Δ/sfu1Δ*: Strains were grown in MD with the addition of 100 µM $CuCl_2$, 100 µM BPS, 100 µM $FeCl_3$, and 100 µM BCS as indicated for 5-6 hours to mid-exponential phase at 30°C with shaking (200rpm). The β-galactosidase activity was then determined as described in Materials and Methods section 2.11. The mean values of three assays are shown and the error bars represent standard deviation.

A. shows changes in expression in response to iron . B.shows changes in expression in response to copper. AWP1 is a control strain containing the placpoly plasmid without any promoter inserted (see Materials and Methods table 2.1).

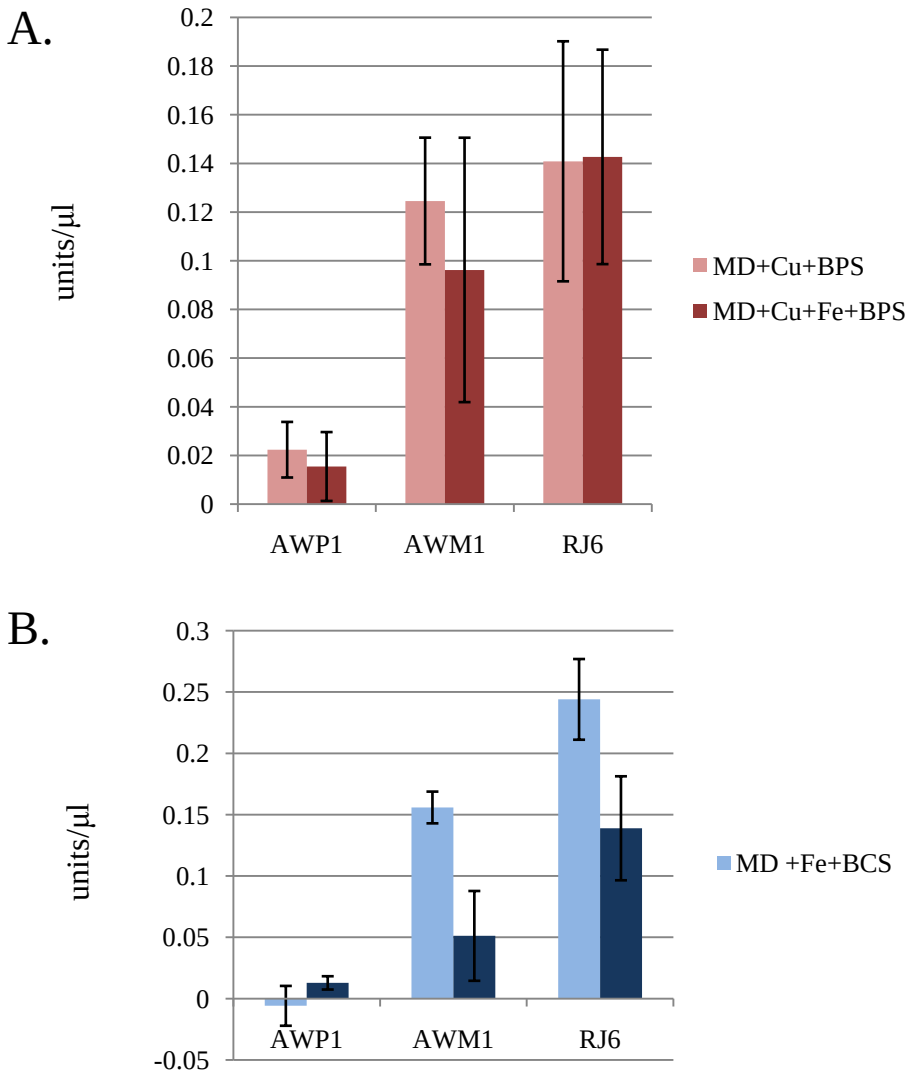


Figure 4.7: A *CaMAC1* Reporter Construct in CNA6 *sfuΔ/sfu1Δ*:

Strains were grown in MD with the addition of 100µM $CuCl_2$, 100µM BPS, 100µM $FeCl_3$, and 100µM BCS as indicated for 5-6 hours to mid-exponential phase at 30°C with shaking (200rpm). The β-galactosidase activity was then determined as described in Materials and Methods section 2.11. The mean values of three assays are shown and the error bars represent standard deviation. A. shows changes in expression in response to iron. B. shows changes in expression in response to copper. AWP1 is a control strain containing the placpoly plasmid without any promoter inserted (see Materials and Methods table 2.1).

increased in response to low copper conditions and that expression was increased in a *Camac1Δ/Camac1Δ* mutant compared to a wildtype strain (Woodacre *et al.*, 2008). Given that CaMac1p has been shown to regulate *SFU1* in some way, it was clear that the expression of these two transcription factors was linked. Consequently the regulation of *CaMAC1* by Sfu1p was investigated by transformation of a *CaMAC1* reporter construct into CNA6 (*sfu1Δ/sfu1Δ*). It was found that in CNA6 (*sfu1Δ/sfu1Δ*) the expression of *CaMAC1* was increased ~1.7 fold (figure 4.7 B). The ~2 fold increase in response to copper was significant (Tukey's one way ANOVA, $P<0.001$) in both AWM1 (wildtype) and RJ6 (*sfu1Δ/sfu1Δ*). No changes in response to iron levels were detected.

4.9: Discussion:

The data from the *sfu1Δ/sfu1Δ* mutant indicates that high affinity iron uptake is regulated using a GATA type transcription factor in a manner comparable to that found in a number of other fungi. The product of the *URBS1* gene that was used to identify *SFU1* (Lan *et al.*, 2004) has been shown to repress *fer1*, a multicopper oxidase, *fer2*, a high affinity iron permease and *fer9* a ferric reductase in *U. maydis* (Eichhorn *et al.*, 2006). The *SREA* gene encodes a GATA-type transcription factor in *A. nidulans*, when it is deleted this causes an increase in iron uptake in a similar manner to iron uptake in the CNA6 *sfu1Δ/sfu1Δ* mutant (Haas *et al.*, 1999b). Furthermore in an *SREA* deletion mutant the expression of *FREA*, a ferric reductase, is increased compared to a wildtype strain. However the metalloregulation is still present, indicating another regulatory element at work (Oberegger *et al.*, 2002). This other system of iron responsive regulation in *A. nidulans* was identified as a complex that binds the consensus sequence

CCAAT called the CBF binding complex. When *HAPC*, a gene encoding a CCAAT-Binding subunit, or *HAPX* in *A. nidulans* is knocked out a number of iron regulated genes are deregulated including *SREA* itself.

In the *sfu1Δ/sfu1Δ* mutant iron responsive cupric reductase activity is still detected, indicating that Sfu1p is not the only source of iron responsive regulation of cell surface cupric reductase activity in *C. albicans*. It is possible that this iron responsive regulation is attributable to the recruitment of Hap43p to the CBF binding complex which has previously been shown to activate expression of *FRP1* (a putative ferric reductase) in response to low iron conditions (Baek *et al.*, 2008). To investigate this further the construction of a *sfu1ΔΔ/hap43ΔΔ* mutant would be desirable to see if the iron responsive regulation of cupric reductase activity was completely abolished.

The higher level of *CaFRE10* transcription in the *sfu1Δ/sfu1Δ* mutant (figure 3.1) was similar to the finding previously that *CaFRE10* transcription is higher and metalloregulation is lost in a *Catup1Δ/Catup1Δ* mutant (Knight *et al.*, 2002). The gene *CaTUP1* encodes a regulator involved in the expression of genes with a wide variety of functions, however it tends to work in concert with other proteins such as Ssn6p and Nrg1p (Garcia-Sanchez *et al.*, 2005). In *S. pombe* Fep1p, the GATA-type transcription factor, has been shown to require Tup11p or Tup12p and iron to bind DNA and this is postulated to be how iron sensitive regulation functions in this organism (Pelletier *et al.*, 2002; Mercier *et al.*, 2006; Znaidi *et al.*, 2004). The protein encoded by the *CaTUP1* gene, performs an analogous global regulator role to *S. pombe TUP11* (Knight *et al.*, 2002; Braun *et al.*, 2000). In the heterologous *S. pombe* system Sfu1p has been shown to interact with the Tup11 and Tup12 proteins (Pelletier *et al.*, 2007). This provides

evidence to support the hypothesis that *CaFRE10* is regulated by both Sfu1p and Tup1p working together as is seen in the homologous *S. pombe* system.

As the iron uptake system is so intimately linked with that of copper uptake (Knight *et al.,* 2002; Stearman *et al.,* 1996; Ardon *et al.,* 2001; Marvin *et al.,* 2003) it would seem likely that copper sensitive regulation would also play a part in regulating high affinity iron uptake. It has recently been shown in our lab that *SFU1* itself is regulated by CaMac1p and is derepressed in a *mac1Δ/mac1Δ* mutant (Woodacre *et al.,* 2008). These data were confirmed in this study using a reporter construct. The copper sensitive regulation of *SFU1* is not detected in the *Camac1Δ/Camac1Δ* strain, even though the overall levels of expression are higher than in the wildtype indicating that CaMac1p may be responsible for this regulation, but perhaps not in its usual role as an activator of transcription. The copper sensitive regulation is not observed in the *sfu1Δ/sfu1Δ* strain and the expression of *SFU1* is higher than in wildtype indicating that *SFU1* is also self regulated. This suggests that the response to low iron conditions may be part of a wider regulatory cascade.

The regulation of these two intimately linked transcription factors is further complicated by the increase in expression of *CaMAC1* in an *sfu1Δ/sfu1Δ* mutant strain indicating that Sfu1p directly or indirectly regulates *CaMAC1*. In an *SFU1* microarray study (Lan *et al.,* 2004) it was shown that the multicopper oxidases *CaFET34* and *CaFET35* were derepressed in the *sfu1Δ/sfu1Δ* mutant and it is known levels of multicopper oxidase protein produced is increased because they are required for the uptake of iron and radioactive iron uptake is greatly increased in *sfu1Δ/sfu1Δ* mutant. The increase in multicopper oxidase activity in the cell would increase demand for copper since the

multicopper oxidase proteins require copper to function. CaMac1p is an activator of transcription and one of the genes it activates is *CaCTR1* which encodes a copper transporter which has increased expression in low copper conditions to transport copper into the cell. In the *sfu1Δ/sfu1Δ* mutant the increase in iron uptake would lead to an increase in multicopper oxidase activity that may lead to copper starvation and this in turn may lead to an increase in *CaMAC1* expression as the CaMac1p is required to activate *CaCTR1*.

Regulation in response to iron and copper is clearly very complex and interdependent. Further studies involving identifying the regulatory element responsible for the increase in *SFU1* expression in response to high iron conditions and investigating increases in expression in response to high iron conditions in general is required to give a fuller picture of iron responsive regulation in *C. albicans*. Further investigation of the role of the global repressor Tup1p would also provide more insight into the nature of iron responsive regulation in *C. albicans* since Tup1p has been shown to repress iron responsive genes, is a repressor of filamentation and has been shown to be involved in the regulation of *CaFRE7*, the copper regulated ferric reductase (Knight *et al.*, 2002; Levitin & Whiteway, 2007). Furthermore microarray studies investigating the genome wide iron responsive regulation by the CBF complex would shed light on what iron responsive regulation can be attributed to repression by Sfu1p and what can be attributed to activation by the recruitment of Hap43p to the CBF complex.

Chapter 5: The Role of Ferric Reductases in the Hyphal Form of *Candida albicans*.

5.1: Introduction:

All the previous work to characterize the high affinity iron uptake system in *Candida albicans* has been carried out in the yeast form. However, this is only one morphological form of *C. albicans* and all three morphological forms; yeast, hyphae and pseudohyphae are required for virulence (Braun & Johnson, 1997; Lo *et al.* 1997; Rocha *et al.* 2001). Furthermore the regulatory networks that control the yeast hyphal switch have also been shown to regulate other virulence determinants which are unrelated to morphology, such as adhesins and extracellular hydrolytic enzymes (Biswas *et al.* 2007; Kumamoto & Vinces, 2005). It has been suggested that the different morphological forms are required for different stages of infection; the yeast form of *C. albicans* for dissemination in the blood stream and the hyphal form for penetration of host tissues and cells because of pressure exerted by the growing hyphal tip (Kumamoto & Vinces, 2005; Gow *et al.* 2002; Whiteway & Oberholzer, 2004).

The iron sensitive regulation of the major ferric reductase and the major iron permease has been shown to be mediated by Sfu1p, which is thought to act by binding CaTup1p (Knight *et al.* 2002; Knight *et al.* 2005; Mason, 2006, Lan *et al.* 2004; Pelletier *et al.* 2007; this study, chapter 4). The global repressor CaTup1p has been shown to be recruited to Nrg1p, Mig1p and Rfg1p to repress filamentation in non inducing

conditions (Biswas *et al.* 2007; Murad *et al.* 2001; Murad *et al.* 2001). Given that both the yeast hyphal switch and high affinity iron uptake have been shown to have an effect on the virulence of *C. albicans* and that regulation of both pathways share the regulator CaTup1p (Braun & Johnson, 1997; Knight *et al.* 2002; Pelletier *et al.* 2007; Braun *et al.* 2000; Braun & Johnson, 2000; Ramanan & Wang, 2000), it is possible that the genes encoding the high affinity iron uptake system may be expressed differently in the hyphal form of *C. albicans* compared with the yeast form.

5.2: Expression of Ferric Reductases in Hyphae:

The two main cell surface ferric and cupric reductases are *CaFRE10* and *CaFRE7*. To see if the expression of these was altered in any way in hyphae compared with yeast, Northern blot experiments were performed for the yeast and hyphal forms of a wild type strain. In addition northern blot analysis of *CaFRE10* and *CaFRE7* was carried out in the CNA6 (*sfu1Δ/sfu1Δ*) and MEM-m2 (*Camac1Δ/Camac1Δ*) mutants in yeast and hyphae. The RNA extraction method used previously for yeast (see Materials and Methods, section 2.4), when applied to hyphae resulted in a much lower yield, this was overcome by increasing the amount of biomass used for each extraction and by increasing the number of freeze thaw steps in the protocol. The expression of *CaFRE10* in the SC5314 wild type strain in low iron conditions is reduced in the hyphal form of *C. albicans* compared to the yeast form (see figure 5.1 A). In both yeast and hyphae the overall expression of *CaFRE10* is higher in CNA6 compared to wild type and iron responsive regulation cannot be detected, indicating Sfu1p is still responsible for iron responsive repression of *CaFRE10* in both yeast and hyphae (figure 5.1 B). The expression of *CaFRE7* has previously been demonstrated to be increased in response to

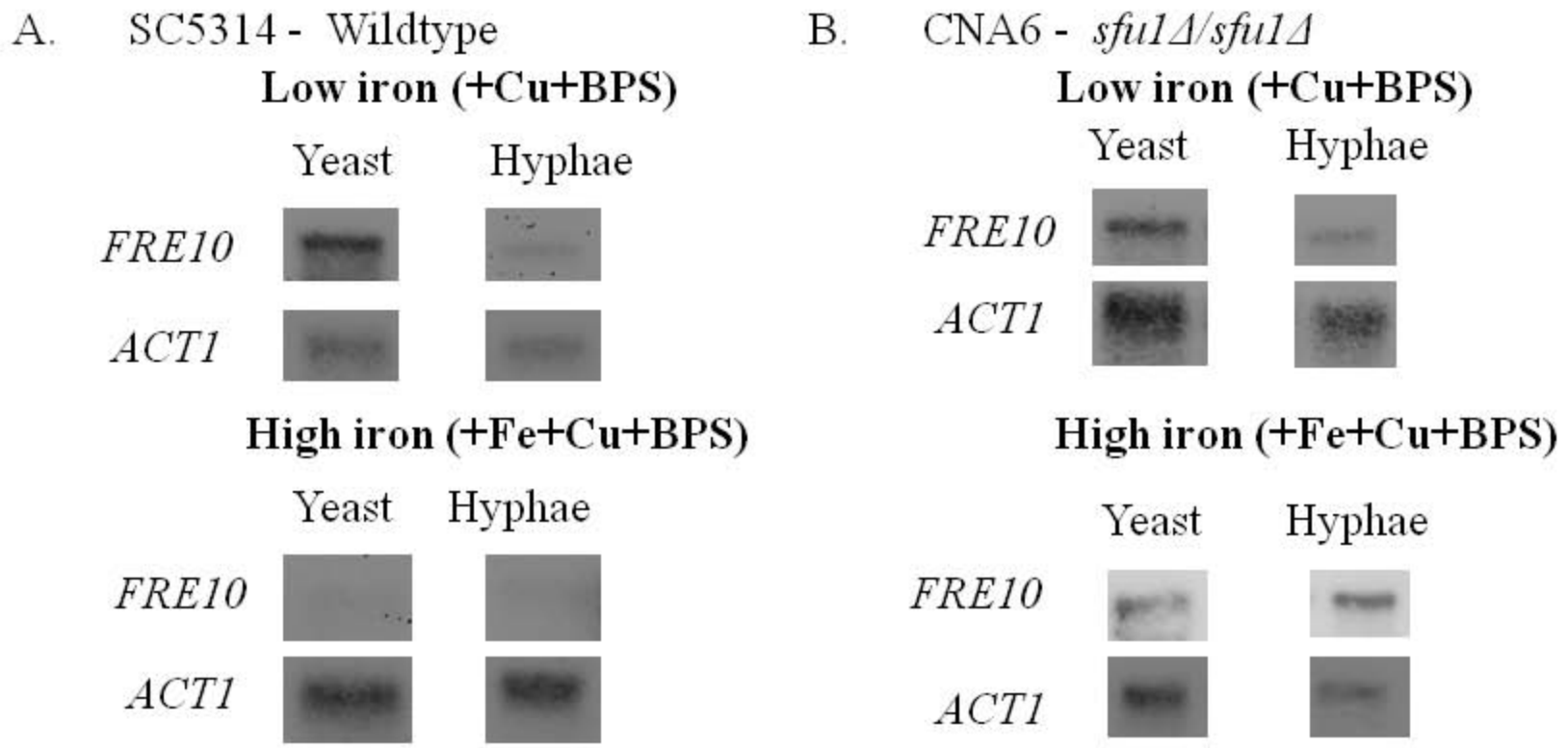


Figure 5.1: Expression of *CaFRE10* in Yeast and Hyphae: Strains were grown in YPD for 6 hours with the addition of 100 μM $CuCl_2$, 100 μM $FeCl_3$ and 100 μM BPS as indicated above. Yeast cells were grown for 6 hours at 30°C, hyphae were induced by the addition of 20% serum and growth at 37°C for 6 hours. Cells were harvested and RNA extracted and run on a formaldehyde gel and blotted as described in Materials and Methods section 2.5. A probe constructed by PCR using either the *CaFRE10F* and *CaFRE10R* primers. The blots were stripped and re-probed with a probe generated by PCR using the *CaACT1F* and *CaACT1R* primers as a loading control. A. Compares expression in yeast and in hyphae in wildtype. B. Compares expression in yeast and hyphae in *sfu1Δ/sfu1Δ*.

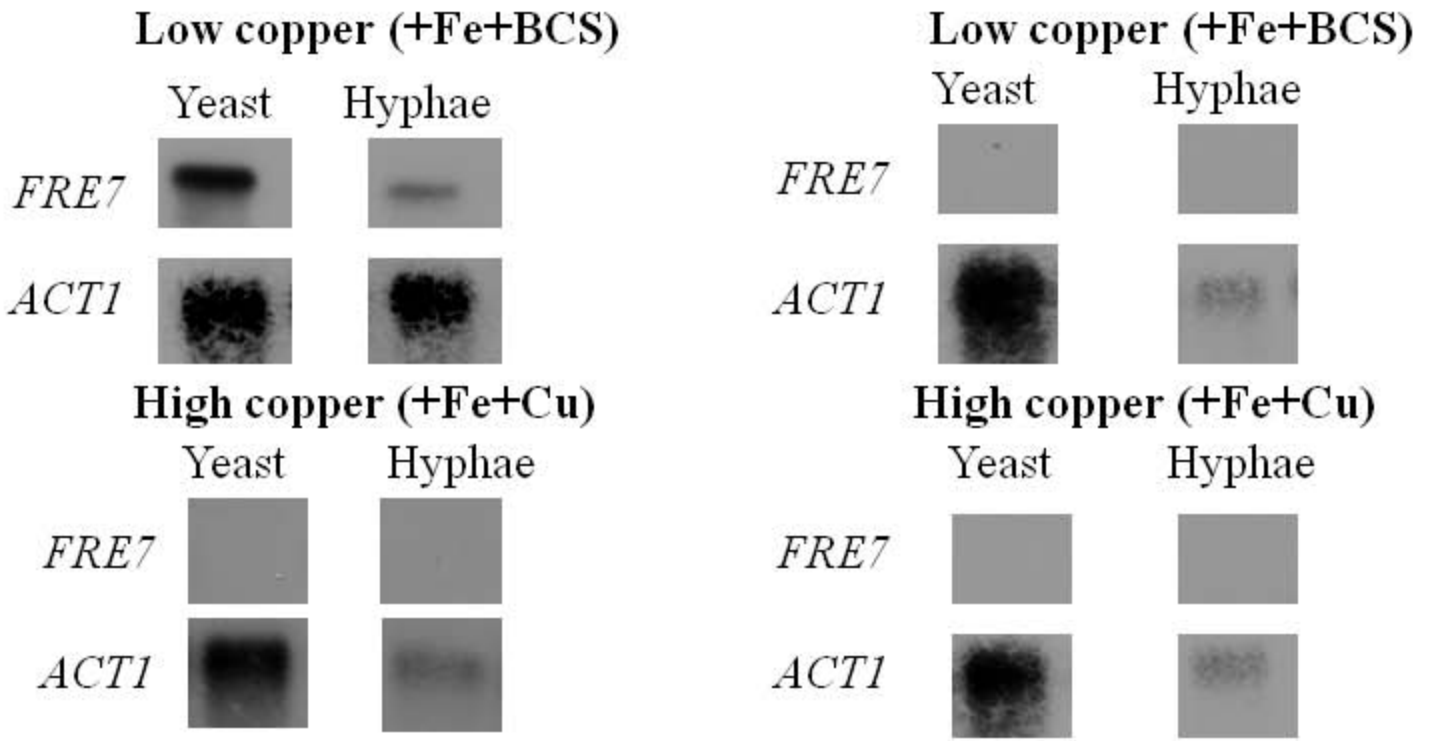


Figure 5.2: Expression of *CaFRE7* in Yeast and Hyphae: Strains were grown in YPD for 6 hours with the addition of 100 μM $CuCl_2$, 100 μM $FeCl_3$ and 600 μM BCS as indicated above. Yeast cells were grown for 6 hours at 30°C, hyphae were induced by the addition of 20% serum and growth at 37°C for 6 hours. Cells were harvested and RNA extracted and run on a formaldehyde gel and blotted as described in Materials and Methods section 2.5. A probe constructed by PCR using either the *CaFRE7F* and *CaFRE7R*. The blots were stripped and re-probed with a probe generated by PCR using the *CaACT1F* and *CaACT1R* primers as a loading control. A. Compares expression in yeast and in hyphae in wildtype. B. Compares expression in yeast and hyphae in *Camac1Δ/Camac1Δ*.

low copper conditions (Levitin & Whiteway, 2007; Woodacre *et al.* 2008). The expression of *CaFRE7* in the wild type SC5314 strain is lower in hyphae compared to yeast (figure 5.2 A). No expression of *CaFRE7* is detected in the MEM-m2 (*Camac1Δ/Camac1Δ*) mutant in yeast or hyphae (figure 5.3 B).

5.3: Comparison of Cell Surface Ferric and Cupric Reductase Activity in Yeast and Hyphae:

Ferric Reductase Activity

In the wild type CAF-2 strain, the cell surface ferric reductase activity is increased in response to low iron conditions. In low iron conditions the activity is approximately 2 fold lower in hyphae compared to yeast, although the increase compared to high iron is still significant (Tukey's one way ANOVA, $P<0.001$) in both yeast and hyphae (figure 5.3 A). The RM10.5 (*Cafre10Δ/Cafre10Δ*) mutant shows a significant decrease in cell surface ferric reductase activity in both yeast and hyphae compared to a wild type strain, although some iron responsive activity remains (figure 5.3 A). The RJ1.9 (*Cafre7Δ/Cafre7Δ*) mutant shows no significant differences in iron responsive cell surface ferric reductase activity between yeast and hyphae, which is surprising given that the wild type CAF-2 shows a smaller increase in activity in response to low iron in hyphae.

In the wild type CAF-2 strain in yeast there is a significant (Tukey's one way ANOVA,

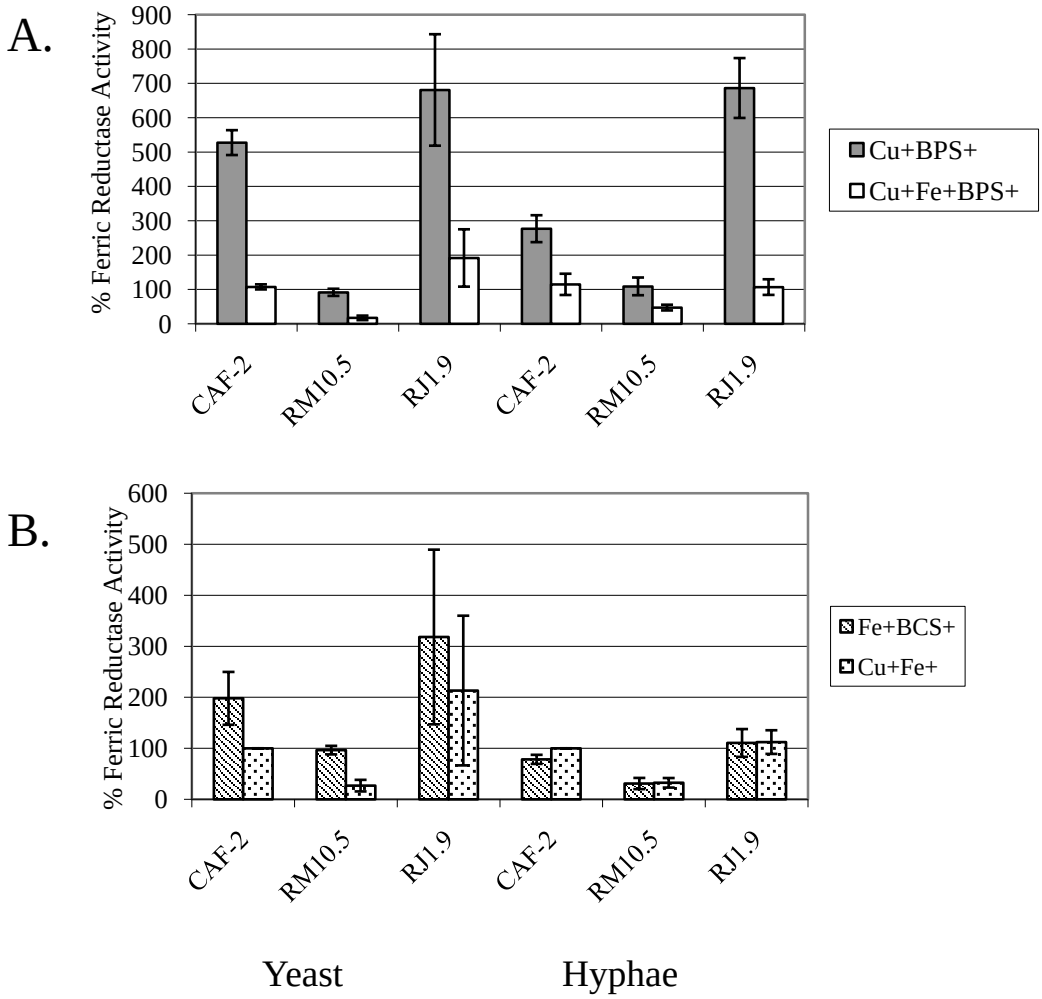


Figure 5.3: Differences in Cell Surface Ferric Reductase Activity in Yeast and Hyphae in Ferric Reductase Mutants: Strains were grown in YPD for 6 hours with the addition of 100 μM $CuCl_2$, 100 μM $FeCl_3$, 100 μM BPS and 300 μM BCS as indicated above. Yeast cells were grown for 6 hours at 30ºC, hyphae were induced by the addition of 20% serum and growth at 37ºC for 6 hours. Once cells were grown up a ferric reductase assay was performed as described in Materials and Methods section 2.9. Results are shown as a % of the ferric reductase activity of CAF-2 grown as yeast with the additions of 100μM $CuCl_2$ and 100μM $FeCl_3$. A mean of 3 repeats is shown, the error bars represent standard deviation. A. Iron responsive ferric reductase activity. B. Copper responsive ferric reductase activity. CAF-2 is the wildtype, RM10.5 is *Cafre10Δ/Cafre10Δ* and RJ1.9 is *Cafre7Δ/Cafre7Δ*.

P<0.001) increase in cell surface ferric reductase activity in response to low copper conditions (figure 5.3 B). In hyphae this increase in response to low copper is not detected. In yeast the RM10.5 (*Cafre10Δ/Cafre10Δ*) mutant shows lower cell surface ferric reductase activity than wild type, but the activity still increases significantly in response to low copper conditions, in hyphae RM10.5 cell surface ferric reductase activity is still lower than wild type and again the increase in response to low copper is not detected. In its yeast form the RJ1.9 (*Cafre7Δ/Cafre7Δ*) mutant does not show the same increase in response to low copper conditions as the wild type has and the response to low copper is also absent in its hyphal form.

Cupric Reductase Activity

In the wild type CAF-2 strain, when grown as yeast, the cell surface cupric reductase activity increases significantly (Tukey's one way ANOVA, P<0.001) in response to low iron conditions, in hyphae this increase in response to low iron is still significant but cupric reductase activity is approximately 1.5 fold higher in low iron conditions (figure 5.4 A). In the yeast form, the RM10.5 (*Cafre10Δ/Cafre10Δ*) mutant shows much lower cupric reductase activity than the wild type strain and iron responsive regulation is not detected. However, in hyphae the cell surface cupric reductase activity is comparable to wild type in the RM10.5 (*Cafre10Δ/Cafre10Δ*) mutant, indicating that *CaFRE10* is not responsible for cell surface cupric reductase activity in hyphae. RJ1.9 (*Cafre7Δ/CAfre7Δ*) mutant shows no difference to wild type in either yeast or hyphae in response to iron.

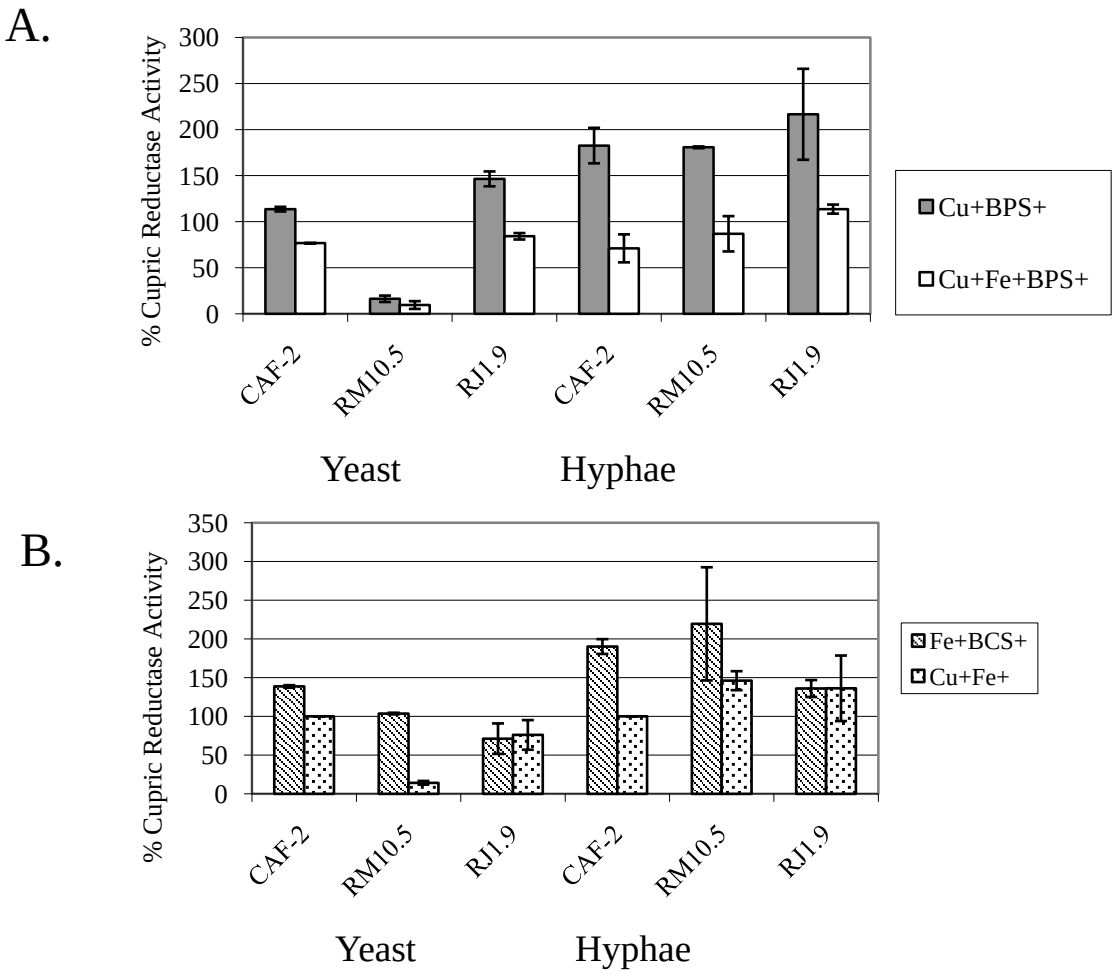


Figure 5.4: Differences in Cell Surface Cupric Reductase Activity in Yeast and Hyphae in Ferric Reductase Mutants: Strains were grown in YPD for 6 hours with the addition of 100 μM $CuCl_2$, 100 μM $FeCl_3$, 100 μM BPS and 300 μM BCS as indicated above. Yeast cells were grown for 6 hours at 30ºC, hyphae were induced by the addition of 20% serum and growth at 37ºC for 6 hours. Once cells were grown up a cupric reductase assay was performed as described in Materials and Methods section 2.9. Results are expressed as a % of cell surface cupric reductase activity of CAF-2 grown as yeast with the addition of of 100 μM $CuCl_2$ and 100 μM $FeCl_3$. A mean of two repeats is shown with error bars representing standard deviation. A. Iron responsive cupric reductase activity. B. Copper responsive cupric reductase activity. CAF-2 is the wildtype, RM10.5 is *Cafre10Δ/Cafre10Δ* and RJ1.9 is *Cafre7Δ/Cafre7Δ*.

In the wild type CAF-2 strain, when grown as the yeast form, the cell surface cupric reductase activity increases in response to copper restricted conditions, in hyphae this increase in response to low copper is still seen but overall cupric reductase activity is higher (figure 5.3 A). In yeast in the RM10.5 (*Cafre10Δ/Cafre10Δ*) mutant cupric reductase activity is much lower than in the wild type, but increase in response to low copper is significant (Tukey's one way ANOVA, P=0.001). However, in hyphae the cell surface cupric reductase activity is comparable to wild type in the RM10.5 (*Cafre10Δ/Cafre10Δ*) mutant. When grown as both yeast and hyphae, the RJ1.9 (*Cafre7Δ/Cafre7Δ*) mutant does not show a copper responsive increase in cell surface cupric reductase activity, indicating that CaFre7p is important for copper responsive cupric reductase activity in both yeast and hyphae.

5.4: Comparison of the Regulation of Cell Surface Ferric and Cupric Reductase Activity in Yeast and Hyphae:

Ferric Reductase Activity

Cell surface ferric reductase activity in a CNA6 (*sfu1Δ/sfu1Δ*) mutant is increased significantly (Tukey's one way ANOVA, P=0.001) compared to the wild type in low iron and low copper conditions in both yeast and hyphae indicating that Sfu1p still has a role to play in iron responsive regulation in the hyphal form of *C. albicans* (figure 5.5). In the MEM-m2 (*Camac1Δ/Camac1Δ*) mutant, in the yeast form, the cell surface ferric reductase activity is increased significantly (Tukey's one way ANOVA, P<0.001) compared to wild type, this has been postulated to be because the inactivation of the

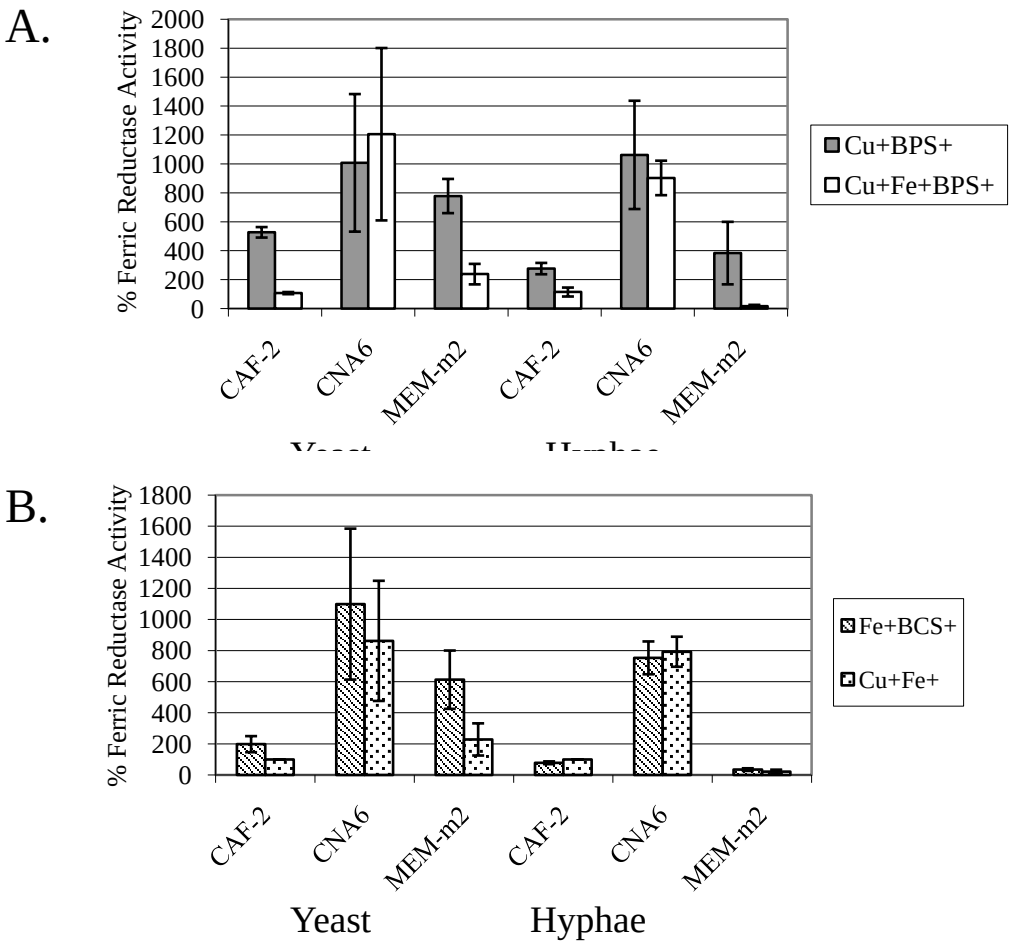


Figure 5.5: Differences in Cell Surface Ferric Reductase Activity in Yeast and Hyphae in CNA6 and MEM-m2: Strains were grown in YPD for 6 hours with the addition of 100 μM $CuCl_2$, 100 μM $FeCl_3$, 100 μM BPS and 300 μM BCS as indicated above. Yeast cells were grown for 6 hours at 30°C, hyphae were induced by the addition of 20% serum and growth at 37°C for 6 hours. Once cells were grown up a ferric reductase assay was performed as described in Materials and Methods section 2.9. Results are shown as a % of the ferric reductase activity of CAF-2 grown as yeast with the additions of 100 μM $CuCl_2$ and 100 μM $FeCl_3$. A mean of 3 repeats is shown, the error bars represent standard deviation. A. Iron responsive ferric reductase activity. B. Copper responsive ferric reductase activity. CAF-2 is the wildtype, CNA6 is *sfu1Δ/sfu1Δ* and MEM-m2 is *Camac1Δ/Camac1Δ*.

copper transporter CaCtr1p in the MEM-m2 (*Camac1Δ/Camac1Δ*) strain causes copper deficiency which in turn causes an iron deficiency due to the requirement of the high affinity iron uptake system for copper (see Chapter 1, section 1.5) and this causes an increase in the ferric and cupric reductase activity (Mason, 2006). In hyphae cell surface ferric reductase activity is much lower in the MEM-m2 (*Camac1Δ/Camac1Δ*) mutant, which may be due to the decrease in expression of *CaFRE10* in hyphae. The increase in response to low copper conditions not observed in the MEM-m2 (*Camac1Δ/Camac1Δ*) mutant in hyphae, although there is still a significant (Tukey's one way ANOVA, $P<0.001$) increase in response to low iron conditions.

Cupric Reductase Activity

In the CNA6 (*sfu1Δ/sfu1Δ*) mutant, when grown as yeast, the cell surface cupric reductase activity is approximately 2 fold higher than it is in wild type and the regulation in response to iron is not detected, however in hyphae the cell surface cupric reductase activity is comparable to wild type with a clear increase in response to low iron conditions (figure 5.6 A). This provides further evidence that *CaFRE10*, which is regulated by Sfu1p, is not responsible for iron responsive cell surface cupric reductase activity found in hyphae. In both the CNA6 (*sfu1Δ/sfu1Δ*) mutant and the MEM-m2 (*Camac1Δ/Camac1Δ*) mutant the copper responsive cell surface cupric reductase activity is not detected in either yeast or hyphae (figure 5.6 B).

5.5: Comparison of the Regulation of *CaMAC1* and *SFU1* in Yeast and Hyphae:

To see if any of the changes in the expression of the cell surface ferric reductases in

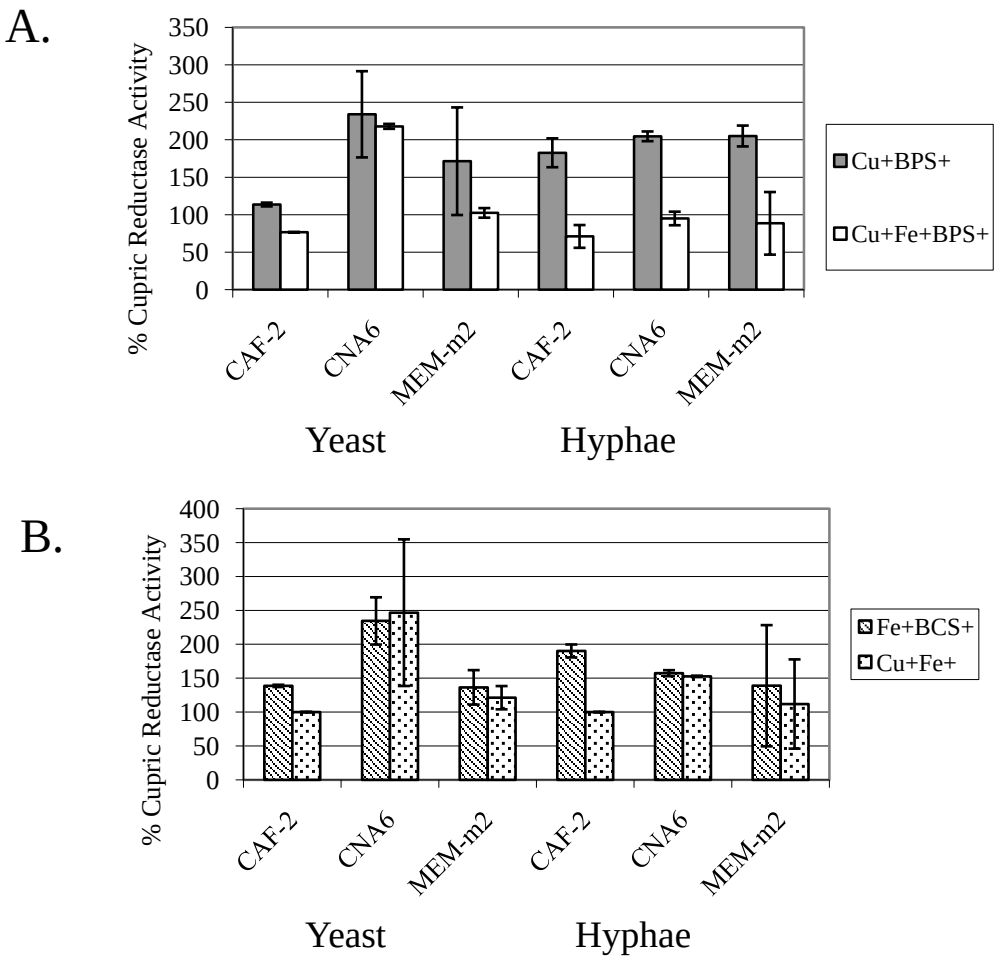
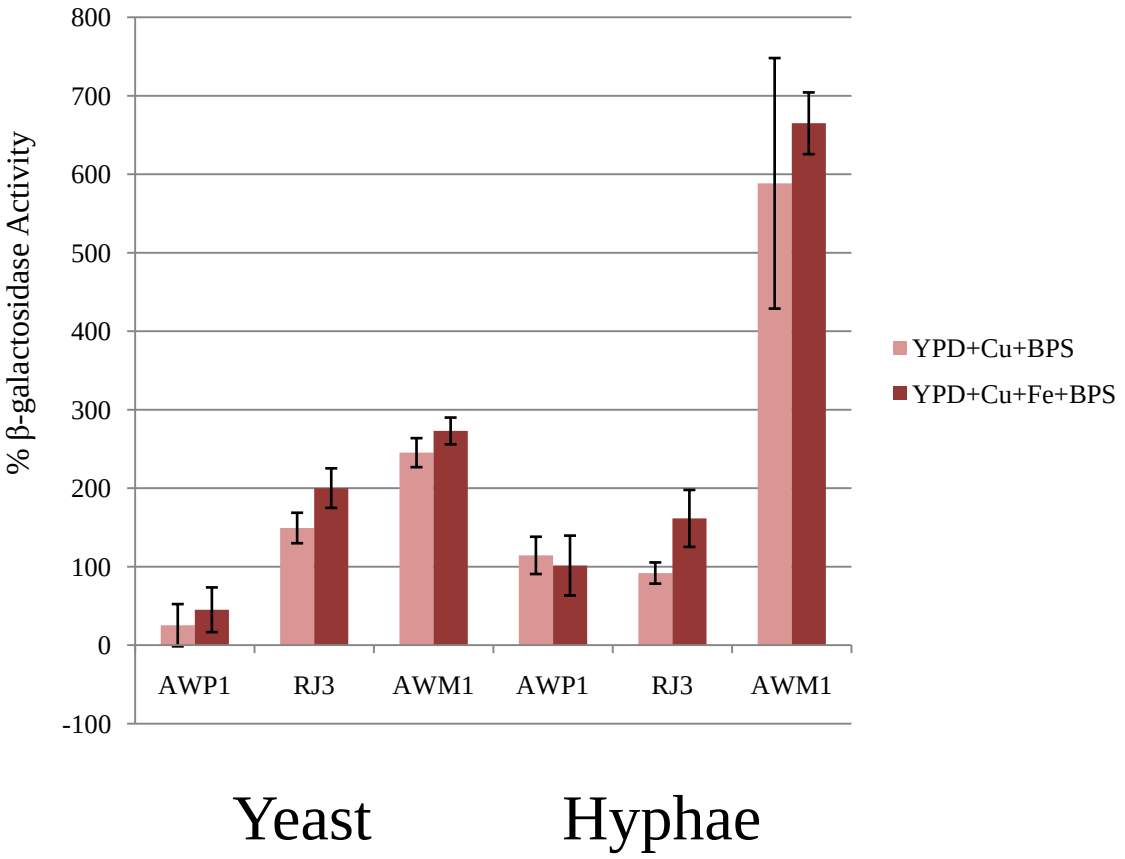


Figure 5.6: Differences in Cell Surface Cupric Reductase Activity in Yeast and Hyphae in CNA6 and MEM-m2: Strains were grown in YPD for 6 hours with the addition of 100μM $CuCl_2$, 100μM $FeCl_3$, 100μM BPS and 300μM BCS as indicated above. Yeast cells were grown for 6 hours at 30°C, hyphae were induced by the addition of 20% serum and growth at 37°C for 6 hours. Once cells were grown up a cupric reductase assay was performed as described in Materials and Methods section 2.9. Results are shown as a % of the cupric reductase activity of CAF-2 grown as yeast with the additions of 100μM $CuCl_2$ and 100μM $FeCl_3$. A mean of 2 repeats is shown, the error bars represent standard deviation. A. Iron responsive cupric reductase activity. B. Copper responsive cupric reductase activity. CAF-2 is the wildtype, CNA6 is *sfu1Δ/sfu1Δ* and MEM-m2 is *Camac1Δ/Camac1Δ*.

A.


800
700
600
500
400
300
200
100
0
-100
% β-galactosidase Activity
AWP1
RJ3
AWM1
AWP1
RJ3
AWM1
YPD+Cu+BPS
YPD+Cu+Fe+BPS
Yeast
Hyphae

B.

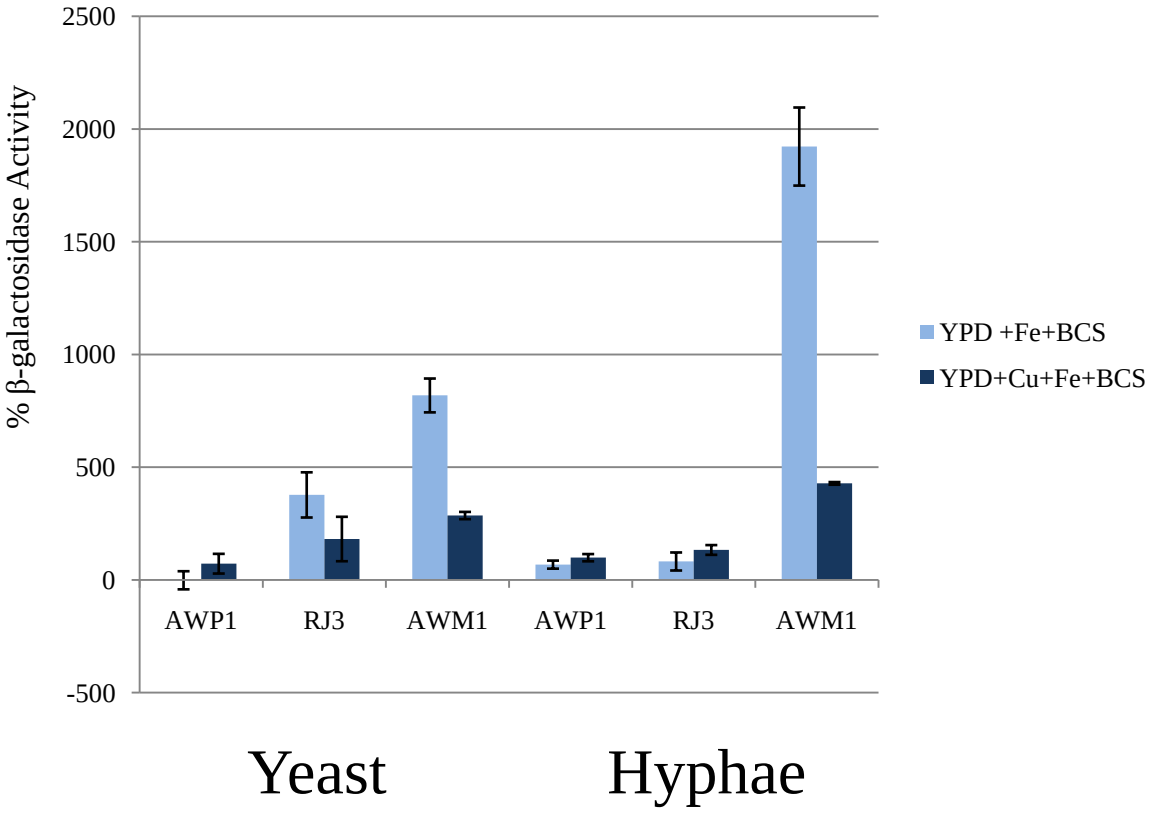

2500
2000
1500
1000
500
0
-500
% β-galactosidase Activity
AWP1
RJ3
AWM1
AWP1
RJ3
AWM1
YPD +Fe+BCS
YPD+Cu+Fe+BCS
Yeast
Hyphae

Figure 5.7: Expression of *SFU1* and *CaMAC1* in Hyphae Compared to Yeast:

Strains were grown in YPD for 5-6 hours to mid-exponential phase at 30°C with shaking (200 rpm) for yeast and in YPD with the addition of 20% serum or 5-6 hours at 37°C with shaking (200 rpm) for hyphae. To generate low iron conditions 100 μM $CuCl_2$ and 100 μM BPS was added, for a high iron condition 100 μM $FeCl_3$, 100 μM $CuCl_2$ and 100 μM BPS was added. To generate a low copper condition 100 μM $FeCl_3$ and 600 μM BCS was added and to generate high copper conditions 100 μM $FeCl_3$, 600 μM $CuCl_2$ and 600 μM BCS was added. The β-galactosidase activity was then determined as described in Materials and Methods section 2.11. Data is expressed as a % of the β-galactosidase activity of AWP1 in YPD with the addition of 100 μM $CuCl_2$ and 100 μM $FeCl_3$ grown at 30°C for yeast and addition of 20% serum, 100 μM $CuCl_2$ and 100 μM $FeCl_3$ grown at 37°C for hyphae. The mean values of three assays are shown and the error bars represent standard deviation. A. shows changes in expression in response to iron. B. shows changes in expression in response to copper. AWP1 is a control, AMW1 is the *CaMAC1* reporter construct in the wildtype BWP17 strain and RJ3 is the *SFU1* reporter construct in the wildtype BWP17 strain (see Materials and Methods table 2.1).

hyphae compared with yeast were attributable to changes in expression of the transcriptional regulator genes *CaMAC1* and *SFU1*, *lacZ* reporter constructs of the promoters of the genes encoding these regulators were grown as both yeast and hyphae in copper and iron restricted conditions and the β-galactosidase activity was determined. The *C. albicans* strain AWP1 is a control containing the placpoly plasmid without any promoter insert, AWM1 is the *CaMAC1* reporter construct in the wild type BWP17 and RJ3 is the *SFU1* reporter construct in the wild type BWP17 (see Materials and Methods table 2.1). In yeast, *CaMAC1* expression is increased approximately 2 fold in response to low copper conditions (Woodacre *et al.* 2008). A significant (Tukey's one way ANOVA, $P<0.001$) increase in *CaMAC1* expression was seen across all conditions in hyphae compared to yeast and in hyphae there was a approximately 3 fold increase in activity in response to low copper rather than the 2 fold increase seen in yeast (figure 5.6). In yeast, *SFU1* expression decreases in response to low iron conditions and increases in response low copper conditions and the copper reponsive regulation was attributable to CaMac1p (Chapter 4, figure 4.7). In hyphae, *SFU1* expression is significantly (Students t-test at the 5% level) lower compared to yeast in response to low iron conditions (figure 5.6). Expression of *SFU1* in hyphae is also decreased in low copper conditions and no copper responsive increase in expression can be detected, however this may be simply because the assay is not sensitive enough to detect such low levels of expression. The *SFU1* gene was shown to be repressed in some way by CaMac1p in Chapter 4 (figure 4.7) and this may account for the decrease in *SFU1* expression in hyphae compared to yeast since there is an increase in the levels of CaMac1p in hyphae which could further repress *SFU1*.

5.6: Discussion:

The Northern blot data provided a very interesting result; the expression of both *CaFRE10* and *CaFRE7* is reduced in hyphae compared with yeast. This decrease may be due to the fact that the high affinity iron uptake is less important in hyphae than it is in yeast. This idea is further supported by the previous finding that expression of *CaFRE1* is reduced in response to serum and that a serum induced yeast hyphal switch resulted in an increase in siderophore uptake (Lesuisse *et al.,* 2002). Hyphae are induced by the addition of serum and growth at a temperature of 37˚C (Sudbery *et al.,* 2004), i.e. the conditions found in the blood of the human host. The expression of the heam oxygenase *HMX1* is induced under these conditions (Pendrak *et al.,* 2004). Since *C. albicans* can lyse erythrocytes and utilise iron bound in haemoglobin it would fit that this would be the primary iron source for hyphae and that the haem iron uptake system would be active (Moors *et al.,* 1992; Casanova *et al.,* 1997; Pendrak *et al.,* 2004). Further weight is added to this argument from the fact that the levels of free iron (which the high affinity iron uptake system utilises) are incredibly low in the blood of the human host because it is tightly bound in transferrin (Edison *et al.,* 2008), making the high affinity iron uptake system a less effective means of iron acquisition. Whereas in other environments within the host, such as the liver, where some free iron is available it may be that the high affinity iron uptake system is more efficient since the iron can be simply reduced and taken up, rather than having to go through the process of erythrocyte lysis and extraction of iron from haem. It is interesting to note that despite the high levels of transferrin in serum, induction of hyphae using serum results in a decrease in expression of *CaFRE10*, which has been shown to be involved in uptake of transferrin bound iron (Knight *et al.,* 2005). In this study we routinely used serum to

induce hyphae and so it is not possible to tell if the decrease in expression of *CaFRE10* and *CaFRE7* is as a result of the hyphal form or as a result of some factor contained in the serum. Further experiments are planned in our laboratory to induce hyphae by other means, such as addition of GlcNAc, and see if the same results are generated.

The smaller increase in cell surface ferric reductase activity in low iron conditions in hyphae compared with yeast (figure 5.2) may be explained by the decrease in *CaFRE10* and *CaFRE7* expression in hyphae compared to yeast (figure 5.1). However, cupric reductase activity is higher in hyphae than it is in yeast (figure 5.3) and *CaFRE7* is clearly still important, as the increase in cupric reductase activity in response to low copper in hyphae is still lost in the RJ1.9 (*Cafre7Δ/Cafre7Δ*) mutant (figure 5.3). Furthermore, none of the cupric reductase activity in hyphae appears to be as a result of CaFre10p, since no difference is observed compared to wild type in a *Cafre10Δ/Cafre10Δ* mutant in hyphae. This, combined with the decrease in *CaFRE7* expression indicates that another cupric reductase at work. Proteins tend to have both ferric and cupric reductase activity since both metals are very similar and the function of the protein is simply to donate electrons (Van Ho *et al.* 2002). The decrease in ferric reductase activity and the increase in cupric reductase activity indicates a protein which preferentially reduces copper over iron. In addition to the increase of cell surface cupric reductase activity in hyphae, this increase does not seem to be attributable to *CaFRE10* (figure 5.3), this is also supported by the decrease in expression of *CaFRE10* in hyphae compared with yeast (figure 5.1).

The lower expression of *CaFRE7* in hyphae compared to yeast is surprising given that *CaFRE7* is regulated by CaMac1p (Levitin & Whiteway, 2007; Woodacre *et al.* 2008) and the expression of *CaMAC1* is increased in hyphae compared to yeast. This indicates that CaMac1p is not the only regulatory element involved in the regulation of *CaFRE7* and copper-responsive cell surface cupric reductase activity. Furthermore, it has previously been shown that *CaFRE7* is negatively regulated by CaTup1p in response to prostaglandin E (Levitin & Whiteway, 2007), indicating that it is not just copper involved in the regulation of this gene. CaTup1p has also been shown to complex with Sfu1p and has been shown to be involved in regulating *CaFRE10* (Knight *et al.* 2002; Pelletier *et al.* 2007). The CaTup1 protein also binds to Nrg1, Mig1 and Rfg1 to repress hyphal formation in non inducing conditions (Biswas *et al.* 2007; Murad *et al.* 2001; Murad *et al.* 2001), providing another link between the regulation of high affinity iron uptake and the morphological form of *C. albicans*. It is possible that the changes in expression in *CaFRE10* in hyphae compared with yeast are attributable to the action of CaTup1p. Future work involving indentifying the regulatory element involved in the decrease of expression of the ferric reductases in hyphae is required.

It is possible that the decrease in expression of ferric reductases in hyphae is due to the increase in pH that addition of serum causes, the YPD medium used is a pH 4 and serum is at ~pH 7, so addition of 20% serum will alter the pH to some extent. Change in pH which not only causes changes in morphology via Rim101p, the pH sensing regulator, independently of the serum (Biswas *et al.,* 2007), but a higher pH has also has been shown to result in the increase in expression of the ferric reductase genes *FRE1* and *FRP2* (Bensen *et al.,* 2004). However, without further experiments buffering the

pH of the medium and inducing hyphae by means other than serum it is difficult to draw conclusions.

The link between iron and morphology had previously been indicated in a microarray study which demonstrated that 6 genes which show lower expression and 7 genes that show higher expression during hyphal induction are regulated by iron availability (Lan *et al.*, 2004). Predominately genes that are upregulated in the yeast-hyphal transition are also regulated by low iron conditions, indicating a tendency to form hyphae in response to low iron. However, this does not provide any insight into the link between the use of iron uptake systems in the different hyphal forms as the microarray was only performed in the yeast form of *C. albicans*. The work presented here demonstrates differential regulation of an iron uptake system in different morphological forms, rather than regulation of morphology by iron. Together these studies demonstrate that the regulation of morphology and regulation in response to iron is linked and this study shows that regulation of the ferric reductases is complex and is linked to the morphological form of the organism. This is probably due to the requirement of this pathogen to respond to the changing environment within the host.

Chapter 6: Discussion

Candida albicans is an opportunistic pathogen of humans causing superficial mucosal infections and more serious blood borne disease (Weinberger *et al.*, 2005). High affinity iron uptake in *C. albicans* is dependent on copper and has been shown to be required for the virulence of the organism (Ramanan & Wang, 2000; Van Ho *et al.*, 2002). The first stage of both high affinity iron and copper uptake is to reduce the element at the cell surface to a more soluble valence and this is achieved by ferric reductase proteins (Van Ho *et al.*, 2002). The aims of this project were to characterize the role of *CaFRE7* in cell surface iron and copper reduction, to further characterize the role of Sfu1p in iron responsive regulation of the high affinity iron and copper uptake systems in *C. albicans* and to investigate the possibility that high affinity iron uptake may be different in hyphae compared to yeast. Previous studies had shown that the major ferric reductase encoded by *CaFRE10* was regulated only by iron and was responsible for iron responsive cell surface ferric and cupric reductase activity (Knight *et al.*, 2005; Mason, 2006). It was demonstrated in Chapter 3 that the copper regulated CaFre7p is responsible for copper responsive cell surface ferric and cupric reductase activity. This data shows that CaFre10p is responsible for iron responsive cell surface ferric and cupric reductase activity and CaFre7p is responsible for copper responsive cell surface cupric reductase activity. Both CaFre10p and CaFre7p reduce iron and copper, so it follows that their expression should be regulated by the concentration of these elements in the environment. The *CaFRE10* and *CaFRE7* genes are regulated by one of these elements each, iron in the case of CaFre10p and copper in the case of

CaFre7p. Using this regulation *C. albicans* can respond to a deficiency of iron or copper by the increase in the expression of one or the other of these two proteins.

In the model organism *Saccharomyces cerevisiae* ScFre7p is the homolog of CaFre7p and is regulated only by copper levels. Overexpression studies have implicated ScFre7p in cell surface ferric reductase activity in *S. cerevisiae.* However, unlike in *C. albicans*, CaFre7p is not responsible for the majority of cell surface ferric reductase activity in response to copper. Copper responsive cell surface ferric reductase activity in *S. cerevisiae* is attributable to ScFre1p, which is regulated by both the iron and copper content of the growth medium and accounts for 80% of cell surface ferric reductase activity. *S. cerevisiae* has a further cell surface ferric reductase ScFre2p which is regulated only by iron (Bakel *et al.,* 2005; Georgatsou & Alexandraki, 1999; Martins *et al.,* 1998). Very little is known about the regulation of the NADPH-dependent ferric reductase genes found in other yeast, although the expression of the *Schizzosaccahromyces pombe* ferric reductase, *FRP1* is increased in response to low iron conditions (Roman *et al.,* 1993). Furthermore *FRP1* expression is increased in response to high copper conditions and is repressed, along with the rest of the high affinity iron uptake system in low copper conditions (Labbe *et al.,* 1999). In addition ferric reductases have been found to be iron regulated in *Aspergillus nidulans* and *Ustillago maydis*, but the role of copper has not yet been investigated (Eichhorn *et al.,* 2006; Oberegger *et al.,* 2002).

Activation of *CaFRE7* expression in response to low copper conditions has been shown to be mediated by the copper sensing transcription factor CaMac1p. It was previously

demonstrated that *CaMAC1* was autoregulated, rather than constitutively expressed as is the case for *ScMAC1* (Woodacre *et al.,* 2008). The differences in regulation of the major cell surface ferric reductases in *C. albicans* compared to *S. cerevisiae* and the difference in the regulation of *CaMAC1* demonstrates that the high affinity iron and copper uptake systems in *C. albicans* and *S. cerevisiae* are regulated very differently. This may be as a result of the very different environmental niches they inhabit, including the fact that *C. albicans* is pathogenic and *S. cerevisiae* is not.

The two cell surface ferric reductases investigated in this study, CaFre10p and CaFre7p are regulated by iron and copper respectively. The iron responsive transcriptional regulation is thought to be achieved by a complex of Sfu1p and CaTup1p and the copper responsive regulation is achieved by CaMac1p (Woodacre *et al.,* 2008; Pelletier *et al.,* 2007; Knight *et al.,* 2002; Lan *et al.,* 2004). The protein Sfu1p is a GATA type transcriptional repressor homologous to a number of other iron sensing transcription factors found across the fungal kingdom (see Introduction section 1.9). This mechanism of regulation is in contrast to that found in *S. cerevisiae*, which regulates the high affinity iron uptake system in response to iron via the transcriptional activator ScAft1p (Yamaguchi-Iwai *et al.,* 1996). Again, this difference in regulation consolidates the idea that although the high affinity iron uptake system in *C. albicans* is functionally homologous to that found in *S. cerevisiae*, the regulation of these two systems is very different, with *C. albicans* having an iron responsive regulatory system more akin to that found in the other fungi that have been investigated.

The second aim of this project was to further characterise the role of Sfu1p in its regulation of the high affinity iron uptake system in *C. albicans.* There is an increase in *CaFRE10* transcription in the *sfu1Δ/sfu1Δ* mutant and in Chapter 4 it was shown that there is a corresponding increase in cell surface ferric and cupric reductase activity. Although iron responsive ferric reductase activity was not detected in the *sfu1Δ/sfu1Δ* mutant, the iron responsive increase in cupric reductase activity was still detected. One possible explanation is that the iron responsive regulation seen in the absence of Sfu1p is attributable to a system similar to that which regulates *CaFRP1*, which is regulated by iron starvation independently of Sfu1p. This regulation is achieved by the recruitment of Hap43p to the CBF complex, which binds the CCAAT motif, in iron starvation conditions (Baek *et al.* 2008).

The observation that radioactive iron uptake is increased in an *sfu1Δ/sfu1Δ* mutant is consistent with the increase in transcription of the major iron permease *CaFTR1* in an *sfu1Δ/sfu1Δ* mutant (see Chapter 4, figure 4.4). Interestingly, this increase in iron uptake does not result in an increase in iron toxicity (Mansi Vora, personal communication) and this begs a very important question, where does all the iron go? Most fungi detoxify iron by storing it in a complex with siderophores until it is required (Haas, 2003; Schrettl *et al.,* 2004), but since the evidence that *C. albicans* can produce siderophores is unconvincing (Howard, 1999), it is unlikely that these are the mechanism for iron storage and detoxification.

One possible explanation for this lack of toxicity, despite a five fold increase in iron uptake, is that excess iron is sequestered to the vacuole for storage. ScCcc1p is responsible for the transport of iron into the vacuole in *S. cerevisiae*, it localizes to the vacuolar membrane and it's over expression results in depletion of iron in the cytosol (Li *et al.*, 2001). Once the iron is stored in the vacuole there is both a low affinity and a high affinity system for returning the iron to the cytosol when it is required. Low affinity iron uptake from the vacuole to the cytosol is achieved using Smf3p, which is homologous to Smf1 (Cohen *et al.*, 2000; Portnoy *et al.*, 2000). The high affinity transport of iron from the vacuole across the vacuolar membrane to the cytosol involves the reduction of ferric iron on the vacuolar membrane by ScFre6p and transport of ferrous iron by ScFth1p, which forms a complex with the ScFet5p mutilcopper oxidase (Spizzo *et al.*, 1997; Urbanowski & Piper, 1999; Singh *et al.*, 2007). *C. albicans* has homologs of these proteins. Furthermore, one of the genes encoding a putative iron transport protein (*CaFTR2*), when knocked out, did not show a phenotypic effect on radioactive iron uptake into the cell (Ramanan & Wang, 2000). Since CaFtr2p is not transporting iron across the cell surface membrane into the cell, it may be functioning to transport iron across an intracellular membrane. In support of this, the expression of *CaFTR2* was increased in response to high iron conditions (Ramanan & Wang, 2000), which may indicate a role in iron detoxification. It could be located on the inside of the cell surface membrane to transport iron out of the cell when cellular iron concentrations are too high, or it could be located on the vacuolar membrane and transport iron into the vacuole for storage in iron replete conditions.

The GATA-type transcriptional repressor Sfu1p regulates key components of the high affinity iron uptake system in an iron responsive manner (Lan *et al.,* 2004). Expression of *SFU1* has previously been shown by Northern blot analysis to be increased in response to high iron and high copper conditions and that expression was increased in a *Camac1Δ/Camac1Δ* mutant. To further investigate the regulation of *SFU1* in this study an *SFU1* promoter reporter was constructed and introduced in to a wildtype strain, an *sfu1Δ/sfu1Δ* mutant and a *Camac1Δ/Camac1Δ* mutant (Chapter 4, Section 4.7). It was shown in this study that *SFU1* expression is increased in response to high iron conditions and that expression is higher in a *Camac1Δ/Camac1Δ* mutant, which is consistent with the previous Northern blot data. However, contrary to the Northern blot analysis, it was shown here that *SFU1* expression is increased in response to low copper conditions. An increase in Sfu1p in low copper conditions should decrease the expression of the high affinity iron uptake system (as Sfu1p is a repressor). This may have two functions, the first could be to inhibit uptake of iron in a low copper, but iron replete environment as this may lead to iron toxicity. The second function could be to utilise another, copper independent, iron uptake system. *C. albicans* has several mechanisms of iron uptake and in times of copper scarcity it could switch to using alternative method of iron uptake to the high affinity system, since the high affinity system contains multicopper oxidases which require copper to function.

In the *Camac1Δ/Camac1Δ* mutant overall expression of *SFU1* was higher, indicating that CaMac1p directly or indirectly represses *SFU1*. This finding supports the data from previous Northern blot experiments (Woodacre *et al.,* 2008). Furthermore, in the *Camac1Δ/Camac1Δ* mutant copper responsive regulation of *SFU1* was lost, indicating

that the copper responsive regulation is mediated by CaMac1p, although to see if this regulation is direct either mutation of the Copper Responsive Element (CuRE) in the *SFU1* promoter or EMSA binding studies are required. Interestingly, in a *sfu1Δ/sfu1Δ* mutant, *SFU1* expression was increased and its metal ion regulation was not detected indicating that *SFU1* is in part autoregulated, but again, whether this regulation is direct or mediated via some other pathway is not clear.

To see if Sfu1p regulates *CaMAC1*, a *CaMAC1* promoter reporter construct was transformed into the *sfu1Δ/sfu1Δ* mutant. When assayed it was found that *CaMAC1* expression was increased in the *sfu1Δ/sfu1Δ* mutant. This may be as a result of the increase in iron transport observed in the *sfu1Δ/sfu1Δ* mutant. If indeed the increase in radioactive iron uptake is as a result of the increase in the expression of the major iron permease, CaFtr1p, then this would cause an increase in the cellular demand for copper, which would in turn increase expression of *CaMAC1*. This is because iron transport proteins like CaFtr1p must complex with a multicopper oxidase in order to function and the multicopper oxidase proteins require copper to function (Van Ho *et al.*, 2002).

All the work examined so far in this discussion has been performed in the yeast form of *C. albicans*, but this is only one morphological form of *C. albicans* and the switch between yeast and hyphal forms is required for virulence (Sudbery *et al.*, 2004). It was as a result of this that the third aim of this project was to see if there were any differences in expression of the ferric reductases in hyphae compared with yeast.

This study found that expression of the two cell surface ferric and cupric reductases, *CaFRE10* and *CaFRE7* is decreased in hyphae compared to yeast. This is the first time that the expression of the iron uptake genes has been linked to the morphological form of *C. albicans*. A previous study had shown that in the yeast form of *C. albicans*, in a *Catup1Δ/Catup1Δ* mutant expression of *CaFRE10* was higher than in wildtype and the iron responsive regulation of *CaFRE10* was not detected, demonstrating that CaTup1p represses *CaFRE10* (Knight *et al.*, 2002). Furthermore, another study has shown in yeast form, *CaFRE7* is also repressed by Tup1p in response to the immune protein prostaglandin E (Levitin & Whiteway, 2007). Although *CaFRE7* is regulated by CaMac1p in response to copper and *CaFRE10* is regulated by Sfu1p and iron, they are both regulated by the transcriptional repressor CaTup1p. The CaTup1 protein binds to Nrg1p, Mig1p and Rfg1p to repress filamentation in non inducing conditions (Murad *et al.*, 2001; Murad *et al.*, 2001; Biswas *et al.*, 2007). In hyphal inducing conditions it may be that the free CaTup1p is available to recruit other transcription factors and could be involved in the decrease in expression of *CaFRE10* and *CaFRE7* in hyphae compared to yeast.

The expression of *CaFRE7* was shown to still be activated by CaMac1p and *CaFRE10* was shown to be repressed by Sfu1p in hyphae, this indicates that the differences in regulation of *CaFRE10* and *CaFRE7* in yeast and hyphae are not mediated by Sfu1p and CaMac1p. The reporter constructs demonstrate that *CaMAC1* expression is higher in hyphae than it is in yeast (and *SFU1* expression is lower), which would be expected to lead to increase, not the decrease that is seen in *CaFRE7* expression. It is possible that the explanation for this lies with *CaTUP1* which has been previously been shown to

regulate both *CaFRE10* and *CaFRE7* and is a known co-repressor of hyphal formation in non-inducting conditions (Levitin & Whiteway, 2007; Pelletier *et al.,* 2007; Knight *et al.,* 2002; Murad *et al.,* 2001; Murad *et al.,* 2001; Biswas *et al.,* 2007).

Transcription of *CaFRE10* and *CaFRE7* is lower in hyphae than in yeast. This results in a corresponding decrease in cell surface ferric reducatase activity. However, cupric reductase activity is increased. The decrease in transcription of the ferric reductases *CaFRE10* and *CaFRE7* and the corresponding decrease in ferric reductase activity in hyphae may be because the high affinity iron uptake is less important for iron acquisition in hyphae than it is in yeast. However, no other mechanism for copper uptake in low copper conditions has yet been identified in *C. albicans* and so it may be that the high affinity copper uptake system is equally important in both yeast and hyphae. If this is the case, a method of copper reduction independent of the high affinity iron uptake system may be required in hyphae to reduce copper for CaCtr1p to take up in low copper conditions. Whilst this is an explanation for why the cell may have this mechanism, it does pose a problem chemically. Ferric and cupric reductases in *C. albicans* act by simply using NADPH to provide electrons for reduction (Anderson *et al.,* 1992), they are indiscriminate, so why are we seeing this increase in cupric reductase activity which is independent of an increase in ferric reductase activity? It is tempting to propose an unknown mechanism by which copper is specifically reduced, especially since the iron responsive cupric reductase activity in hyphae cannot be attributed to the major cell surface ferric reductase in yeast, CaFre10p. But such a bold claim cannot be inferred simply from the data presented

here; further study involving the identification of the source of the cupric reductase activity in hyphae and characterisation of its chemistry is required.

This study demonstrates that the regulation of iron uptake and the morphological form of *C. albicans* are intimately linked. This may indicate that as the organism changes niche within the host environment different iron uptake systems are required, therefore the regulation of these iron uptake systems has to respond to the changes in environment in a sensitive, comprehensive and ultimately complex way.

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