Identification and Analysis of Ferric Reductase Genes in C. albicans

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by

Robert Peter Mason BSc, MRes (University of Leicester)
Department of Genetics
University of Leicester

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Abstract
Identification and Analysis of Ferric Reductase Genes in *C. albicans*

*Robert Peter Mason*

Many factors are involved in the pathogenicity of *Candida albicans*, including the acquisition of iron. *C. albicans* uses a variety of strategies to acquire iron. One strategy involves the reduction of ferric iron at the plasma membrane. Ferric reductases carry out this reaction and also play an important role in the reduction of copper prior to its uptake. Previous work in our laboratory has identified two ferric reductases encoded by *CaFRE1* and *CaFRE2*. The aims of this project were to identify further ferric reductases in *C. albicans* and examine their expression and role.

In this study a further 15 putative ferric reductases have been identified in the *C. albicans* genome sequence. A number of these genes were studied further and *CaFRE10* was shown to encode the major cell surface ferric reductase. The *CaFRE2* gene was also shown to encode a functional cell surface ferric reductase.

The acquisition of iron and copper are intimately linked and it has previously been shown that, in *S. cerevisiae*, some of the ferric reductases are regulated in an iron and/or copper responsive way. During this study the expression of *CaFRE10* was shown to increase significantly in response to iron limitation. In contrast, the transcript levels of the putative reductase gene, *CaFRE12*, do not change in response to iron levels but do increase in response to copper limitation. This increase was dependent on the copper-sensing transcription factor CaMac1p.

A potential iron responsive transcriptional regulator was also identified. However, analysis of the putative *CaAFT* showed that it was not a functional homologue of iron-sensing transcription factors in *S. cerevisiae*. Work to further study the expression, regulation, and role of the genes identified in this study will give us insights into iron metabolism and its role in the pathogenicity of *C. albicans*. 
Acknowledgments

Throughout the course of my PhD there have been a great many people who have helped and supported me and they deserve a great share of the credit for this thesis. The Genetics department has been a fantastic and friendly environment in which to carry out my PhD and I would like to thank all of its members past and present that have made my studies so enjoyable. I would especially like to thank Annette for initially employing me and then providing a great deal of help and support during the course of my studies. Her numerous last minute appeals for demonstrating have also been of great assistance in providing money for food, clothes, accommodation, and most importantly beer. Annette also deserves my thanks for employing and introducing me to my fellow PhD student and wife to be Alex. I would also like to thank Pete Meacock for all of his help and advice in the fields of science as well as in my occasional forays into the world of trout fishing.

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### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ACD</td>
<td>Anaemia of chronic disease</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired immuno-deficiency syndrome</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>ATPase</td>
<td>Adenosine triphosphatase</td>
</tr>
<tr>
<td>BCS</td>
<td>Bathocuproine-disulphonic acid</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>BPS</td>
<td>Bathophenanthroline-disulphonic acid</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CuRE</td>
<td>Copper response element</td>
</tr>
<tr>
<td>dATP</td>
<td>Deoxyadenosine 5’-triphosphate</td>
</tr>
<tr>
<td>dCTP</td>
<td>Deoxycytidine 5’-triphosphate</td>
</tr>
<tr>
<td>DEPC</td>
<td>Diethyl pyrocarbonate</td>
</tr>
<tr>
<td>dGTP</td>
<td>Deoxyguanosine 5’-triphosphate</td>
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<tr>
<td>DNA</td>
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<tr>
<td>dNTP</td>
<td>Deoxynucleotide 5’-triphosphate</td>
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<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>dTTP</td>
<td>Deoxythymidine 5’-triphosphate</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetra acetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethyleneglycol-bis(β-aminoethyl)-N,N,N,N-tetraacetic acid</td>
</tr>
<tr>
<td>EMSA</td>
<td>Electrophoretic mobility shift assay</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ERQC</td>
<td>ER quality control</td>
</tr>
<tr>
<td>FAD</td>
<td>Flavin adenine dinucleotide</td>
</tr>
<tr>
<td>5-FOA</td>
<td>5-Fluoroorotic acid</td>
</tr>
<tr>
<td>GSH</td>
<td>Reduced glutathione</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>IAA</td>
<td>Isoamyl alcohol</td>
</tr>
<tr>
<td>kb</td>
<td>Kilobase pairs</td>
</tr>
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<td>kDa</td>
<td>Kilodaltons</td>
</tr>
<tr>
<td>LA</td>
<td>Luria agar</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>LB</td>
<td>Luria broth</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>MD</td>
<td>Minimal defined</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-(N-morpholino) propanesulphonic acid</td>
</tr>
<tr>
<td>μM</td>
<td>Micromolar</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NES</td>
<td>Nuclear export sequence</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear localisation signal</td>
</tr>
<tr>
<td>nM</td>
<td>Nanomolar</td>
</tr>
<tr>
<td>nt</td>
<td>Nucleotides</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>pmol</td>
<td>Picomolar</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNase</td>
<td>Ribonuclease</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>SAP</td>
<td>Shrimp alkaline phosphatase</td>
</tr>
<tr>
<td>SD</td>
<td>Synthetic defined</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SSC</td>
<td>Standard saline citrate</td>
</tr>
<tr>
<td>TE</td>
<td>Tris-EDTA buffer</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-Acetate-EDTA</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>TMD</td>
<td>Transmembrane domains</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume by volume</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight by volume</td>
</tr>
</tbody>
</table>

**Strain Nomenclature**

The conventional nomenclature for the naming of *C. albicans* strains containing gene deletions is not followed in this thesis. The name of the disrupted gene is instead followed by Δ or ΔΔ to indicate the disruption of one or two alleles respectively.
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Chapter 1
Introduction

The opportunistic pathogen *Candida albicans* is part of the normal microbial flora of most healthy humans. Over the last few decades the incidence of *C. albicans* infections has risen dramatically, making it an increasingly relevant human pathogen (Clark & Hajjeh, 2002). These infections range from minor to life threatening, and are often associated with changes in the host immune system or microbial flora. A growing number of virulence determinants have also been identified in *C. albicans* that are required for the establishment of an infection (Calderone & Fonzi, 2001; Yang et al., 2003). As the human body maintains the essential nutrient iron at very low levels the acquisition of iron is essential for pathogenicity (Ramanan & Wang, 2000). Iron limitation also affects the expression of known virulence determinants in *C. albicans* that are not directly involved in iron acquisition (Lan et al., 2004). Due to the importance of iron in virulence one of the aims of the work in our laboratory has been to identify components of the iron uptake pathway in *C. albicans*. In many organisms the iron acquisition is linked to the uptake of copper (Dancis et al., 1994) and we previously shown that this link exists in *C. albicans* (Marvin et al., 2004). We are therefore also investigating this link between iron and copper uptake further.

Prior to this study work in our laboratory demonstrated that *C. albicans* is able to reduce iron and copper at the cell surface (Morrissey et al., 1996). The reduction of these two metals increases their solubility and allows their efficient transport into the cell. We have also identified two ferric reductases that are thought to be involved in this process (Hammacott et al., 2000). The work presented in this thesis describes the identification of further ferric reductases in the *C. albicans* genome. The expression and function of a number of the ferric reductases was also examined to confirm their precise role in iron and copper uptake and metabolism.

The introduction of this thesis outlines the importance of iron in *C. albicans* infections, and the processes involved in its acquisition. The importance of iron and copper in biological systems is discussed, followed by a review of the links between iron and virulence. The acquisition of iron and copper in yeast, and the link between
the two processes, is then discussed. Because this thesis is based on the study of ferric reductases this section focuses on reductive iron and copper uptake and processes in the cell requiring the reduction of these transition metals. Because the best studied examples of these mechanisms come from *Saccharomyces cerevisiae* the majority of the information in this section comes from this model organism. The regulation of iron and copper uptake in *S. cerevisiae* and other fungi is then reviewed. Finally, the background to this project and the aims and objectives are outlined.

### 1.1 *Candida albicans*

The opportunistic fungal pathogen *C. albicans* is part of the normal flora associated with the gastro-intestinal tract, oral cavity, skin, and female genitalia of most healthy individuals. It is a dimorphic fungus that can adopt yeast, hyphal, or pseudohyphal morphologies. Until recently *C. albicans* was thought to be an obligate diploid due to the lack of a sexual cycle. However, studies in the last few years have demonstrated mating can occur under very specific conditions (Hull *et al.*, 2000; Magee *et al.*, 2000). This process is still extremely poorly understood and its relevance *in vivo* unclear (reviewed Bennett & Johnson, 2005). The *C. albicans* genome is approximately 15 Mb in size and is carried on 7 chromosomes (Braun *et al.*, 2005; Jones *et al.*, 2004).

### 1.2 *Candida albicans* and Disease

*C. albicans* is an opportunistic human pathogen that can cause both minor and major infections. The switch that triggers pathogenesis is often thought to be associated with changes in the host environment such as immune suppression, debilitation, and alterations in the microbial flora (reviewed in Maertens *et al.*, 2001). This can be due to alterations in the body’s normal processes or medical interventions such as surgery, catheterisation, and treatment with drugs such as antibiotics and immunosuppressants. The majority of *Candida* infections observed in people with these risk factors are relatively minor mucocutaneous infections of the skin, nails, and mucosal surfaces.

The incidence of subcutaneous *C. albicans* infections is hard to quantify, as there is little data available. However, it is thought they may represent one of the most common human infections with approximately 75% of women having at least one
vaginal thrush infection during their life (Ferrer, 2000). While the majority of *C. albicans* infections are relatively minor they can also result in life-threatening candidiasis, where the fungus is disseminated throughout the body and invades organs. These systemic infections are becoming increasingly important, with *Candida* species being the 3rd or 4th most common organism isolated from the blood of hospital patients in the United States (Edmond *et al.*, 1999). While figures vary widely between geographical locations the majority of *Candida* infections are due to *C. albicans*. The overall number of *C. albicans* infections continues to rise but the proportion of *Candida* infections caused by non- *albicans* species is increasing (Hobson, 2003). The increasing number of systemic fungal infections is also of concern because even with antifungal treatment they prove fatal in as many as 55% of cases (Weinberger *et al.*, 2005). The economic burden of systemic *Candida* infections on healthcare services is also extremely significant, with estimates suggesting they cost the US authorities at least 1 billion dollars a year (Miller *et al.*, 2001).

Until the last few decades fungal infections were relatively uncommon and regarded as a minor health risk. However, due largely to recent advances in healthcare, serious fungal infections have increased greatly and are becoming a serious problem in hospitals (Richardson, 2005). One of the main reasons for the increase in the number of fungal infections is the rising in population of individuals who are at risk. Increasing numbers of patients are undergoing organ transplants, cancer treatment, and immunosuppressive therapy. The survival times of these patients are also improving and immunosuppressive treatments are becoming increasingly aggressive and lasting for longer periods.

Another problem associated with *C. albicans* infections is the increase of resistance to antifungal drugs (Reviewed Akins, 2005; Morschhauser, 2002). This is of particular importance as there are a limited number of drugs available for the treatment of *C. albicans* and many of them work by targeting similar fungal processes. Two of the main drugs that have been utilised for the treatment of *C. albicans* infections are fluconazole and amphotericin B. These agents target fungal ergosterol synthesis and are both highly effective, and unlike many antifungals have few side effects. However, *C. albicans* appears to be able to gain resistance to fluconazole much more easily than some other modern drugs such as caspofungin. One explanation for the
susceptability of fluconazole to resistance is that it is fungistatic, so the surviving cells often have resistance (Anderson et al., 2005; Morschhauser et al., 2002). Another problem associated with fluconazole is that there are a number of different mechanisms that can result in fluconazole resistance (Anderson et al., 2005).

1.3 Importance of Iron and Copper

Due to their chemical properties iron and copper play an important and often vital role in many cellular processes. Probably the most important aspect of iron and copper chemistry is their ability to switch between the oxidised (Fe³⁺ & Cu²⁺) and reduced (Fe²⁺ & Cu⁺) forms allowing them to participate in numerous single electron transfer reactions.

Iron

With the exception of Lactobacillus and a few Bacillus species iron is indispensable for the survival of all life (Archibald, 1983; Crichton, 2001). The widespread use of iron in biological processes can be attributed to a number of different factors including the fact that it is the second most abundant metal, and the fourth most abundant element, in the earth's crust (Crichton & Pierre, 2001). Iron is also suitable for use by organisms as ferrous salts are soluble, which allows their transport across biological membranes. The chemical properties of iron also mean that it is the most versatile transition metal used in redox reactions. While it is theoretically able to adopt a wide variety of oxidation states ranging from Fe²⁺ to Fe⁵⁺ it is predominantly found as Fe²⁺ and Fe³⁺ in aqueous solutions. It can be easily converted between Fe³⁺ and Fe²⁺ through the reduction of ferric iron by many common reducing agents or by the oxidation of ferrous iron by dioxygen. The range of redox potentials spanned by iron is extremely broad and covers almost the entire range observed in biological reactions. The redox potential of iron containing proteins can also be altered and refined by the binding of a range of specific ligands to iron. In addition to its importance in electron transfer reactions iron plays an important role in the storage and activation of molecular oxygen (Pierre et al., 2002). Iron has a particular affinity with oxygen as Fe³⁺ is a hard acid that prefers interactions with hard ligands such as oxygen (Pierre et al., 2002). Therefore iron is the active site in molecules such as
haem that are found in proteins involved in oxygen binding and oxygen requiring enzymatic reactions (Radisky et al., 1999).

One of the reasons iron is used so extensively in nature is that early in the earth's history the atmosphere contained relatively little oxygen, leading to the predominance of relatively soluble ferrous iron (Crichton & Pierre, 2001). However, the evolution of cyanobacteria and other oxygen-producing organisms created an oxidising environment, converting iron to relatively insoluble ferric oxides and hydroxides. The contrast in solubility between ferric and ferrous iron is vast with Fe$^{3+}$ having a solubility of $10^{-17}$ M at neutral pH while Fe$^{2+}$ is $10^{-1}$ M (Arredondo & Nunez, 2005). Organisms therefore needed to evolve methods to solubilise iron to allow its transport across the membrane. One solution used by bacteria and eukaryotes involves the reduction of extracellular iron to the more soluble ferrous form by a class of enzymes known as ferric reductases (Pierre et al., 2002).

Copper
Like iron, copper is an essential element for the survival of most organisms. For example in S. cerevisiae copper is required for the function of the essential enzyme cytochrome C oxidase, which is required for the use of non-fermentable carbon sources (reviewed Hamza & Gitlin, 2002). Copper is also required for protecting the cell from oxidative stress during aerobic growth, as it is an essential cofactor for superoxide dismutase (reviewed Fridovich, 1995). The transport of iron into the S. cerevisiae cell is also dependent on copper as a multicopper oxidase, Fet3p, is an essential component of the high affinity uptake system (Askwith et al., 1994). In other species, copper is similarly required for the function of a wide variety of oxidases and oxygenases (Crichton & Pierre, 2001). Copper is particularly useful as it has a higher redox potential than iron, between 0.25 V and 0.75 V, which allows it to participate in the oxidation of compounds such as superoxide, ascorbate, and catechol (Crichton & Pierre, 2001).

One of the areas where the chemistry of iron and copper differs widely is in the solubility of compounds containing the various redox states. In contrast to iron the cuprous form of copper is less soluble than the cupric form and often forms sulphides that precipitate (Crichton & Pierre, 2001). This means that the ancient anoxic
reducing environment presumably made copper difficult to acquire by early organisms. Therefore, copper only became widely available following the appearance of dioxygen in the environment. The availability problems associated with copper in early times provides an explanation for why copper is less widely used in ancient prokaryotes and archaea compared with many eukaryotes.

Toxicity of Iron and Copper Use
While iron and copper are essential for nearly all forms of life the accumulation of large quantities within the cell can have disastrous consequences. If free iron and copper are available within the cell they are able to react with reactive oxygen species such as hydrogen peroxide and molecular dioxygen via the Harber-Weiss/Fenton reaction as shown in Figure 1.1 (Halliwell & Gutteridge, 1984). This reaction produces extremely toxic hydroxyl radicals that can damage almost all biological compounds such as lipids, DNA, and proteins (Halliwell & Gutteridge, 1984; Imlay & Linn, 1988). The accumulation of excess levels of iron and copper can also cause problems by competing for similar biological sites that can result in the inappropriate loading of the incorrect metal onto a protein (Jungmann et al., 1993). Therefore, due to the importance of maintaining sufficient but non-toxic levels of iron and copper within the cell, organisms have developed complex homeostatic mechanisms to regulate levels of both metals (Hantke, 2001; Hentze et al., 2004).

1.4 Iron and Virulence
Iron is an essential nutrient for both the host and potential pathogens, so it is not surprising that the battle for control of this nutrient plays a central role in the infection process. It has long been known that the acquisition of iron is directly linked to the virulence of many pathogenic organisms (reviewed Howard, 1999; Ratledge & Dover, 2000; Schaible & Kaufmann, 2004). Due to the toxicity of iron at high concentrations, higher eukaryotes have developed complex systems for maintaining free iron within the body at very low levels. Indeed it has been estimated that levels of free ionic iron in the human body are maintained at approximately $10^{-18}$ M (Bullen et al., 2000). As most microbes require iron concentrations of at least $10^{-6}$ to $10^{-7}$ M for growth (Weinberg, 1978) the maintenance of these extremely low free ionic iron concentrations in the host plays an important role in preventing colonisation by
Figure 1.1 Harber-Weiss-Fenton Reactions. (1) Hydrogen peroxide can be formed by the donation of two electrons and protons to superoxide, which is a by-product of a variety of cellular reactions. (2) The donation of an electron from ferrous iron to hydrogen peroxide results in the formation of ferric iron, hydroxyl anions, and harmful hydroxyl radicals. These hydroxyl radicals are capable of damaging a number of cellular components including DNA, proteins, and lipids. (3) One of the sources of the ferrous iron necessary for the second reaction comes from the reduction of ferric iron by superoxide.
harmful pathogens. Microbes have therefore needed to develop complex iron acquisition strategies to enable them to colonise the human host.

The levels of free ionic iron within the human host are very low because the majority of iron is bound to proteins with a very high affinity for this essential metal (Reviewed in Aisen et al., 2001). After being absorbed into the blood stream dietary iron is immediately bound by transferrin. Transferrin is an 80-kDa bilobular glycoprotein able to bind two molecules of ferric iron, whose function is to transport iron from plasma and extracellular fluid to cells within the body (reviewed Aisen et al., 1999; Aisen et al., 2001). Alternative forms of transferrin are found in milk and tears (lactoferrin) and avian eggs (ovotransferrin) where they play an essential role in preventing microbial colonisation (Ratledge & Dover. 2000). In a healthy individual transferrin and lactoferrin are present in excess and are only 30-40% iron saturated, enabling free iron levels to be maintained at very low levels (Griffiths, 1999). Once transferrin has bound iron it is recognised by cellular transferrin receptors and internalised to the early endosome, where the acidic nature of this compartment triggers the release of iron (Aisen et al., 1999; Aisen et al., 2001). Transferrin and its receptor are then recycled to the cell surface where transferrin is released into the external environment to bind more iron.

Whilst some iron is located extracellularly bound to proteins including transferrin and lactoferrin, the majority is located within cells (Aisen et al., 2001). Intracellular iron is bound to ferritin or haem containing proteins such as haemoglobin. Ferritin is the principal protein involved in the storage and detoxification of iron and is found in organisms ranging from bacteria to humans (Aisen et al., 2001). Human apoferritin is a 440-kDa spherical molecule that is able to store as many as 4500 iron atoms as insoluble ferric salts in its core (Harrison & Arosio, 1996). The apoferritin molecule is constructed from 24 oblong subunits that can be classified as either heavy or light. The subunit types share high homology, although only the heavy subunit contains a site essential for oxidation of ferrous iron while the light subunit promotes iron core formation. The composition of ferritin can therefore be altered depending on the cells requirements. Molecules that contain more heavy subunits are more active in iron metabolism while those with more light chains are often used for long term iron storage.
In addition to maintaining low levels of iron to prevent colonisation by potentially harmful microbes the human body is able to lower iron levels even further in response to infection (Reviewed in Weiss, 2005). This process occurs through a number of mechanisms and is collectively called the hyperferraemic response or the anaemia of chronic disease (ACD). It is the most common form of anaemia observed among hospital patients and occurs in individuals suffering from conditions such as cancer, autoimmune disease, and long-term infections. During hyperferraemia the reticuloendothelial system activates host defence mechanisms that lower the levels of iron in the serum, and in intracellular pools. The concentration of iron in serum is lowered by increasing the transfer of iron to cells, where increased ferritin synthesis leads to the storage of large quantities of iron. This phenomenon can be seen in volunteers infected with Francisella tularensis, where the saturation of transferrin drops from 30-40% to 10% (Pekarek et al., 1969). Intracellular stores of iron are also lowered by degrading ferritin to produce insoluble haemosiderin (Aisen et al., 2001). The process behind this change in iron metabolism is poorly understood but can be mediated by interleukin-1 in response to fever caused by infection (Kluger & Rothenburg, 1979).

The effect of iron on microbial virulence and human disease can be observed when iron levels in the body are altered. As early as 1872 it was observed that patients with quiescent tuberculosis often suffered fatal relapses if given iron supplements (Ratledge, 2004). A direct correlation between increases in iron within the body and virulence has subsequently been demonstrated for at least 18 bacterial species (Griffiths, 1999). For example guinea pigs rapidly die of septic shock if injected with iron in conjunction with normally harmless doses of E. coli (Bullen et al., 1968). Medical conditions such as hereditary iron overload (haemocromatosis) have also long been associated with an increased susceptibility to infections from a wide range of organisms (Moalem et al., 2004).

A link between iron and virulence in C. albicans has long been suspected, and there is a growing amount of evidence to support this link. Clinical evidence includes the observation that patients suffering from acute leukaemia, which often causes severe iron overload, are highly susceptible to C. albicans infections (Caroline et al., 1969).
This situation is exacerbated by chemotherapy (Karp & Metz., 1986) as this can cause a sharp increase in transferrin saturation to over 95%, a reduction in overall iron binding, and the presence of low molecular weight iron compounds in the circulation (Halliwell et al., 1988). The increase in infections observed in neutropenic patients may also be at least partially due to the increased availability of iron for *C. albicans* (Bullen et al., 2006).

Laboratory based research has also provided evidence for a strong link between iron and pathogenicity in *C. albicans*. Exposure to low iron conditions results in a slower growth rate and changes in the profile of cell surface proteins (Sweet & Douglas, 1991a). Early studies also reported that serum inhibits the growth of *C. albicans* due to the presence of transferrin, and that this inhibition could be reversed by the addition of haemin or haemoglobin (Moors et al., 1992). Growth inhibition is also observed when similar iron binding compounds including lactoferrin and ovotransferrin are added to cultures (Kirkpatrick et al., 1971; Valenti et al., 1985). Changes in hyphal and germ tube formation have also been observed, with very low iron conditions being inhibitory to hyphal formation, while the addition of haemin to media induces hyphal formation (Casanova et al., 1997; Sweet & Douglas, 1991b).

Virulence studies of strains containing deletions of genes involved in iron metabolism have also highlighted the importance of iron uptake in infection. The deletion of a high affinity iron permease, *CaFTR1*, results in attenuated virulence in a systemic mouse model (Ramanan & Wang, 2000). A gene involved in siderophore transport, *CaARN1*, is also essential for the invasion of epithelial cells but is not necessary for full virulence in systemic mouse models (Heymann et al., 2002). A recent study has also identified a gene, *MNN5*, which encodes a mannosyltransferase that was initially implicated in iron metabolism, as it was capable of complementing a *ScaflAl* mutant (Bai et al., 2005). Although a recent study suggests that its role in *C. albicans* iron metabolism is less clear its activity is regulated by iron and *Camnn5Al* mutants display attenuated virulence and resistance to lactoferrin (Bai et al., 2006). The authors of this study have proposed that CaMnn5p may regulate the function of certain proteins by altering their glycosylation in response to iron limitation although this theory has not been extensively tested.
The completion of the *C. albicans* genome sequence, and the development of microarray technology, has allowed large-scale changes in gene expression to be analysed. A recent study has shown that 626 ORFs are more highly expressed in high iron conditions, and 526 transcripts are more abundant in low iron (Lan *et al.*, 2004). Interestingly, the ORFs expressed more highly in low iron conditions include not only genes encoding components of iron uptake and metabolism pathways but also those associated with cell surface components, mitochondrial function, secreted hydrolases, and drug resistance. A number of the iron responsive genes have also been previously implicated in virulence such as those encoding secreted aspartyl proteinases, host cell adhesion proteins, and proteins involved in the yeast to hyphal transition. Widespread transcriptional changes in response to iron limitation are also observed in numerous bacterial pathogens, and are vital for the establishment and progression of infections (Litwin & Calderwood, 1993). This suggests that iron availability is an important factor in the pathogenesis of *C. albicans*.

### 1.5 Iron Acquisition in *S. cerevisiae* and other fungi

Although a great deal is known about the acquisition of transition metals in a wide variety of prokaryotes (reviewed in Ratledge & Dover, 2000) our understanding of these processes in eukaryotes is relatively poor. Much of our knowledge of transition metal acquisition in not only fungi, but higher eukaryotes as well, comes from extensive studies in the model organism *S. cerevisiae*. The relative ease of performing classical genetics and molecular biology experiments in *S. cerevisiae*, combined with a fully sequenced and annotated genome, has resulted in the identification of many genes and pathways involved in iron and copper uptake and metabolism. Studies in other eukaryotes have since shown that there is a remarkable degree of conservation in the processes of iron acquisition, storage, and metabolism between *S. cerevisiae* and higher eukaryotes (Himmelbau & Amasino, 2000; Valentine & Grailla, 1997).

In *S. cerevisiae* at least four distinct mechanisms have been identified for the acquisition of iron from the external environment (van Ho *et al.*, 2002). These processes are tightly regulated in response to iron levels and involve the high affinity uptake of reduced iron via an iron specific permease, the acquisition of siderophore
bound iron, and the uptake of reduced iron via two non-specific low affinity metal ion transporters (Reviewed in DeFreitas et al., 2003; Kosman, 2003). To prevent the accumulation of dangerous levels of iron only the low affinity systems are utilised in iron replete conditions ($K_m = 30 \mu M$ to $40 \mu M$), with the high affinity systems being induced in response to iron deprivation ($K_m = 0.1 \mu M$ to $2 \mu M$) (Eide et al., 1992). The systems used for the acquisition of iron and copper in $S$. cerevisiae are illustrated in Figure 1.2.

**Ferric Reductases**

One mechanism of iron uptake involves the reduction of ferric iron to the soluble ferrous form, which can easily be transported across the cell membrane (Pierre et al., 2002). Ferric iron reduction in many organisms is performed by a group of enzymes known as ferric reductases, which are found in prokaryotes and eukaryotes (Pierre et al., 2002). Bacterial ferric reductases are soluble flavin reductases, which catalyse the reduction of free flavins using NADPH or NADH as an electron donor. The reduced flavin can then be used to reduce iron bound to siderophores, facilitating its release (Fontecave et al., 1994). This is due to the fact that siderophores have a much higher affinity for ferric versus ferrous iron. In contrast to bacterial flavin reductases eukaryotes utilise membrane associated cytochrome b-like enzymes for the reduction of iron (Pierre et al., 2002). The only examples of these enzymes to be extensively studied come from $S$. cerevisiae, although similar enzymes have been identified in mice and plants (McKie et al., 2001; Waters et al., 2002). Ferric reductases have also been identified in a range of other fungi including *C. albicans*, *Schizosaccharomyces pombe*, *Aspergillus nidulans*, and *Cryptococcus neoformans* (Hammacott et al., 2000; Nyhus & Jacobson, 1999; Oberegger et al., 2002; Roman et al., 1993).

Early studies of iron uptake in $S$. cerevisiae demonstrated that it possessed a cell surface mechanism that was able to reduce iron from compounds such as ferricyanide, ferric citrate, ferric-EDTA chelates, and siderophores (Crane et al., 1982; Lesuisse et al., 1987). Further analysis revealed that the cell surface ferric reductase activity associated with cells was regulated in response to iron concentrations, with reduction of iron compounds being maximal in iron depleted conditions (Dancis et al., 1990; Lesuisse et al., 1987). Fractionation experiments also demonstrated that while the
Figure 1.2 Iron and copper uptake in the model organism *S. cerevisiae*. In *S. cerevisiae* the uptake of copper and iron are intricately linked with several proteins being involved in the transport of both metals. Prior to their uptake iron and copper are reduced at the cell surface by the reductases Fre1p and Fre2p. Iron is then transported across the cell membrane by a high affinity transport complex comprising of the iron permease Ftr1p, and the multicopper oxidase Fet3p that requires copper for its correct functioning. During its transport iron is first oxidised by Fet3p, which allows it to be recognised and transported by Ftr1p. A wide range of ferri-siderophores can also be transported across the membrane by a family of proteins, Arn1-4p, that have different siderophore specificities. The GPI-anchored proteins Fitl-3p bind iron at the cell surface and are known to increase the efficiency of siderophore transport. High affinity copper transport occurs through two redundant transporters, Ctr1p and Ctr3p, and is then transported to its site of use by the chaperones Atx1p, Lys7p, and Cox17p. Lys7p and Cox17p transport copper for incorporation into Cu,Zn superoxide dismutase and via Sco1p into cytochrome c oxidase. Of particular relevance is Atx1p that delivers copper to Ccc2p in the membrane of the Golgi for incorporation into Fet3p. When iron and copper are abundant their transport mainly relies on a number of non-specific low affinity transporters including Fet4p and Smf1p. Following entry into the cell iron is safely stored in the vacuole where a number of transporters are responsible for its import and export. Iron can be imported into the vacuole by Ccc1p and possibly Cot1p. The ferric reductase Fre6p is though to be located in the vacuole and this may reduce iron prior to its export from the vacuole via the iron permease/multicopper oxidase complex formed by Fth1p/Fet5p, or the Nram transporter Smf3p. Iron can also be transported into the mitochondria by Mrs3/4p and possible Smf2p where the ferric reductase Fre5p is thought to be located. Genes encoding components of the high affinity iron uptake mechanism are transcriptionally regulated by a pair of paralogous transcription factors, Aft1p and Aft2p, which activate transcription in response to low iron conditions. Proteins whose genes are regulated in by Aft1/2p are shown in red. High affinity copper uptake is regulated in response to low copper conditions by the transcriptional activator Mac1p and the proteins whose genes it regulates are shown in blue. A further link between iron and copper uptake is provided by the fact that Fre1p is regulated by Mac1p and Aft1/2p. Figure adapted from Bakel *et al.*, (2005) and DeFreitas *et al.*, (2003).
The majority of cellular ferric reductase activity is associated with the plasma membrane, lower levels are found in cellular compartments such as the mitochondria (Lesuisse et al., 1990).

The first components of the cell associated ferric reduction system in *S. cerevisiae* were identified following mutagenesis experiments that created a mutant with negligible levels of cell surface ferric reductase activity (Dancis et al., 1990). A single genomic clone was then identified that was able to restore ferric reductase activity when reintroduced into the mutant strain. This clone encoded a protein, Fre1p, which was 686 amino acids long and contained features that were consistent with its role as a ferric reductase. The Fre1p sequence contains a number of putative transmembrane domains and a 22 amino acid N-terminal leader peptide (Anderson et al., 1992). The final 402 amino acids of Fre1p also share 17.9 % identity and 62.2 % similarity with the large subunit of the human cytochrome b558, gp91phox, which is an oxidoreductase involved in the respiratory burst in phagocytes (Chanock et al., 1994). The cytochrome b558 oxidoreductase transfers a single electron from cytoplasmic NADPH across the membrane to molecular oxygen via FAD and heme (Wientjes & Segal, 1995). This results in the production of superoxide, which accumulates in the lumen of the vacuole and aids in microbial killing.

The majority of the homology between Fre1p and gp91phox is found in a number of highly conserved regions. These regions include a HPFTXXS motif that has been implicated in FAD binding in cytochrome b558 (Taylor et al., 1993). A glycine rich motif and a cysteine-glycine couplet, which form peptide loops thought to be involved in NADPH binding, are also found in both proteins (Segal & Abo, 1993). The protein sequences also contain four conserved histidine residues that are required for the binding of the essential co-factor b-haem (Finegold et al., 1996; Lesuisse & Labbe, 1989; Shatwell et al., 1996).

Experimental studies in *S. cerevisiae* have confirmed the role of Fre1p in the extracellular reduction and uptake of iron. The expression of *FRE1* mirrors the profile of surface reductase activity, with both being induced in low iron conditions and repressed in response to iron (Dancis et al., 1992). Deletion of *FRE1* also results in an 80 % reduction in ferric reductase activity and retarded growth in low iron conditions.
The importance of ferric iron reduction prior to uptake is also shown by the fact that the deletion of FRE1 causes defects in the uptake of ferric, but not ferrous, iron (Dancis et al., 1990).

Following the sequencing of chromosome XI a second ferric reductase was identified on the basis of its homology to Fre1p (Cassamayor et al., 1995). The FRE2 gene encodes a protein 711 amino acids in length and contains a 23 amino acid N-terminal signal sequence and a number of putative transmembrane domains in a similar fashion to Fre1p (Georgatsou & Alexandraki, 1994). While the overall level of homology is relatively low (24.5 % identity) a number of important motifs are highly conserved between the two proteins, including regions implicated in the binding of FAD and NADPH. The Fre2p protein also contains four conserved histidine residues that have been implicated in bis-heme binding in Fre1p, although reports suggest Fre2p does not bind heme (Lesuisse et al., 1996).

Deletion of FRE2 shows that it is responsible for the majority of the residual ferric reductase activity observed in the fre1Δ mutant, suggesting that they encode the major cell surface reductases in S. cerevisiae (Georgatsou & Alexandraki, 1994). The deletion of both FRE1 and FRE2 causes severe growth defects in low iron conditions, severely reduces iron uptake, and decreases cell surface reductase activity by 90-98 % (Georgatsou & Alexandraki, 1994). It has been suggested that the residual reductase activity in the fre1Δfre2Δ mutant is due to other reductases or to the secretion of phenolic compounds capable of reducing iron, although their precise importance in iron uptake is unknown (Lesuisse et al., 1992). The relative importance of FRE1 and FRE2 in ferric reduction is complicated by the observation that they have different reductase activity profiles (Georgatsou & Alexandraki, 1994). Analysis of fre1Δ and fre2Δ mutant strains reveals that Fre1p activity is maximal in early growth while Fre2p plays a more significant role later in growth.

In addition to their ability to reduce iron Fre1p and Fre2p are able to reduce copper (Georgatsou et al., 1997; Hassett & Kosman, 1995; Lesuisse and Labbe, 1992). The deletion of both FRE1 and FRE2 abolishes the majority of cell associated cupric reductase activity (Georgatsou et al., 1997). This reduction of copper at the cell
surface is an important part of the uptake process as the rate of radioactive copper uptake is reduced by 50 to 70% in \textit{fre1\textDelta} mutants (Hassett & Kosman, 1995). The expression of Ctrlp also increases in \textit{fre1\textDelta} and \textit{fre1\textDeltafre2\textDelta} mutants, presumably in an attempt to compensate for the decrease in copper uptake (Georgatsou \textit{et al.}, 1997). However, while the majority of cupric reductase activity is abolished in \textit{fre1\textDeltafre2\textDelta} mutants there is still some residual activity. This suggests the presence of a further ferric reductase with a role in the reduction of copper.

Following the completion of the \textit{S. cerevisiae} genome sequence a further 7 ORFs have been identified with significant homology to \textit{FRE1} and \textit{FRE2} (Georgatsou & Alexandraki, 1999). The majority of these ORFs encode proteins with a higher degree of similarity to Fre2p, especially Fre3p (75.9 % identity; 86.9 % similarity) and Fre4p (57.3 % identity; 74.8 % similarity), and the slightly less similar Fre5p (38.5 % identity; 60.1 % similarity) and Fre6p (38 % identity; 61 % similarity). Another ORF encodes Fre7p, which is equally distantly related to both Fre1p (23.9 % identity; 51.9 % similarity), and Fre2p (23.2 % identity; 52.9 % similarity). The other two ORFs identified, \textit{YGL160w} and \textit{YLR047c}, are distantly related to the other ferric reductases and are most similar to each other. The seven putative ferric reductases share a number of important features with Fre1p and Fre2p (Georgatsou & Alexandraki, 1999). All of the proteins contain numerous putative transmembrane domains and only Fre7p, Ygl160p, and Ylr047p do not have a hydrophobic N-terminal signal sequence. They also contain conserved binding sites for FAD and heme, and only Ylr047p does not have a predicted NADPH binding site. Analysis of the expression of these seven ORFs using reporter constructs and northern blot analysis has shown that \textit{FRE3-6} are regulated by the levels of iron, but not copper, in the growth media in a similar fashion to \textit{FRE2} (Georgatsou & Alexandraki, 1994; Georgatsou & Alexandraki, 1999; Martins \textit{et al.}, 1998). In contrast the expression of \textit{FRE7} is only regulated in response to copper levels and \textit{YML160w} and \textit{YLR047c} transcript levels are unaffected by either iron or copper (Georgatsou & Alexandraki, 1999; Martins \textit{et al.}, 1998).

In contrast to Fre1p and Fre2p little is known about the precise role of the other ferric reductases in \textit{S. cerevisiae}. One possibility is that the other reductases may play a role in the reduction of specific iron substrates such as iron bound to siderophores. The
ability of *S. cerevisiae* to reduce siderophores is well known and evidence suggests that Fre1-4p play a role in this process (Heymann *et al.*, 2000b; Lesuisse *et al.*, 1989; Lesuisse *et al.*, 1987; Yun *et al.*, 2001). Both Fre1p and Fre2p are able to reduce a wide range of hydroxamate and catechol class siderophores and Fre3-4p are also able to reduce iron bound to specific siderophores (Yun *et al.*, 2001). Analysis of *fre1-2Δ* and *fre1-3Δ* mutants shows that Fre3p is able to reduce iron bound to the hydroxamate siderophores ferrioxamine B, ferrichrome, triacetylfusarinine C, and rhodotorulic acid. Although the levels of siderophore reduction by Fre3p are 40 times lower than for Fre1-2p they are sufficient to allow the *fre1-2Δ* mutant to grow using siderophores as their sole iron source. The analysis of *fre1-3Δ* and *fre1-4Δ* mutants shows that Fre4p is able to reduce iron bound to rhodotorulic acid, and that the *fre1-3Δ* mutant was able to use this as its sole iron source. This study also showed that Fre1-2p are required for the reduction of iron bound to the catechol enterochelin in addition to their ability to reduce all of the siderophore types that act as substrates for Fre3-4p.

There is currently no evidence to explain the specificity of Fre1-4p for their ferri-siderophore substrates but it may simply reflect the differing reducing powers of the four proteins. The available data shows that Fre1p and Fre2p are the strongest reducers and Fre4p the weakest. Therefore, the strongest reducers have the broadest range of substrate.

Although the precise roles of Fre5-7p, Ygl160p, and Ylr047p are unknown a number of potential functions exist for these proteins. In common with Fre3-4p these proteins may be involved in the reduction of specific substrates. Alternatively they may be involved in the intracellular trafficking of iron. Early experiments demonstrated that intracellular compartments such as the mitochondria are able to reduce iron (Lesuisse *et al.*, 1990). Recent large-scale experiments in *S. cerevisiae* provide some evidence that Fre5p and Fre6p may function in intracellular compartments. A study of the mitochondrial proteome revealed the presence of Fre5p, suggesting it is involved in iron metabolism within this important organelle (Sickmann *et al.*, 2003). A genome wide localisation study has also shown that Fre6p is located in the vacuolar membrane (Huh *et al.*, 2003). The presence of a ferric reductase situated in the vacuolar membrane is not unexpected, as homologues of the Ftr1p/Fet3p cell surface high affinity iron transport complex are involved in the transport of vacuolar iron, and presumably require the reduction of iron prior to its transport (Urbanowski *et al.*, 2003).
1999). The function of Fre7p is unclear, although its regulation in response to copper depletion suggests its involvement in copper transport or metabolism. However, fre7Δ mutants do not display defects in the extracellular reduction of copper or iron (Hassett & Kosman, 1995). It has therefore been proposed that Fre7p is involved in copper trafficking and metabolism within the cell.

As described, the possible functions of many of the ferric reductases are known but the role of YGL160w and YLR047c is still unclear. Although northern blot analysis of the ferric reductases showed changes in copper or iron levels do not alter the expression of these genes (Georgatsou & Alexandraki, 1999) the levels of YLR047c transcript increase in a strain carrying a deletion of the copper sensing transactivator Mac1p (DeFreitas et al., 2004). This study also demonstrated that Ylr047cΔ mutant strains exhibit phenotypes that are consistent with defects in iron metabolism. These include reduced growth on iron or copper limited media and the inability to utilise non-fermentable carbon sources.

**High Affinity Iron Transport**

The uptake of iron in *S. cerevisiae* can occur through high affinity (K_m = 0.15 μM), or low affinity (K_m = 30-40 μM), iron uptake mechanisms (Eide et al., 1992). High affinity iron uptake occurs through a transport complex containing a multicopper oxidase (Fet3p) and a permease (Ftr1p), which specifically transports ferric iron (Stearman et al., 1996). During transport the reduced iron produced by the cell surface ferric reductases is oxidised by the multicopper oxidase and transported through the iron permease. Similar high affinity systems appear to function in the majority of yeast and fungi, with homologues of the transport complex having been identified and characterised in *C. albicans*, *Schizosaccharomyces pombe*, *Pichia pastoris*, *Cryptococcus neoformans*, *Rhizopus oryzae* and *Arxula adeninivorans* (Askwith & Kaplan, 1997; Eck et al., 1999; Fu et al., 2004; Paronetto et al., 2001; Ramanan & Wang, 2000; Lian et al., 2005; Wartmann et al., 2002). This oxidase/permease system of transport is also found in other eukaryotes including the algae *Chlamydomonas reinhardtii* (LaFontaine et al., 2002) and bacteria such as a magnetotactic strain of a marine vibrio (Dubbels et al., 2004).
The first component of the high affinity iron transport complex to be isolated in *S. cerevisiae* was a multicopper oxidase encoded by *FET3* (Askwith *et al.*, 1994). It was initially identified following a screen for mutants showing defects in high affinity iron uptake, which were therefore only able to grow in iron rich media. The *FET3* gene was then isolated from a genomic library due to its ability to restore high affinity iron uptake to one of the mutants. The involvement of the *FET3* gene product in iron uptake was confirmed by the creation and analysis of a *fet3Δ* mutant. This mutant was unable to grow on non-fermentable carbon sources and had a severe growth defect in low iron conditions due a lack of high affinity iron uptake. These phenotypes are consistent with Fet3p being a vital component of the high affinity uptake system.

The *FET3* gene encodes a 686 amino acid type I membrane protein with an N-terminal hydrophobic signal peptide and a single predicted transmembrane domain located in the C-terminus (Askwith *et al.*, 1994). It contains several conserved motifs that are found in the multicopper oxidase family of proteins and shows significant overall homology to many members of this family. The multicopper oxidases include proteins such as ascorbate oxidase, plant and fungal laccases, and the mammalian protein ceruloplasmin (Askwith *et al.*, 1994). These proteins have the ability to catalyse the oxidation of 4 moles of substrate while reducing molecular oxygen to produce water. This process is unique as they are the only enzymes known to reduce both atoms of $O_2$ to give water, and thus avoid the possible production of harmful superoxide radicals. Of particular interest is ceruloplasmin, which is involved in the oxidation of iron prior to its insertion into transferrin and ferritin using the reaction shown below (Osaki *et al.*, 1966).

$$4 \text{Fe (II)} + \text{O}_2 + 4 \text{H}^+ \rightarrow 4 \text{Fe (III)} + 2 \text{H}_2\text{O}$$

Further characterisation of the Fet3p protein has proved it is involved in iron uptake and functions as a multicopper oxidase in a similar fashion to ceruloplasmin. Early evidence for its role as a multicopper oxidase included the observation that high affinity iron uptake is reduced in copper limiting conditions and in strains that have defects in copper uptake (Askwith *et al.*, 1994; Dancis *et al.*, 1994). This link between iron and copper uptake is due to the absolute requirement of copper for multicopper

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oxidases such as Fet3p to catalyse the oxidation of iron. Interestingly, humans with defects in copper uptake and metabolism also display symptoms that are consistent with decreased iron uptake and metabolism (Pierre & Crichton, 2001). The consumption of oxygen by yeast cells also demonstrates the role of Fet3p in iron uptake, as it increases in iron limiting conditions (DeSilva et al., 1995). The stoichiometry of iron transport to O₂ consumption has been determined as approximately 4:1, which supports the involvement of a multicopper oxidase in iron transport. This change in oxygen consumption is Fet3p dependent, as it is not observed in fet3Δ mutants. Purification of intact Fet3p has also conclusively shown that Fet3p is able to oxidise ferrous iron in a copper dependent manner (De Silva et al., 1997). Spectroscopic analysis of purified Fet3p has revealed the presence of four copper molecules bound to the protein in a similar fashion to ceruloplasmin (Blackburn et al., 2000; Hassett et al., 1998). These copper molecules form binding sites with different functions. A type I copper binding site is responsible for the reduction of ferrous iron while a tri-nuclear cluster formed by a type II and two type III coppers is involved in the reduction of oxygen to produce water (Blackburn et al., 2000).

Although Fet3p is essential for high affinity iron uptake it lacks many of the features of a classic membrane transport protein (Askwith et al., 1994). For example, Fet3p lacks the multiple membrane spanning domains classically associated with many membrane transport proteins. It was therefore suggested that Fet3p might act as a complex with other proteins such as a membrane permease. This membrane permease, encoded by FTR1, was later identified in genetic screens for essential components of the high affinity iron uptake pathway (Stearman et al., 1996). Mutant strains lacking the FTR1 gene have identical phenotypes to a fet3Δ strain with impaired growth in low iron conditions, the inability to utilise non-fermentable carbon sources, and a lack of high affinity iron uptake. The FTR1 gene is regulated in response to iron and encodes a 404 amino acid polypeptide that has an N-terminal leader sequence and seven transmembrane domains. The membrane topology of Ftr1p has been extensively studied by tagging the protein in several locations and studying its localisation (Severance et al., 2004). Comparisons of its localisation in permeabilised and un-permeabilised cells revealed that the N-terminus is extracellular.
and the C-terminus cytosolic. The location of several extracellular loops was also examined and their localisation was consistent with the presence of 7 transmembrane domains.

The current evidence supports the initial hypothesis that Fet3p and Ftrlp interact to form a high affinity iron transport complex (Stearman et al., 1996). In normal cells both proteins are localised to the cell surface, but in fet3Δ strains Ftrlp accumulates in the endoplasmic reticulum. A similar result is observed in ftr1Δ mutants where Fet3p fails to reach the cell surface. Further evidence for the cooperation of these two proteins comes from the study of homologous proteins in S. pombe (Askwith et al., 1997). The multicopper oxidase, Fiop, and the iron permease, Fip1p, from S. pombe are not able to functionally rescue S. cerevisiae fet3Δ or ftr1Δ mutant strains. However, if both S. pombe genes are co-expressed in a fet3Δ mutant high affinity iron uptake is restored. This demonstrates that the oxidase and permease function together and suggests that they are the only membrane proteins required to facilitate high affinity iron uptake. Further evidence for an interaction between Ftrlp and Fet3p came from the study of a pair of homologous proteins in S. cerevisiae, Fth1p and Fet5p, which are involved in vacuolar iron transport (Urbanowski et al., 1999). Co-immunoprecipitation experiments were used to show a direct interaction between Fth1p and Fet5p in vivo. Subsequent analysis of the Ftr1p and Fet3p homologues in Pichia pastoris also demonstrated that these proteins associate as a complex in cross-linked membranes (Bonaccorsi di Patti et al., 2005). As this is the only study to have successfully shown an interaction between Fet3p and Ftrlp the authors of this study suggest that the interaction between the two proteins is probably relatively weak, and is therefore easily disrupted by protein preparation and electrophoresis methods.

As discussed earlier if both Fet3p and Ftrlp are present they are transported to the cell surface via a post-Golgi compartment (Stearman et al., 1996). Without the presence of their respective partners Ftrlp and Fet3p are localised to the endoplasmic reticulum. The retention of these proteins is thought to occur through the ER quality control (ERQC) system, which prevents the transport of immature proteins beyond the ER (Sato et al., 2004). Analysis of Fet3p retention shows that without Ftrlp it is able to enter the Golgi but is retrieved and returned to the ER. This process is mediated by
Rerlp, which recognises specific polar residues within the Fet3p transmembrane domains and returns it to the endoplasmic reticulum. The interaction of Fet3p with Ftrlp is theorised to block Rerlp recognition sites, allowing the proteins to continue to the cell surface. The return of Fet3p to the ER is unusual as recognition of proteins by the ERQC mechanism normally results in their rapid destruction by the ER associated degradation pathway. It is therefore thought that the return of Fet3p to the ER is used to increase the chances of Fet3p/Ftrlp interactions occurring.

The intracellular transport of Fet3p is of particular importance, as its function as an oxidase requires loading with copper (Askwith et al., 1994; Dancis et al., 2004; Yuan et al., 2005). The loading of apoFet3p with copper occurs in a post-Golgi compartment (Yuan et al., 1995) and a number of proteins that are essential for this process, but do not affect normal vesicular trafficking, have been identified (reviewed van Ho, 2002). Mutations in the genes encoding these proteins result in an accumulation of apo-Fet3p at the cell surface and thus abolish high affinity iron uptake. Following transport into the cell cytosolic copper is captured by a small cytosolic chaperone, Atx1p, and transported to the late-Golgi vesicle (Lin et al., 1997). A small amount of copper is also able to reach the vesicle by endocytosis but this process is much less efficient. Following copper binding, Atx1p specifically delivers it to Ccc2p, which is located in apo-Fet3p containing intracellular compartments (Lin et al., 1997). Ccc2p is an ATP-dependent copper transporter that transports Atx1p derived copper across the vesicle membrane to the lumen where it can be incorporated into Fet3p (Yuan et al., 1995; Yuan et al., 1997). The ScCcc2p protein is a homologue of the human Menkes and Wilson proteins, which are both capable of rescuing an S. cerevisiae ccc2Δ (Hung et al., 1997; Payne & Gitlin, 1998). In humans defects in the Menkes or Wilsons proteins have severe effects (Reviewed in Mercer et al., 2001) with the lack of Menkes causing infant death due to defective copper transport from intestinal cells to the plasma. The absence of the Wilsons protein causes copper accumulation in the liver and defective ceruloplasmin function due to the inability to load it with copper.

A number of environmental factors within the vesicle are also important in the insertion of copper into apoFet3p. The acidity of the vacuole is important for copper
loading of apoFet3p, and mutations in a number of genes that maintain vesicular pH prevent loading of apoFet3p (Davis-Kaplan et al., 2004; Gaxiola et al., 1998). These genes include CHW36 and TFP1, which encode subunits of an H\(^+\)-ATPase involved in vesicular acidification (Davis-Kaplan et al., 2004; Gaxiola et al., 1998). The loading of apoFet3p also requires Cl\(^-\) ions and a voltage gated chloride channel, Gep1p, is essential for functional Fet3p production (Davis-Kaplan et al., 1998; Gaxiola et al., 1998). The transport of Cl\(^-\) ions into the vesicle by Gep1p is also important in maintaining the net electronegativity of the organelle (Gaxiola et al., 1998). Without Gep1p the acidification of the vesicle by the H\(^+\)-ATPase and the transport of cations such as copper into the vesicle increase the membrane potential and impede further cation transport.

As mentioned homologues of the S. cerevisiae iron permease and multicopper oxidase have recently been identified in C. albicans (Eck et al., 1999; Ramanan & Wang, 2000). The permeases, CaFtrlp and CaFtr2p, are 83% identical and share ~54% identity with ScFtrlp (Ramanan & Wang, 2000). Comparisons of the characterised oxidases CaFet3p and CaFet99p with ScFet3p show they are very similar and share 55% and 60% identity at the amino acid level respectively (Eck et al., 1999). Analysis has shown that CaFtrlp and CaFet3p probably have similar functions to their S. cerevisiae homologues (Eck et al., 1999; Ramanan & Wang, 2001). CAFTR1 and CAFET3A mutants show growth defects after prolonged exposure to low iron conditions and the CAFTR1 mutant has reduced levels of ferric iron uptake. The expression pattern of CAFTR1 is also similar to that of SCFTR1 as they are both maximally expressed in low iron levels (Ramanan & Wang, 2000). When placed under the control of an inducible promoter CAFTR1 is also able to complement the iron uptake defects associated with a SCFTR1 mutant. In contrast the role of CAFTR2 is unclear as it is maximally expressed in iron replete conditions (Knight et al., 2002; Ramanan & Wang, 2000). Although its role is unclear CaFtr2p is a functional iron transporter as it is able to complement a CAFTR1 mutant when under the control of the CAFTR1 promoter (Ramanan et al., 2000). Its functionality has also been shown in S. cerevisiae where it is able to complement a SCFTR1 mutant when placed under the control of an inducible promoter (Knight et al., 2002). The role of CaFet99p is also unknown as it as it has not been as extensively studied as CaFtr1/2p and CaFet3p.
However, it is known that *CaFET99* transcript levels increase in response to low iron conditions in a similar fashion to *ScFET3*.

The affect of the deletion of *CaFTR1*, *CaFTR2*, and *CaFET3* on the virulence of *C. albicans* has also been studied and gives conflicting results. Only the deletion of *CaFTR1* affects the virulence of *C. albicans* in mouse models, with its loss rendering the strain avirulent (Eck *et al.*, 1999; Ramanan & Wang, 2000). Therefore only CaFtr1p is essential for high affinity iron uptake and during the establishment of an infection. This is confusing as in *S. cerevisiae* both proteins are required for the correct localisation and functioning of each other, therefore *Cafet3Δ* would be expected to have an identical virulence phenotype to the *Cafr1Δ*. One explanation is that CaFet3p does not interact with CaFtr1p in the main iron transport complex. It is also possible that other oxidases can interact with CaFtr1p, as there are multiple CaFet3p homologues in *C. albicans*. However, CaFet3p appears to play a role in some aspects of the infection as its deletion results in a 44% reduction in the ability of cells to bind to fibroblasts (Eck *et al.*, 1999).

**Siderophore Uptake**

Another system of acquiring iron from the environment used by a wide variety of organisms is through the use of siderophores (Reviewed in Haas, 2003; Kosman, 2003). Siderophores are low molecular weight organic compounds that have very high affinities for ferric iron (Reviewed in Neilands *et al.*, 1995). The iron free forms of these compounds are synthesised and secreted into the surrounding environment where they bind and solubilise ferric iron. The iron siderophore complex, or the iron bound to the siderophores, is recognised and transported across the membrane by specialised uptake systems on the cell surface. The majority of bacteria and fungi produce at least one type of siderophore and commonly show the ability to utilise different siderophores produce by other organisms (Byers & Arceneaux, 1998). While *S. cerevisiae* is able to utilise siderophores from a wide range of other organisms it is unable to produce its own siderophores (Lesuisse *et al.*, 1987; Neilands *et al.*, 1995).

In *S. cerevisiae*, iron chelated by siderophores can be transported into the cell via reductive or non-reductive pathways (Lessuise *et al.*, 1998; Yun *et al.*, 2001). The first of these involves the extracellular reduction and release of iron from the siderophores,
followed by its transport via the high affinity ferrous uptake system (Lesuisse et al., 1998; Yun et al., 2001). The reduction step is performed by the ferric reductases Fre1p, Fre2p, Fre3p, and Fre4p, and the ferrous iron transported into the cell by the Fet3p/Ftr1p transport complex (Yun et al., 2001). Studies using strains defective in the non-reductive mechanism of siderophores uptake have shown that the reductases involved in this process show a degree of substrate specificity, with Fre1/2p facilitating the reduction of trihydroxamate and dihydroxamate type siderophores. When neither of these reductases is present Fre3p is required for the reduction of ferrioxamine B, ferrichrome, triacetylfusarinine C, and rhodotorulic acid. Similarly Fre4p is required for the utilisation of rhodotorulic acid in the absence of Fre1-3p.

The non-reductive mechanism for ferri-siderophore transport involves a number of homologous transporters of the major facilitator superfamily encoded by ARNI-4, which are able to specifically transport siderophores of the catechol and hydroxamate classes (Lesuisse et al., 1998; Heymann et al., 1999; Heymann et al., 2000a; Heymann et al., 2000b; Yun et al., 2000a; Yun et al., 2000b). The transporters show different specificities with Arn1p being able to transport the ferrichrome type siderophores ferrirubin, ferrirhodin, and ferrichrome A (Heymann et al., 2000b). The Arn2p transporter is specific for triacetylfusarinine C, Arn3p for ferrioxamine B, and Arn4p recognises enterochelin (Heymann et al., 1999; Heymann et al., 2000a; Lesuisse et al., 1998). However, the substrate specificity of the transporters is not absolute as a number of them, especially Arn2p and Arn3p, show competition for the uptake of some siderophores types (Lesuisse et al., 2001).

The precise mechanism by which the Arn1-4 proteins transport siderophores across the cell membrane is still poorly understood, but studies of Arn1p have provided a model for uptake. Localisation studies have shown that Arn1p and Arn3p are located in multiple intracellular vesicles similar to the late endosomal compartments (Yun et al., 2000a; Yun et al., 2000b). This is supported by data showing that Arn1p containing vesicles co-migrate with Pep12p, a late endosomal protein, in density gradient centrifugation experiments (Becherer et al., 1996). Recent studies have shown that in the presence of low concentrations of ferrichrome Arn1p re-localises to the plasma membrane, although no uptake of the siderophores is detected (Heymann et al., 2000b; Yun et al., 2000b; Kim et al., 2002). When the concentration of
Figure 1.3 Proposed model for Arn1p mediated siderophore uptake in *S. cerevisiae*. The Arn1p siderophore transporter initially enters the late endosome from the Golgi. Ferrichrome (■) binds iron (●) and enters the cell via fluid phase endocytosis and binds to a high affinity site on Arn1p. This causes a conformational change that results in the relocalisation of Arn1p to the plasma membrane. At the cell surface the binding of ferrichrome to a second lower affinity binding site in Arn1p triggers its internalisation. Following internalisation the siderophore is released from Arn1p, and the iron liberated for use within the cell. Diagram adapted from Kim *et al.*, (2002).
ferrichrome increases Arn1p cycles between the plasma membrane and the late endosomal compartments, where the iron is presumably released.

The mechanism by which the changes in localisation occur is unknown but mutagenesis and kinetic experiments in *S. cerevisiae* involving Arn1p, as well as comparisons with other transport proteins has allowed a model to be produced that is illustrated in Figure 1.3 (Kim *et al.*, 2002; Kim *et al.*, 2005). This model proposes that ferrichrome initially enters the endosome via fluid-phase endocytosis and binds to a high affinity binding site on Arn1p. Ferrichrome binding causes a conformational change in the C-terminal region of Arn1p, which results in its re-localisation to the plasma membrane. Upon localisation at the plasma membrane Arn1p binds another ferrichrome molecule at a second lower affinity binding site, causing internalisation of the fully loaded protein to the endosome. The movement of Arn1p to the endosome then results in the release of the siderophores from Arn1p, possibly by the reversion of the protein to its original conformation.

While a wealth of information is present regarding siderophores uptake in *S. cerevisiae* our knowledge of the same process in *C. albicans* is poor. A large number of studies have been performed in an attempt to confirm if *C. albicans* is able to produce and excrete siderophores but they have produced conflicting results although the majority of studies report the production of siderophores of an uncharacterised type (Holzberg & Artis, 1983; Ismail *et al.*, 1985; Sweet & Douglas, 1991). In contrast, analysis of the *C. albicans* genome suggests that it lacks vital components of siderophore biosynthesis pathways (Haas, 2003). Some possible explanations provided for these discrepancies include the production of false positives in the assays commonly used, or that there is a large degree of strain variation. However, the latter explanation is strongly supported by a recent study where 81% of the 32 isolates examined displayed the production of siderophores (Hannula *et al.*, 2000).

Although the production of siderophores by *C. albicans* is still debatable, its ability to utilise a wide variety of siderophores is well known. Multiple studies have shown that *C. albicans* can efficiently utilise the hydroxamate siderophores ferrichrome, ferricrocin, ferrichrysin, ferrirubin, and to a lesser extent triacetylfusarinine C, COP, ferrioxamine E and ferrioxamine B (Arden *et al.*, 2001; Heymann *et al.*, 2002;
Lesuisse et al., 2002). Recent studies have also identified a single homologue of the ARN family of siderophore transporters in *C. albicans* that shares 46% homology with ScArn1p (Arden et al., 2001; Heymann et al., 2002; Lesuisse et al., 2002). The CaArn1p protein is necessary for the uptake of most of the siderophores types utilised by *C. albicans* with the exception of ferrioxamine E and B, which require a functional reductive uptake system for transport (Heymann et al., 2002; Hu et al., 2002). In contrast to ScArn1p, localisation studies show that the CaArn1p is predominately found in the plasma membrane and becomes increasingly prominent in the cytoplasm upon exposure to siderophores (Hu et al., 2002). A functional CaArn1p protein is also required for full virulence in epithelial invasion assays, although a Caarn1ΔΔ is fully virulent in a systemic mouse model (Heymann et al., 2002; Hu et al., 2002). The variation in these two virulence studies is not uncommon and may suggest that siderophores uptake is only vital in specific areas of the infection process and this may not be easily seen in a systemic model.

**Cell Wall Facilitators of Iron Uptake**

In order for iron to reach the plasma membrane based uptake systems it must first cross the cell wall. Global analysis of iron regulated genes has identified three cell wall proteins that are negatively regulated by iron and facilitate the transport of iron across the cell wall to the plasma membrane (Protchenko et al., 2001). The FIT1-3 genes are the most strongly induced in response to iron limitation with their mRNA transcript levels increasing 60, 140, and 230 fold respectively. The Fit1-3p proteins each have an amino terminal signal sequence and a GPI signal. The GPI family of proteins are anchored to the cell wall following cleavage of the GPI signal at the plasma membrane and subsequent attachment to β-1,6-glucan (Caro et al., 1997; Protchenki et al., 2001).

The phenotypic analysis of individual and multiple FIT gene deletion mutants has helped elucidate the role of the three proteins in iron uptake (Protechenko et al., 2001). Interestingly the mutant phenotypes associated with the deletion of single or multiple FIT genes are almost identical, suggesting that the proteins form a complex or function in the same pathway. A fitΔ mutant displays reduced levels of siderophore bound iron uptake via the Arn1p dependent iron uptake pathway, although the
significant levels of uptake remaining demonstrate they are not essential for this process. Levels of iron uptake via the Ftr1p/Fet3p system are unaffected, although FET3 expression is increased in a fitΔ strain. This indirectly suggests that Fit1-3p are also involved in uptake via Ftr1p/Fet3p, as loss of the Fit proteins requires the increased expression of genes encoding other components of the high affinity iron uptake system to compensate for their loss. Deletion of the FIT genes also results in a reduction of cell surface bound iron.

It is currently thought that Fit1-3p facilitate the movement of iron across the cell wall and periplasmic space (Protchenko et al., 2001). The increased amount of iron present in the cell wall in the presence of Fit1-3p may therefore increase the local concentrations available for uptake via the high affinity iron uptake systems. It has also been suggested that Fit1-3p may also store iron at the cell surface that can be used in the event of protracted iron starvation.

**Low Affinity Iron Uptake**

The presence of a low affinity iron uptake pathway in *S. cerevisiae* was suggested by the observation that cells lacking high affinity uptake are still viable when the media is supplemented with iron (Askwith et al., 1994). Early studies identified a divalent metal transporter encoded by FET4 as being the major low affinity iron transporter in *S. cerevisiae* (Dix et al., 1994). Evidence for the role of Fet4p includes the observation that FET4 over-expression increases low affinity uptake. The deletion of FET4 also results in the loss of low affinity uptake while leaving the high affinity system intact. The Fet4p transporter is a 552 amino acid long protein that contains six predicted transmembrane domains and is localised at the plasma membrane (Dix et al., 1994; Dix et al., 1997). In contrast to the high affinity uptake system Fet4p is able to facilitate the transport of a number of metals into the cell including nickel, cobalt, cadmium, zinc, copper, and manganese as well as iron (Hassett et al., 2000; Li et al., 1998; Jensen et al., 2002). Indeed recent evidence has revealed that Fet4p is a physiologically relevant copper and zinc transporter (Hassett et al., 2000; Portnoy et al., 2001, Waters et al., 2002).
Northern blot analysis of FET4 expression has shown that its expression is negatively regulated in response to iron, with expression increasing threefold in iron deplete conditions (Dix et al., 1997). However, until recently the complex mechanisms involved in FET4 regulation were unclear. The regulation of FET4 initially appeared to be different to the high affinity uptake system as its expression was unaltered in strains carrying a AFT1Δp allele, which leads to the constitutive expression of high affinity iron uptake genes (Dix et al., 1997). However, subsequent analysis revealed that FET4 is regulated by AFT1 in an iron dependent manner (Jensen et al., 2002; Waters et al., 2002). The FET4 gene is also regulated in response to zinc levels by the transcription factor Zaplp, which is consistent with the observation that Fet4p functions as a physiologically relevant zinc transporter (Waters et al., 2002). However, while Fet4p functions as a low affinity copper transporter it is not regulated by copper, or by the copper responsive transcription factor Mac1p.

In addition to its regulation by levels of iron and zinc, FET4 expression increases under anaerobic conditions (Linde et al., 1999). These changes in FET4 expression are due to the transcription factor Rox1p, which represses a range of target genes in aerobic conditions by recruiting the general transcriptional repressors Tup1p and Ssn6p (Zhang et al., 1999; Zitomer et al., 1997). Analysis of the FET4 promoter shows that Rox1p functions to attenuate the expression of FET4 in response to zinc or iron deprivation in aerobic conditions (Waters et al., 2002). A number of theories have been suggested to explain the tight regulation of FET4 in response to oxygen. The low affinity uptake system may be tightly regulated in aerobic conditions to prevent the influx of harmful levels of metals such as iron and copper, which show decreased toxicity in anaerobic conditions. Evidence for this theory included the increased sensitivity of fet3Δ mutant strains, which express higher levels of FET4, to cobalt, copper, manganese, and zinc (Li et al., 1998). The deletion of Rox1, which results in a 10-fold increase in FET4 expression in aerobic conditions, also results in an increased sensitivity to metals such as cadmium (Jensen et al., 2002). Another possibility is that FET4 may become the dominant iron transporter under anaerobic conditions. In anaerobic conditions the high affinity uptake system is not functional due to the requirement of oxygen for Fet3p activity. Therefore FET4 may be derepressed to compensate for the loss of the more efficient high affinity uptake system.
Evidence for this theory includes the observation that *fet4Δ* mutant strains accumulate lower levels of iron than wild-type strains under anaerobic conditions and that many genes encoding components of the high affinity uptake system are repressed in anaerobic conditions (Jensen *et al.*, 2002; Linde *et al.*, 2002). Analysis of other iron uptake genes has also identified *SMF1* as being regulated by oxygen and iron in a similar fashion to *FET4*. *SMF1* is a member of the major facilitator superfamily and is responsible for vacuolar trafficking of iron. This suggests that under anaerobic conditions a whole new system of iron transport may function.

### 1.6 Intracellular Iron Storage and Transport

#### Iron Storage

Due to the catastrophic consequences of free iron accumulating within the cell, iron must be safely stored in a non-toxic manner following import to prevent cellular damage (Schrettl *et al.*, 2004). The storage of iron also provides a cellular pool that can be utilised when this essential metal is no longer abundant. Studies have shown that animal, plants, and even many bacteria utilise ferritin, or its plant and bacterial derivatives phytopherritin and bacterioferritin, as iron storage molecules (Chiancone *et al.*, 2004; Schrettl *et al.*, 2005). In contrast the only fungi that utilise ferritin like molecules for iron storage are members of the *Zygomyceta* (Schrettl *et al.*, 2004). These fungal ferritins can be separated into groups that resemble mammalian ferritin (Bozarth *et al.*, 1972), bacterial ferritin (Carrano *et al.*, 1996), or are unique (David *et al.*, 1971).

Instead of using ferritin a number of fungi utilise siderophores to not only acquire iron from the surrounding environment but to also store it safely within the cell (Schrettl *et al.*, 2004). For example *Aspergillus nidulans* and *Neurospora crassa* produce multiple siderophores in low iron conditions including ferrocrocin, which is used as an intracellular iron storage molecule (Eisendle *et al.*, 2003). Following transport of siderophore bound iron across the membrane it is transferred to desferri-ferrocrocin. Another example is *S. pombe*, which has recently been shown to produce ferrichrome (Schrettl *et al.*, 2004). It was noted that the production of ferrichrome did not alter in response to changes in iron availability unlike the siderophore transporters, which are negatively regulated in response to iron (Pelletier *et al.*, 2003). However, during high
iron conditions intracellular ferrichrome is primarily in the ferri form, while it is mainly iron free in low iron conditions (Schrettl et al., 2004). It has therefore been suggested that ferrichrome can function as an iron storage molecule in \textit{S. pombe}.

In contrast to many other aspects of cellular iron metabolism, the storage of iron in \textit{S. cerevisiae} is poorly understood. Instead of using ferritin-like molecules for storage \textit{S. cerevisiae} prevents the accumulation of toxic levels of iron by storing it in the vacuole (Raguzzi et al., 1988). Shortly after being transported into the cell, iron is found in the vacuole where it is thought to bind to polyphosphates. The iron within the vacuole can then be stored for later use or mobilised for use in compartments such as the mitochondria. Although there is little direct evidence for this theory there are a number of observations that support it. For example, iron is found within the vacuole shortly after transport into the cell (Raguzzi et al., 1988) and a number of mechanisms have been identified for transporting iron out of the vacuole (Li et al., 2001; Portnoy et al., 2000; Urbanowski et al., 1999). Mutants that carry defects in vacuole formation and trafficking are also more sensitive to iron within the environment (Bode et al., 1995; Szczypka et al., 1997)

As mentioned the presence of iron transport systems in the vacuolar membrane is compelling evidence that the vacuole is the major iron storage compartment in \textit{S. cerevisiae}. The presence of transport mechanisms for the import of iron into the vacuole was first suggested when it was found that \textit{end4A} mutants can still accumulate iron in the vacuole (Li et al., 2001). The \textit{end4A} mutant is defective in endocytosis at the stage of internalisation but has vacuoles that are indistinguishable from other strains. This was an important finding because many early studies on vacuolar iron accumulation could not discount the possibility that the presence of iron in the vacuole was not simply a result of endocytosis, which is a constitutive process. This accumulation of iron in the vacuole is at least partially due to Ccc1p, which was originally identified as a Mn\textsuperscript{2+} transporter (Fu et al., 1994). Further investigation has shown that Ccc1p localises to the vacuolar membrane and over-expression of \textit{CCC1} results in the accumulation of increased concentrations of iron and manganese within the vacuole (Li et al., 2001). The transport of both metals by Ccc1p is not surprising as many other Mn\textsuperscript{2+} transporters are also able to facilitate the transport of Fe\textsuperscript{2+} (Cohen et al., 2000). The \textit{CCC1} gene was also identified as a suppressor of the respiratory
deficiency associated with some mutants that accumulate toxic concentrations of iron within the mitochondria (Chen et al., 2000). This is thought to be because higher levels of Ccc1p increase vacuolar iron accumulation, thus lowering cytosolic iron levels and preventing accumulation of iron within the mitochondria (Li et al., 2001). The evidence available therefore suggests that Ccc1p is responsible for the transport of iron and manganese into the vacuole in a process that does not require endocytosis. However, it is not clear whether Ccc1p is the only vacuolar iron importer as end4Δccc1Δ mutants grow very poorly, resulting in inconclusive results regarding the presence of other import mechanisms.

The ability of the cell to mobilise iron stored within the vacuole initially suggested the presence of a iron exporter in the vacuolar membrane. Subsequent investigation has revealed the presence of at least two vacuolar iron export systems, the Fth1p/Fet5p complex and the Nramp transporter Smf3p (Portnoy et al., 2000; Urbanowski et al., 1999). The Fth1p and Fet5p proteins are homologues of the multicopper oxidase Fet3p and the iron permease Ftr1p, which form the cell surface high affinity iron transport complex (Urbanowski et al., 1999). In contrast to their cell surface homologues, Fth1p and Fet5p localise to the vacuolar membrane, and in common with other vacuolar proteins they are not degraded by the vacuolar protease Pep4p. Examination of cellular fractions containing Fth1p that was C-terminally labelled with GFP has shown that the C-terminal region is located within the cytosol, as it is susceptible to proteolysis. This therefore suggests that Fth1p functions to transport iron from the vacuole into the cytosol. The Fth1p and Fet5p appear to form a complex as immuno-precipitation experiments show that they directly interact. The deletion of FET5 also results in the accumulation of Fth1p in the endoplasmic reticulum (Urbanowski et al., 1999), in a similar fashion to Ftr1p and Fet3p when only one of the proteins is expressed (Stearman et al., 1996).

The current model regarding the function of the Fth1p/Fet5p complex is that they are involved in the mobilisation of iron from the vacuole in response to low iron conditions and all the current evidence supports this theory (Urbanowski et al., 1999). The expression of both FET5 and FTH1 increases in response to iron starvation and Fet5p protein levels show a similar pattern (Spizzo et al., 1997; Urbanowski et al., 1999; Yamaguchi-Iwai et al., 1996). The expression of FET3 is also activated in
moderate iron conditions in *fet5Δ* mutants suggesting that cellular iron levels are low due to the inability to mobilise iron stored within the vacuole (Urbanowski *et al.*, 1999). Further evidence for the role of the Fet5p/Fthlp complex is provided by the fact that *fet5Δ* mutants show an abnormal lag in growth when transferred from fermentative to respiratory carbon sources. It is known that vacuolar levels of iron decrease during this transition, presumably due to the need for large amounts of iron in the synthesis of iron containing proteins such as the cytochrome oxidases that are required for respiratory growth.

The second vacuolar iron exporter to be identified was Smf3p, which is a member of the Nramp family of metal transporters (Portnoy *et al.*, 2000). Localisation experiments have shown it is present in the vacuolar membrane, and its function as a vacuolar protein is supported by the observation that the localisation of Smf3p is unaffected in a *pep4A* mutant (Portnoy *et al.*, 2000; Portnoy *et al.*, 2002). Subsequent analysis of Smf3p expression and protein turnover has shown that the primary function of Smf3p is probably as a vacuolar iron exporter. Evidence for this includes the observation that *SMF3* expression is unaffected by manganese levels but increases in response to iron limitation (Portnoy *et al.*, 2002). In high manganese conditions Smf1p and Smf2p are directed to the vacuole and degraded (Liu *et al.*, 1999a; Liu *et al.*, 1999b; Portnoy *et al.*, 1999). In contrast, Smf3p protein levels and localisation are unaffected by metal levels within the medium (Portnoy *et al.*, 2002). Further evidence to support the role of Smf3p in iron trafficking is provided by the observation that in low iron conditions the expression of *FET3* is much more strongly induced in a *smf3Δ* mutant, suggesting that the cell requires the uptake of greater quantities of exogenous iron to compensate for defects in the mobilisation of vacuolar iron (Portnoy *et al.*, 2000).

**Mitochondrial Iron Metabolism**

Iron within the mitochondria is used for a number of processes including the formation of Fe-S clusters and haem (Chitambar, 2005). The best understood of these processes is the formation of Fe-S clusters and their insertion into proteins. After synthesis in the mitochondrion 2Fe-2S or 4Fe-4S clusters are attached to cysteine residues in a variety of proteins (Beinert *et al.*, 1987; Beinert *et al.*, 2000). The iron
sulphur proteins formed by this process have an essential role in organisms ranging from bacteria to humans (Beinert et al., 2000). At least 100 proteins containing Fe-S clusters have been identified and these are involved in processes such as electron transfer, enzymatic reactions, and the regulation of biological processes (Johnson et al., 1987). The process of Fe-S cluster biosynthesis is of particular importance as it is currently thought to provide the signal responsible for sensing the levels of iron within the cell (Chen et al., 2004; Rutherford et al., 2005).

The presence of Fe-S proteins in bacteria and mammals has been known since the 1960s (Beinert et al., 1997). However, the processes involved in the assembly of Fe-S clusters, and their insertion in proteins, is only now becoming clear. The vast majority of early work focused on bacterial Fe-S cluster synthesis but it is now becoming apparent that this process is highly conserved among prokaryotes and eukaryotes. Extensive studies in bacteria have identified three distinct mechanisms for the synthesis of Fe-S clusters. The majority of cellular Fe-S proteins are produced by the ISC (iron sulphur cluster) pathway, which currently contains 10 proteins (Takahashi & Nakamura, 1999; Zheng et al., 1998). The second pathway responsible for Fe-S protein synthesis, SUF (sulphur mobilisation) pathway, appears to be particularly important under iron limiting and oxidative stress conditions in many bacteria (Outten et al., 2004; Takahashi & Tokumoto, 2002; Tokomoto et al., 2004). A third Fe-S protein synthesis mechanism, the NIF (nitrogen fixation) pathway, is found mostly in nitrogen fixing bacteria where it is involved in the synthesis of the complex Fe-S protein nitrogenase (Frazzon & Dean, 2003; Zheng et al., 1993). However, the recent identification of NIF proteins in Helicobacter pylori shows that this system can produce Fe-S clusters for use in a number of non-nitrogenase proteins (Olson et al., 2000). Examination of bacterial and eukaryotic genomes reveals an interesting distribution of the various Fe-S cluster biosynthesis pathways. Many bacterial species contain both the ISC and SFU pathways (Takahashi & Tokumoto, 2002), and it is thought that this allows organisms to adapt to a wider range of environmental conditions (Outten et al., 2004). In contrast the NIF system is mostly confined to nitrogen fixating species, although the NIF system is present in the non-nitrogen fixing e-proteobacteria H. pylori and Campylobacter jejuni (Olson et al., 2000). The phylogenetic distribution of the NIF system, and the observation that it is extremely
sensitive to oxygen has led to the theory that its distribution is limited due to the
greater efficiency of the ISC and SFU pathways in aerobic conditions (Tokumoto et
al., 2004).

Subsequent examination of eukaryotic genomes and experimental analysis of Fe-S
protein synthesis has revealed that a number of proteins involved in the bacterial ISC
pathway appear to be present in eukaryotes, and display a remarkable degree of
sequence conservation (Lill et al., 2000). Homologues of proteins from the SFU
pathway have also been identified in a number of photosynthetic eukaryotic
organisms, where they are located within the plastids (Ali et al., 2004; Takahashi et
al., 2002). A functional NIF system of Fe-S cluster assembly has also been identified
in the amaeobic intestinal parasite Entamoeba histolytica, which raises a number of
important questions concerning its uses and inheritance (Ali et al., 2004). Sequence
comparisons between NIF proteins found in various species suggests that E.
histolytica may have inherited this system via horizontal gene transfer form a e-
proteobacteria (Ali et al., 2004). The identification of a NIF pathway in E. histolytica
also provides further evidence for the theory that this system is important for the
synthesis of many non-nitrogenase proteins.

As with many other eukaryotic processes our knowledge of the formation of Fe-S
proteins is most complete in S. cerevisiae and the current model proposed for this
process is illustrated in Figure 1.4 (Reviewed in Lill & Muhlenhoff, 2005). Initially
homologues of bacterial ISC proteins were identified within the genome, and their
involvement in Fe-S synthesis confirmed by studying their localisation and the effect
of gene deletions. The deletion of some of these genes proved lethal, confirming the
importance of Fe-S cluster synthesis within the cell. The depletion of many of these
essential genes confirmed their role in Fe-S cluster synthesis as it resulted in the
accumulation of mitochondrial iron, impaired haem synthesis, oxidative stress, and
depleted cytochromes (Lessuise et al., 2003; Rostig et al., 2002).

The first step in the de novo synthesis of Fe-S clusters involves the transfer of iron
and sulphur from donors to the scaffold proteins Isu1p and Isu2p (reviewed in Lill et
al., 2005). When the scaffold proteins are compared between bacteria and eukaryotes
they are among the most evolutionary conserved proteins known (Schilke et
Figure 1.4 Mitochondrial Iron Sulphur Cluster Biosynthesis in *S. cerevisiae*. Within the mitochondria the *de novo* synthesis of Fe-S cluster biosynthesis occurs on the scaffold proteins Isu1p and Isu2p. The sulphur required for Fe-S cluster biosynthesis is generated by Nfs1p, which removes and binds elemental sulphur from cysteine. The sulphur bound to Nfs1p is reduced to sulphide using electrons supplied by Yah1p and Arh1p that require NADH as an electron donor. Reduced iron for Fe-S cluster synthesis enters the mitochondria via the transporters Mrs3p and Mrs4p. The yeast frataxin homologue Yfh1p then assists in the binding of iron to the protein scaffold. Following its assembly the Fe-S cluster is released and incorporated into apoproteins in the mitochondria. The chaperones Ssq1p, Jac1p, and the thiol reductase Grx5p function after synthesis of the transient Fe-S cluster and so may be involved in its release and incorporation. Fe-S clusters are also exported from the mitochondrial for the formation of cytosolic and nuclear Fe-S proteins. This requires the transporter Atm1p, the sulphyl oxidase Erv1p, and reduced glutathione (GSH). Figure adapted from Lill & Muhlenhoff, (2005) and Lill & Kispal, (2000).
One of the best-characterised processes in Fe-S cluster assembly is the generation of sulphur from a donor and its subsequent transfer to the scaffold. The pyridoxyl phosphate-dependent cysteine desulphurase Nfs1p is responsible for the release of sulphur from cysteine and its transfer to Isu1/2p (Kispal et al., 1999). The elemental sulphur produced by Nfs1p binds to cysteine residues within the protein and is then reduced to sulphide. The electrons used in the reduction of sulphur are generated from NADPH and transferred along an electron transport chain containing Yahlp and Arhlp (Lill & Muhlenhoff, 2005). The current model predicts that following the binding of sulphur Nfs1p forms a tetrameric complex with Isu1/2p and the persulphide is transferred to the scaffold (Gerber et al., 2003).

While there is still some debate regarding the order of the reaction chemistry of Fe-S cluster biogenesis it is currently thought that reduced iron binds to the Isu1/2p scaffold following the transfer of sulphur (Lill & Muhlenhoff, 2005). There are a number of potential routes of entry for iron into the mitochondria. The majority of iron enters the mitochondria via the carrier proteins Mrs3/4p, which are located in the mitochondrial inner membrane (Zhang et al., 2005). However, in the absence of Mrs3/4p there appears to be a slower and less efficient route for the entry of iron that is currently uncharacterised. The fate of iron once it has entered the mitochondria is still unclear. The majority of evidence suggests that the majority of mitochondrial iron is bound to Yfhlp (Frataxin), which may act as a storage molecule and chaperone for the delivery of iron to processes such as Fe-S cluster formation and haem synthesis (Yoon et al., 2003).

Following the assembly of the transient Fe-S clusters they are released from the scaffold and inserted into apoproteins (Lill & Muhlenhoff, 2005). Although a number of proteins have been implicated in this process the precise nature of Fe-S cluster release and transfer is still unclear. Both Jac1p and Ssq1p are known to interact with Isu1/2p, with Jac1p targeting the binding of Ssq1p to a Leu-Pro-Pro-Val-Lys motif within Isu1p (Dutkiewicz et al., 2003; Dutkiewicz et al., 2004; Hoff et al., 2003). The binding of ATP to Ssq1p and its subsequent hydrolysis releases Ssq1p from the scaffold (Dutkiewicz et al., 2003). This is also thought to cause the dissociation of the Fe-S clusters from the scaffold. A role for the thiol reductase Grx5p, which maintains cysteines in their reduced state, in iron sulphur cluster biosynthesis has also been
proposed (Rodriguez-Manzaneque et al., 1999; Rodriguez-Manzaneque et al., 2002). However, two conflicting models exist regarding its function. The first proposes that Grx5p acts after Fe-S cluster biosynthesis due to the accumulation of Fe-S clusters on the scaffold proteins in grx5Δ mutants (Muhlenhoff et al., 2003). An alternative theory suggests the phenotypes associated with the grx5Δ mutant are more likely to be associated with a role for Grx5p in the reduction of cysteine residues in Nfs1p or Isu1/2p during Fe-S cluster assembly (Alves et al., 2004).

In addition to their incorporation into mitochondrial proteins Fe-S clusters are exported to the cytosol (Lill & Muhlenhoff, 2005). The formation of cytosolic Fe-S proteins is therefore dependent on mitochondrial Fe-S cluster synthesis (Kispal et al., 1999). A number of components of the Fe-S cluster export system are currently known including the ABC transporter, Atm1p (Kispal et al., 1999). However, while Atm1p is essential for the formation of cytosolic Fe-S proteins the substrate it transports is unknown. The possibility that Atm1p exports a signal molecule or small peptide vital for cytosolic and nuclear Fe-S protein maturation has therefore not been excluded. In addition to Atm1p the sulphyl oxidase Erv1p, which is located in the intermembrane space, is required for the maturation of cytosolic Fe-S proteins (Lange et al., 2001). An absence of glutathione is also known to inhibit the formation of cytosolic Fe-S protein maturation and is thought to be involved in the export of Fe-S clusters (Sipos et al., 2002).

1.7 Copper Uptake in S. cerevisiae and Other Fungi
Among eukaryotes the mechanism of high affinity copper uptake is extremely well conserved, with the majority of organisms containing similar components (reviewed in van Ho, 2002). In this process reduced copper is transported across the plasma-membrane by members of the highly conserved Ctr family of transport proteins (Puig et al., 2002). These proteins are tightly regulated by the levels of copper in the media at both the transcriptional and protein level to avoid the influx of toxic levels of copper into the cell (Reviewed in Ho et al., 2002; Rutherford & Bird, 2004). Once it has entered the cell levels of free copper are extremely tightly controlled as it binds to chaperones for delivery to the site of incorporation into specific proteins (van Ho et
Copper transport and metabolism is also of great importance as defects in its transport impair iron metabolism (Dancis et al., 1994b).

**High Affinity Copper Transport**

In *S. cerevisiae* copper can be transported into the cell by two high affinity transport proteins encoded by *CTR1* and *CTR3* (Dancis et al., 1994a; Knight et al., 1996). The *CTR1* gene was initially identified in a screen for mutants with defects in high affinity iron uptake (Dancis et al., 1994a). The *ctrlA* mutant isolated displayed defects in iron transport that could be corrected by the addition of excess copper to the media. Further analysis revealed that the *ctrlA* strain showed defects in copper uptake, resulting in the inactivation of the copper requiring protein Fet3p, a multicopper oxidase that is an essential component of the high affinity iron uptake system. The *ctrlA* mutant also displayed a number of other phenotypes that are consistent with its role as a copper transporter. These include the inability to grow on respiratory carbon sources, increased sensitivity to oxidative stress, reduced growth in low copper conditions, and increased tolerance to excess copper (Dancis et al., 1994a; Dancis et al., 1994b). The inability to grow on respiratory carbon sources is possibly due to the requirement of copper for a variety of enzymes including cytochrome c oxidase. The *ctrlA* strain also shows decreased levels of Cu, Zn SOD1 activity that may explain the increased sensitivity to oxidative stress. Expression studies also reveal that much higher levels of copper in the media are required to activate the Ace1p transcription factor in a *ctrlA* strain. In excess copper conditions Ace1p is activated by the binding of excess intracellular copper and up-regulates genes involved in copper detoxification. Therefore, as *ctrlA* cells are defective in copper transport a higher level of copper in the media is required to reach the threshold concentration of intracellular copper required for Ace1p activation.

The *CTR1* gene was cloned by complementation of the *ctrlA* mutant identified in the screen for high affinity iron uptake mutants (Dancis et al., 1994a). The *CTR1* gene encodes a protein that is 406 amino acids in length and contains three predicted transmembrane domains. The protein is highly glycosylated and localises to the plasma membrane. Localisation studies using C- and N-terminally tagged Ctrl1p constructs demonstrate that the N-terminal region is located extracellularly and the C-
terminal region is located in the cytoplasm (Puig et al., 2002). Analysis of the protein sequence shows the N-terminal domain of Ctrlp is rich in methionine and serine residues and contains multiple MXM and MXXM motifs. Similar motifs have been observed in bacterial proteins involved in copper handling, suggesting a role for these motifs in copper binding (Cha & Cooksey, 1991; Odermatt et al., 1993). The C-terminal domain of Ctrlp is rich in charged amino acids, especially cysteines and histidines towards the extreme C-terminus (Dancis et al., 1994a). Due to the observation that most eukaryotic membrane transport proteins have 6 to 12 transmembrane domains (Serrano, 1991) it was initially suggested that multiple Ctrlp molecules interact to form multimers (Dancis et al., 1994b). This theory was tested by co-transforming a ctrl A strain with plasmids containing CTR1 engineered to contain either a HA or myc epitope tag (Dancis et al., 1994b). Immuno-precipitation experiments were then used to demonstrate that the epitope tagged proteins associate, and therefore co-precipitate. The results of sucrose density gradient centrifugation experiments also suggested that the Ctrlp protein migrates as a dimer, due to its larger than expected size.

The CTR3 gene was isolated in a screen to identify mutations that were able to complement the ctrl A mutant phenotype (Knight et al., 1996). A single mutation was identified, CTR3S, which restored copper uptake levels to 50% of the wild-type and complemented the growth phenotypes associated with the ctrl A mutant. Analysis revealed that the mutation occurred within the promoter of a previously sequenced gene, CTR3. It was found that CTR3 was not expressed in the ctrl A strain due to the presence of a 6 kb Ty2 transposable element separating the ORF from the majority of its promoter. In the CTR3S strain only 25 bp of the Ty2 transposable element remained and the expression of the gene increased in response to low levels of copper. Analysis of other yeast strains revealed that only 3 out of 15 commonly used lab strains do not have a Ty2 insertion within the CTR3 promoter, and CTR3 was only expressed in the strains lacking the transposable element. In contrast, all S. cerevisiae clinical isolates tested had functional copies of CTR3 that lacked the Ty2 insertion. Interestingly, the deletion of CTR1 in strains that have a functional CTR3 gene does not result in copper deprivation or the subsequent phenotypes observed in previously described ctrl A mutants. Studies have shown that CTR1 and CTR3 can functionally complement each
other and that the deletion of both is required to produce phenotypes that are consistent with defects in copper transport. However, while the two genes are functionally redundant strains carrying copies of both genes have a distinct growth advantage in low copper conditions over strains carrying only CTR1 or CTR3.

The CTR3 gene encodes a 241 amino acid protein that contains 3 putative transmembrane domains (Knight et al., 1996). Comparison of the copper transporter protein sequences shows that Ctr3p shows only low sequence homology to Ctrlp, except in the transmembrane domains (Knight et al., 1996; Puig et al., 2002). The Ctr3p proteins lacks the MXM and MXXM motifs found in Ctrlp but it does contain 11 cysteine residues, three pairs of which are present in CXC or CXXC motifs (Knight et al., 1996; Pena et al., 2002). Similar motifs in ScMac1p, ScAce1p, ScCcc2p, SpCup1p, CgAmt1p, and ScCox17p are thought to be involved in copper binding (Dancis et al., 1994). Localisation experiments demonstrate that Ctr3p is present in the plasma membrane, and immuno-precipitation experiments show it functions as a multimer (Pena et al., 2000). However, unlike Ctrlp that forms dimers, cross-linking experiments suggest that Ctr3p forms trimeric complexes in the plasma membrane. The possibility that Ctrlp and Ctr3p interact and form complexes has not been investigated, but deletion studies demonstrate that the formation of heteromultimeric complexes is not essential to facilitate copper transport. Interestingly S. pombe also contains a pair of Ctrlp homologues encoded by ctr4+ and ctr5+ that are both required for high affinity copper uptake (Zhou et al., 2001). It is theorised that SpCtr4p and SpCtr5p function as heteromultimeric complexes, as both genes are required to complement a S. cerevisiae ctrl1 Δctr3Δ strain. Both proteins are also required for copper uptake in S. pombe and fail to localise to the plasma-membrane unless both are present. Co-immunoprecipitation experiments have also shown that SpCtr4 and SpCtr5 interact to form high molecular weight complexes.

Interestingly, sequence analysis suggests that Ctrlp and Ctr3p homologues are present in all known eukaryotic organisms (Puig et al., 2002). Experimental studies have also isolated Ctrl/3p homologues in a wide range of eukaryotes including humans, mice, Drosophila melanogaster, lizards, plants, and zebrafish (Lee et al., 2000; Kampfenkel et al., 1995; MacKenzie et al., 2004; Riggio et al., 2002; Zhou & Grischier, 1997).
Our laboratory has identified a Ctrlp copper transporter in *C. albicans* that is essential for iron uptake (Marvin *et al.*, 2003). The *CaCTR1* gene was isolated through its ability to complement a *S. cerevisiae ctrlActr3A* mutant strain. Further analysis has shown that CaCtrlp is required for high affinity iron uptake with its loss producing similar phenotypes to those observed in a *S. cerevisiae ctrlActr3A* mutant (Marvin *et al.*, 2004). Comparison of the Ctr protein sequences reveals the presence of a number of important conserved features, and suggests a conserved mechanism of copper uptake from yeast to humans (Puig *et al.*, 2002). Except for ScCtrlp the transmembrane domains of the Ctr protein family show a high degree of sequence similarity to Ctr3p and they all contain a number of highly conserved residues. In contrast, except for Ctr3p all of the Ctr proteins have N-terminal ectodomains that contain a number of MX₂MXM motifs similar to those in ScCtrlp. These observations have raised the possibility that copper transporters in eukaryotes evolved from the fusion of Ctr1p and Ctr3p in *S. cerevisiae* or a closely related relative (Labbe *et al.*, 1999).

Comparison of the Ctr family of proteins has provided useful information for the identification of specific residues and motifs that are important in Ctr based copper uptake (Puig *et al.*, 2002). As mentioned, except for Ctr3p, all of the Ctr proteins are rich in methionine residues and the position of three of these is highly conserved. The first of these is Ctr1p Met-127, which is located 20 amino acids upstream of the first transmembrane domain in the Ctr family. This residue is essential for ScCtrlp function, as a M₁₂₇A substitution abolishes copper uptake and produces phenotypes that are identical to a *ctrlA* strain. Mutations in hCtrlp also show that the last two methionines, Met-43 and Met-45, before the first transmembrane domain are similarly essential. The substitution of a similarly placed methionine in ScCtrl3p was also examined and had no effect on the respiratory competence of cells. However, strains carrying a M₂₀A substitution in ScCtrl3p displayed reduced growth in low copper conditions compared to wild-type cells, suggesting that Met-20 has an important role in copper uptake. The possible role of ScCtrl1p Met-127 was investigated by substituting it with cysteine, which functions as a copper ligand in many proteins (Koch *et al.*, 1997). The Ctr1p protein carrying a M₁₂₇C substitution was fully functional and restored wild-type levels of growth and copper uptake to a *ctrlA* strain.
(Puig et al., 2002). Therefore Met-127 appears to be involved in the binding of copper close to the transmembrane domains

The remaining two highly conserved methionines in the Ctr protein family are found in a MXXXM motif located in the second transmembrane domain (Puig et al., 2002). Mutagenesis of the methionine residues, Met-256 and Met-260, within the MXXXM motif reveals that only Met-260 is essential for Ctr1p function and a M260C substitution has no effect. In contrast mutation of either of the corresponding methionine residues in ScCtr3p produces a partial phenotype, and mutation of both residues is required to abolish copper transport. A similar result was also obtained with hCtr1p where both methionine residues in the MXXXM motif are critical for protein function.

**Low Affinity Copper Uptake**

The observation that *S. cerevisiae* strains lacking high affinity copper uptake are able to grow normally when excess copper is added to the media suggested the presence of a low affinity uptake system (Dancis et al., 1994). A number of alternative routes for copper entry into the cell have subsequently been identified. The low affinity iron transporter Fet4p is also capable of facilitating the transport of a variety of other metals, including physiologically relevant amounts of copper (Hassett et al., 2000). However, while the expression of *FET4* is regulated in response to iron and zinc levels it is unaffected by copper levels (Waters et al., 2002).

There are also reports that the Nramp transporter Smf1p, which was originally implicated as a manganese transporter, can transport a variety of metals including copper, iron, cadmium, and cobalt (Chen et al., 1999; Liu et al., 1997). Evidence for the involvement of Smf1p in copper uptake includes the observation that copper can rescue the sensitivity of *smf1Δ* mutants to EGTA (Cohen et al., 2000; Supek et al., 1996). However, this is slightly confusing given that the *smf1Δ* mutant possesses multiple other copper transporters including Ctr1p, Ctr3p, and Fet4p (Cohen et al., 2000). Other evidence for the role of Smf1p in copper comes from the observation that *bsd2A* mutants accumulate large amounts of copper (Liu et al., 1997). In wild-type cells Bsd2p regulates Smf1p by targeting it for degradation in the vacuole in response to high levels of manganese (Liu et al., 1999). In metal limiting conditions,
or when Bsd2p is absent, Smf1p localises to the plasma membrane (Liu et al., 1997). However, as the bsd2Δ mutant has abnormally large amounts of Smf1p the physiological relevance of this transporter in copper uptake is unclear (Cohen et al., 2001). The role of Smf1p in copper uptake is also complicated by the observation that it does not contribute significant levels of copper to the metallochaperones within the cell (Portnoy et al., 2001).

1.8 Regulation of Iron Uptake

_Transcriptional Control of Iron Uptake Genes in S. cerevisiae_

The transcriptional response to low iron conditions in _S. cerevisiae_ is mediated by the paralogous iron responsive transcription factors Aftlp and Aft2p (Blaiseau et al., 2001; Yamaguchi-Iwai et al., 1995). In response to low iron these transcription factors up-regulate the expression of a wide variety of genes involved in iron transport, distribution, and utilisation (Rutherford et al., 2004).

The _AFT1_ gene was initially identified in the same genetic screen for iron uptake mutants as _FTR1_ (Stearman et al., 1996). A mutant carrying a dominant _AFT1-1up_ allele was isolated that displayed constitutive activation of iron uptake genes, irrespective of iron levels (Yamaguchi-Iwai et al., 1995). The _AFT1-1up_ mutant displayed constitutively high levels of ferric iron reduction and uptake, and an increased sensitivity to high concentrations of iron. These phenotypes are caused by the inappropriate expression of genes such as _FRE1, FRE2, and FET3_ in iron replete conditions. The deletion of _AFT1_ confirmed its role in iron metabolism as it reduces the ability of _S. cerevisiae_ to grow in low iron conditions. The _aft1Δ_ mutant is also unable to utilise non-fermentable carbon sources (Casas et al., 1997) and is sensitive to oxidative stress (Blaiseau et al., 2001). The iron responsive transcription of a number of genes including _FRE1-6, FET3, and FTR1_ is also drastically reduced or abolished in the _aft1Δ_ strain (Georgatsou et al., 1999; Martine et al., 1998; Yamaguchi-Iwai et al., 1996). Footprinting experiments have shown that Aftlp binds to the consensus sequence PyPuCACCpCu within the promoters of genes involved in iron uptake and metabolism, including _FRE1, FRE2, FTR1, FET3, FTH1, and CCC2_ (Yamaguchi-Iwai et al., 1996).
Analysis of the AFT1 DNA and protein sequences, and comparisons with the AFT1-
$^{1p}$ allele, reveals a number of interesting features. The AFT1 gene encodes a 690
amino acid polypeptide with a predicted molecular weight of 78 kDa (Yamaguchi-
Iwai et al., 1995). The N-terminal region of Aft1p is quite basic, with clusters of basic
amino acids similar to those found in DNA binding domains and nuclear localisation
signals (Yamaguchi-Iwai et al., 1995). This region also contains four cysteine
residues at positions 143, 215, 291, and 293, which are potential iron binding ligands.
Cysteine 291 and 293 are arranged in a CXC motif, and the AFT1-$^{1p}$ allele is the
result of a single G→T mutation resulting in the substitution of cysteine 291 with
phenylalanine. The N- and C-terminal regions of Aft1p also contain an abnormally
high proportion of histidines, which are known to act as metal binding ligands in a
number of other proteins (Adrait et al., 1999). A glutamine rich region similar to
those observed in a number of other transcriptional activators is also found in the C-
terminal portion of Aft1p (Mitchell & Tjian, 1989).

Until recently the mechanism behind the Aft1p mediated activation of genes in
response to changing iron levels was unclear. It is now apparent that the ability of
Aft1p to activate transcription is controlled by its localisation within the cell
(Yamaguchi-Iwai et al., 2002). When iron is abundant Aft1p is located in the
cytoplasm and moves to the nucleus in response to iron deprivation. Analysis of
Aft1p has revealed the presence of a nuclear export sequence (NES) and a pair of
nuclear localisation sequence (NLS) (Blaiseau et al., 2001; Ueta et al., 2003;
Yamaguchi-Iwai et al., 2002). The NES sequence is based around leucine residues at
position 99 and 102 and is similar to leucine rich NES sequences identified in other
proteins (Yamaguchi-Iwai et al., 2002). Mutation of either of the leucine residues
results in the constitutive nuclear localisation of the protein, producing phenotypes
similar to those of the AFT1$^{1p}$ mutant (Yamaguchi-Iwai et al., 2002). The NLS
sequences in Aft1p, KPKKKR (position 202-207) and RKPK (position 352-355), are
recognised by the nuclear import receptor Pse1p and their deletion produces an aft1Δ-
like phenotype (Ueta et al., 2003). Either of the NLS sequences is sufficient for Pse1p
mediated nuclear import of Aft1p as mutations in both NLSs are required to abolish
import. Co-immunoprecipitation experiments have revealed a direct physical
interaction between Pse1p and Aft1p, although the interaction is unaffected by iron status.

Although it is known that Aft1p localisation is altered in response to changes in iron (Yamaguchi-Iwai et al., 2002), the signal responsible for triggering this change is still unclear. Due to the presence of numerous histidine and cysteine residues within the protein it was assumed that Aft1p sensed iron levels by directly binding iron, or iron containing proteins (Yamaguchi-Iwai et al., 1995; Yamaguchi-Iwai et al., 1996). However, a recent study has shown that Aft1p is unable to directly bind iron, and does not co-purify with Fe-S proteins (Rutherford et al., 2005). It has therefore been suggested that Aft1p senses a signal from the mitochondrial Fe-S cluster biosynthesis pathway (Chen et al., 2004). Evidence for this theory includes the loss of iron responsiveness of Aft1p and Aft2p in strains that have mutations in genes involved in mitochondrial Fe-S protein biosynthesis and transport (Chen et al., 2004; Rutherford et al., 2005). Interestingly, this phenotype is not seen in mutants that have defects in cytosolic Fe-S protein maturation (Rutherford et al., 2005). Aft1p may therefore sense the presence of a transient intermediate in Fe-S protein biosynthesis that is transported from the mitochondria (Rutherford et al., 2005).

In addition to Aft1p a second iron responsive transcription factor, encoded by the paralogous gene AFT2, has been identified in S. cerevisiae (Blaiseau et al., 2001; Rutherford et al., 2001). In common with many pairs of paralogous transcription factors in S. cerevisiae, AFT2 is believed to have been generated during an ancient genome duplication event (Wolfe et al., 1997). The Aft1p and Aft2p proteins show 26% identity, although the main regions of homology are contained within the N-terminal portions of both proteins (Blaiseau et al., 2001; Rutherford et al., 2001). In common with Aft1p the N-terminal region of Aft2p is rich in basic amino acids and contains four conserved cysteine residues at positions 86, 109, 187, and 189. Mutation of either of the cysteines within the Aft2p CXC motif results in the loss of iron responsive inhibition (Rutherford et al., 2001). In contrast to Aft1p, the Aft2p protein does not contain C- or N-terminal histidine rich regions or a glutamine rich C-terminal domain (Blaiseau et al., 2001).
In contrast to AFT1, the deletion of AFT2 does not produce noticeable phenotypic changes (Blaiseau et al., 2001; Rutherford et al., 2001). However, deletion of both genes exacerbates the mutant phenotypes associated with the aft1Δ mutant. The aft1Δaft2Δ mutant is unable to grow in low iron conditions and is more sensitive to oxidative stress than the aft1Δ mutant (Blaiseau et al., 2001). The residual expression of iron uptake and metabolism genes such as FET3 and CTH2 in the aft1Δ mutant is also abolished by the additional deletion of AFT2. These observations suggest that Aft1p and Aft2p have overlapping functions, and that in the absence of Aft1p the Aft2p transcription factor is essential for the adaptation of cells to iron limitation.

Although a number of studies have attempted to determine the individual contributions of Aft1p and Aft2p to iron responsive transcription our understanding of this subject is still relatively basic. Early investigations focused on comparing the effect of the AFT1-lup and AFT2-lup alleles upon the expression profile of genes using DNA microarrays (Rutherford et al., 2001; Rutherford et al., 2003). These studies confirmed that the AFT1-lup and AFT2-lup alleles alter the expression of distinct and overlapping groups of genes as shown in Table 1.1 (Rutherford et al., 2003). However, it is still unclear whether a number of the genes identified in this screen are direct targets of Aft1p or Aft2p. Recently a more detailed study has compared the expression profiles of the aft1Δ, aft2Δ, and aft1Δaft2Δ mutants with wild-type cells in response to iron and integrated the result with those obtained from the constitutive mutants (Courel et al., 2005). This confirmed that groups of iron responsive genes are targets for Aft1p, Aft2p, or both transcription factors. However, there are some discrepancies between the two studies, and it has been suggested that these may be the result of the different genetic backgrounds used (Courel et al., 2005).

In general, Courel and colleagues have suggested that Aft1p is the major regulator of genes involved in high affinity iron uptake including FRE1, ARN1-4, and FET3 (Courel et al., 2005). In contrast, Aft2p is the major regulator of genes such as SMF3 and MRS4 that are involved in vacuolar and mitochondrial iron transport and metabolism. Northern blotting and chromatin immuno-precipitation experiments have confirmed the microarray results, and provided insights into the action of the two transcription factors (Courel et al., 2005). While Aft1p and Aft2p are able to bind the same promoter elements in vitro (Rutherford et al., 2003), CHIP experiments
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<th>Gene</th>
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<td>ARN4 (ENB1)</td>
<td>Siderophore transporter</td>
<td>Yun et al., 2000</td>
</tr>
<tr>
<td>ATX1</td>
<td>Copper chaperone, delivers copper to Ccc2p</td>
<td>Lin et al., 1997</td>
</tr>
<tr>
<td>CCC2</td>
<td>Cu²⁺ transporting P-type ATPase, delivers copper to Fet3p</td>
<td>Yamaguchi-Iwai et al., 1996</td>
</tr>
<tr>
<td>FET3</td>
<td>Multicopper oxidase, forms cell surface high affinity iron transporter with Ftr1p</td>
<td>Yamaguchi-Iwai et al., 1995</td>
</tr>
<tr>
<td>FET4</td>
<td>Low affinity iron transporter</td>
<td>Jensen et al., 2002</td>
</tr>
<tr>
<td>FET5</td>
<td>Multicopper oxidase, forms vacuolar iron transporter with Fth1p</td>
<td>Urbanowski &amp; Piper, 1999</td>
</tr>
<tr>
<td>FTR1</td>
<td>Iron permease, forms cell surface high affinity iron transporter with Fet3p</td>
<td>Yamaguchi-Iwai et al., 1996</td>
</tr>
<tr>
<td>FTH1</td>
<td>Iron permease, forms vacuolar affinity iron transporter with Fet5p</td>
<td>Yamaguchi-Iwai et al., 1996</td>
</tr>
<tr>
<td>HMX1</td>
<td>Heme oxygenase</td>
<td>Foury &amp; Talibi, 2001</td>
</tr>
<tr>
<td>ISU1</td>
<td>Mitochondrial Fe-S cluster assembly scaffold proteins</td>
<td>Garland et al., 1999</td>
</tr>
<tr>
<td>ISU2</td>
<td>Mitochondrial Fe-S cluster assembly scaffold proteins</td>
<td>Garland et al., 1999</td>
</tr>
<tr>
<td>MRS4</td>
<td>Mitochondrial iron transporter</td>
<td>DeFreitas et al., 2004</td>
</tr>
<tr>
<td>PCA1</td>
<td>CPX-type ATPase, unknown function</td>
<td>DeFreitas et al., 2004</td>
</tr>
<tr>
<td>SMF3</td>
<td>Nramp transporter, vacuolar iron transporter</td>
<td>Portnoy et al., 2002</td>
</tr>
<tr>
<td>TIS11</td>
<td>Post-transcriptional regulator of iron regulated genes</td>
<td>Foury &amp; Talibi, 2001; Puig et al., 2005</td>
</tr>
</tbody>
</table>

Table 1.1 Aftlp and Aft2p target genes in *S. cerevisiae*. The table shows a list of iron regulated genes whose transcription is directly affected by Aft1/2p in an iron responsive manner. Analysis has shown that some of these genes are preferentially regulated by Aftlp (Yellow), Aft2p (Blue), or are equally responsive to both transcription factors (Green). Genes whose preference for Aftlp or Aft2p has not been investigated are shown in white. Figure adapted from Courrel et al., (2005); DeFreitas et al., (2003); Rutherford et al., (2003).
demonstrate that this is not the case *in vivo* (Courel et al., 2005). For example, in CHIP experiments Aftlp binds well to the promoters of *FET3* and *FTR1*, poorly to *SMF3*, and not at all to *MRS4*. In contrast Aft2p binds well to the promoters of *MRS4*, *SMF3*, and *FTR1*, but poorly to *FET3*. Comparisons of the promoters of these genes suggests that Aftlp binds most strongly to the sequence TGCACCC, while Aft2p binds preferentially to (G/A)CACCC. However, selective binding to these promoter sequences may involve other trans-acting factors because some Aft2p specific promoters contain the TGCACCC motif. Indeed, it has been demonstrated that Aftlp can interact with the HMG box chromatin-associated factor Nhp6p, which facilitates its binding to specific promoters (Fragiadakis et al., 2004).

**Transcriptional Regulation of Iron Uptake in Fungi**

While *S. cerevisiae* uses the iron responsive transcription factors Aftlp and Aft2p to modulate the transcription of genes in response to iron it is becoming apparent that the majority of fungi utilise a different regulatory system. The regulation of iron uptake in most fungi is mediated by GATA like transcription factors that contain two zinc finger domains separated by a short conserved sequence (Rutherford et al., 2004; Scazzocchio, 2000). In contrast to the transcriptional activators Aftlp and Aft2p, the iron sensing GATA transcription factors repress target gene expression in response to iron (Rutherford et al., 2004). This alternative system of iron regulation was first identified in *Ustilago maydis*, where Urbs1p is responsible for the repression of siderophore biosynthesis and uptake in response to iron (Voisard et al., 1993). Iron responsive GATA transcription factors have subsequently been identified in *Aspergillus nidulans* (SREA), *Neurospora crassa* (SRE), and *S. pombe* (Fep1p) (Haas et al., 1999; Pelletier et al., 2002; Zhou et al., 1998). Interestingly, unlike *S. cerevisiae* all of these fungi produce siderophores and the GATA transcription factors play a crucial role in regulating their synthesis and transport (Pelletier et al., 2002). An iron responsive GATA factor, Sfu1p, was recently isolated from *C. albicans* and this raises further questions in the debate about the ability of this opportunistic pathogen to produce siderophores (Lan et al., 2004).

Although the GATA-type transcription factor Fep1p in *S. pombe* has only recently been identified (Pelletier et al., 2002), it is probably the best characterised. The deletion of *fep1*+ causes intracellular iron accumulation and increased levels of ferric
reductase activity that are unresponsive to iron levels (Pelletier et al., 2002). These phenotypes are due to the loss of iron mediated repression of genes encoding a ferric reductase Frp1p, iron permease Fip1p, multicopper oxidase Fio1p, and the siderophore transporters Str1-3p (Pelletier et al., 2002; Pelletier et al., 2003). The promoters of all of these genes contain the consensus sequence 5'-(A/T)GATAA-3', which is required for Fep1p mediated repression (Pelletier et al., 2003). In vitro binding assays have confirmed the ability of purified Fep1 to specifically bind to DNA fragments containing this sequence. Interestingly, purified Fep1 was only able to bind to DNA when the E. coli expressing the protein were cultured in high iron conditions (Pelletier et al., 2002). This suggests that the binding of Fep1p to recognition sequences in DNA may be directly regulated by the binding of iron to Fep1p.

The frp1+, fio1+, and fip1+ promoters each contain two Fep1p recognition sequences that are able to individually support significant levels of Fep1p mediated repression (Pelletier et al., 2002). However, analysis of the sites in the fio1+ promoter suggests that they have different binding affinities for Fep1p (An et al., 1997; Pelletier et al., 2002). Evidence for this includes competition assays that show Frp1p interacts more strongly with the distal recognition sequence in EMSA assays (Pelletier et al., 2002). Mutations in the distal recognition sequence also produce a more significant drop in the repression of fio1+ in response to iron than mutations in the proximal site. Interestingly, the promoters of strl-3+ only contain a single Fep1p recognition sequence that is necessary for iron responsive repression (Pelletier et al., 2003). This is in contrast to Urbs1p, which requires the presence of two binding sites within the SIT promoter for full iron responsive repression (An et al., 1997). However, it must be noted that only a single Urbs1p target gene has been examined in U. maydis, so it is possible that other unidentified target genes require only a single GATA recognition site within their promoters.

One area of Fep1p function that has been extensively studied is the effect of the zinc finger domains on DNA binding. The first 131 residues of Fep1p are the minimal region required for the binding of DNA fragments containing GATA recognition sequences (Pelletier et al., 2003). This region contains both of the Zn-finger domains of Fep1p, which have been named ZF1 and ZF2. The zinc finger motifs are essential
for Fep1p function as the deletion of either one produces phenotypes similar to fepΔ mutants (Pelletier et al., 2005). However, while both domains are essential \textit{in vivo} they have discrete functions \textit{in vitro}. Only ZF2 is able to interact with DNA containing the (A/T)GATAA consensus sequence when tested \textit{in vitro}. While ZF1 is not essential for DNA binding its deletion decreases DNA binding 5-fold. This is sufficient to abrogate the ability of Fep1p to fully repress target genes.

All of the fungal iron responsive GATA transcription factors contain a short sequence containing four highly conserved cysteine residues separating the ZF1 and ZF2 domains (Rutherford et al., 2004). Mutagenesis of the first two, last two, or all four cysteine residues in Fep1p reduces its ability to bind DNA, and cause iron responsive repression (Pelletier et al., 2005). Similar experiments have been performed with the \textit{N. crassa} SRE protein, showing that mutation of the conserved cysteines residues reduces the level of DNA binding (Harrison & Marzluf, 2002). However, when studied \textit{in vivo} the SRE cysteine mutations have an interesting, and different effect, on iron uptake than in Fep1p. Analysis of siderophore synthesis and ornithine oxygenase activity in the cysteine mutants shows that the mutant SRE acts as a dominant repressor of siderophore synthesis. During this investigation of SRE it was also noted that the purified protein had a reddish colour and an absorbance spectrum that suggested it was an iron binding protein. Interestingly the mutant SRE protein was colourless and the absorbance spectrum suggested the lack of bound iron. This raises a number of interesting questions because SRE would be expected to repress its target genes when iron was bound. It is also puzzling that the SRE cysteine mutants have dominant repressor phenotypes when the mutant protein binds DNA less strongly.

The fungal iron responsive GATA factors contain a coiled-coil domain in the C-terminal portion of the protein, which is thought to be involved in protein-protein interactions (Pelletier et al., 2005; Znaidi et al., 2004). Studies of Fep1p have identified a number of interacting proteins that require sequences within or near the coiled-coil domain (Pelletier et al., 2005; Znaidi et al., 2004). Yeast two-hybrid analysis and cross-linking experiments have demonstrated that Fep1 is able to interact with itself to form homodimers (Pelletier et al., 2005). This interaction requires a leucine zipper motif located in the C-terminal region of Fep1p and is necessary for
full repression of fio1+. The yeast two-hybrid technique has also been utilised to demonstrate an interaction between Fep1p and the general transcriptional repressors Tup11p and Tup12 (Znaidi et al., 2004; Pelletier et al., 2005). These interactions have been confirmed using in vitro pull-down assays with purified proteins and with in vivo co-immunoprecipitation experiments. The minimal region of Fep1p required for this interaction has been mapped and encompasses residues 405-541. It is thought that in iron replete conditions Fep1p acts as a mediator to recruit Tup11/12p to specific promoters to suppress transcription. Further evidence for this theory includes the observation that tup11Δtup12Δ mutants have defects in iron transport that are identical to those in fep1Δ mutants (Pelletier et al., 2002). Interestingly, while the deletion of both tup11+ and tup12+ is required to give physical phenotypes that are consistent with loss of iron mediated repression Tup12p alone is responsible for changes in the expression of fio1+ and frp1+ expression (Fagerström-Billai & Wright et al., 2005; Pelletier et al., 2002; Znaidi et al., 2004).

As mentioned an iron responsive GATA type transcriptional factor Sfu1p has recently been identified in C. albicans that appears to have a similar role to Fep1p (Lan et al., 2004). Microarray analysis of the response of wild-type and sfu1ΔΔ mutants to iron limitation suggests that ~140 genes are directly regulated by Sfu1p. A total of 31 genes are more highly expressed in the sfu1ΔΔ mutant, with the majority having potential roles in iron uptake and homeostasis. This suggests that Sfu1p is the major transcriptional regulator of iron uptake in C. albicans. Interestingly, 108 genes are more highly expressed in the wild-type, suggesting Sfu1p could function as both a transcriptional repressor and activator.

**Post-transcriptional Control of Iron Uptake Genes**

In addition to the activity of Aft1p and Aft2p S. cerevisiae has mechanisms for the post-transcriptional regulation of genes in response to both high and low iron conditions. In iron replete conditions the cell possesses a system to monitor and degrade mRNA transcripts from a subset of iron uptake genes including FIT1-3, ARN1-3, FRE2-3 (Lee et al., 2005). This was first noted in strains lacking the S. cerevisiae RNase III orthologue Rnt1p, which display an increased sensitivity to iron due to elevated levels of transcripts from genes whose products are involved in iron
uptake. It is thought that Rnt1p recognises and cleaves RNA hairpins capped with an AGNN tetraloop structure (Chanfreau et al., 2000; Lee et al., 2005). After cleavage the mRNA is processed and degraded by the exonucleases Rntlp and Xmnp (Danin-Kreiselman et al., 2003; Lee et al., 2003; Lee et al., 2005). Analysis of the iron uptake genes up-regulated in rnt1Δ mutants reveals the presence of predicted Rnt1p recognition stem-loop structures in the mRNA of ARN2-4, FIT1-3, and FRE2-3 (Lee et al., 2005). Further analysis of two of these genes, ARNI and FIT2, showed that their mRNAs are in vitro substrates for Rnt1p. While this system of regulation and its targets have been identified the way in which it is controlled by iron is unknown.

A complementary mechanism of post-transcriptional control decreases transcript levels of target genes in low iron conditions (Puig et al., 2005). In response to iron limitation S. cerevisiae not only increases the expression of genes involved in iron uptake, but also down-regulates non-essential iron requiring metabolic processes to conserve iron (Shakoury-Elizeh et al., 2004). This second iron conserving process is regulated post-transcriptionally through the action of Cth2p, which targets selected mRNA for degradation (Puig et al., 2005). Cth2p is a zinc-finger RNA-binding protein also found in plants and mammals that binds to specific sequences within mRNAs and targets them for degradation. The expression of CTH2 in S. cerevisiae is controlled by Aft1p and Aft2p, and is therefore up-regulated in response to iron starvation conditions. In low iron conditions CTH2 is expressed and Cth2p binds to AU-rich elements located within the 3' -UTR of target mRNAs, targeting them for degradation. Interestingly, the half-life of the mRNA decreases with increasing numbers of AU-rich sequences in the 3' -UTR.

The importance of the Cth2p mediated regulation of iron metabolism is shown by the fact that cth2Δ mutants grow poorly in iron limited conditions (Puig et al., 2005). Microarray experiments using a cth2Δ mutant strain have shown that Cth2p regulates the abundance of over 80 different transcripts including those for aconitase, succinate dehydrogenase, α-ketoglutarate dehydrogenase, and a variety of genes involved in Fe-S cluster assembly. The repression of non-essential iron consuming processes by Cth2p is thought to increase the efficiency of iron usage within the cell. The Cth2p mediated post-transcriptional regulation of some genes may also fine-tune the
response of selected genes to low iron as *FIT1-2* are regulated by Aft1p and Aft2p and also targeted by Cth2p.

**Post-translational Regulation of Iron Uptake**

The high affinity iron uptake system *S. cerevisiae* is also post-translationally regulated (Felice et al., 2005). The addition of 50 μM iron to media causes the internalisation and degradation of Fet3p and Frt1p. This process requires endocytosis and the vacuolar protease Pep4p. In common with many post-translationally regulated membrane proteins the ubiquitination of Frt1p is required for the internalisation and degradation of the iron transport complex. However, in contrast to most other post-translationally regulated membrane proteins Frt1p is not ubiquinated by the ubiquitin ligase Rsp5p. Interestingly, the signal responsible for the ubiquitination of Frt1p is thought to be the passage of iron through the iron transport complex, as transport activity is required for internalisation. A similar system is seen in the Nramp transporter Smf1p, where metal transport is thought to cause conformational changes (Hettema et al., 2004). Changes in a hydrophobic domain of Smf1p are then recognised by Rsp5p and Bsd2p, resulting in the ubiquitination, internalisation, and degradation of the transporter. The degradation of Frt1p following transport is hypothesised to be a good means of regulating iron uptake as it allows levels of the transporter to be rapidly changed following alterations in the transcription of *FTR1* and *FET3* (Felice et al., 2005). However, it is unknown why substantial levels of Fet3p and Frt1p internalisation and degradation only occur at very high concentrations of iron (50 μM) when transcription is severely reduced at 10 μM. This is also puzzling given that the high affinity iron transport via Frt1p and Fet3p has a *Kₘ* of 0.15-0.2 μM (Askwith et al., 1994).

**1.9 Regulation of Copper Uptake**

**Transcriptional Regulation of Copper Uptake**

In a number of fungi high affinity copper uptake is regulated transcriptionally by a family of copper sensing transcriptional activators (Rutherford et al., 2004), with the prototype being Mac1p in *S. cerevisiae* (Jungmann et al., 1993). They are zinc finger proteins that sense cellular copper levels and activate the transcription of genes whose protein products are required for high affinity copper uptake. Following the
identification of Maclp in *S. cerevisiae*, homologues have subsequently been identified in *S. pombe* (Cuf1p), *Podospora anserine* (GRiSEA), and *C. glabrata* (CgMaclp) (Borghouts & Osiewacz, 1998; Labbe et al., 1999; Srikantha et al., 2005). Our laboratory has also isolated a Maclp homologue in *C. albicans* that performs a similar function to the other transcriptional activators (Marvin et al., 2004).

The *S. cerevisiae* Maclp protein was initially investigated because it shared significant homology with two transcription factors that had previously been implicated in copper homeostasis, Ace1p from *S. cerevisiae* and Amt1p from *C. glabrata* (Jungmann et al., 1993). Both ScAce1p (Thiele et al., 1988) and CgAmt1p (Zhou & Thiele, 1991) activate the transcription of genes encoding metallothioneins, which bind and sequester excess copper within the cell as part of the cellular response to toxic levels of copper (Perego & Howell, 1997). The significant homology shared by these proteins is mostly confined to the N-terminal portion with Maclp residues 1-40 exhibiting 53% and 48% amino acid identity respectively to the corresponding regions in ScAce1p and CgAmt1p (Jungmann et al., 1993).

The *S. cerevisiae* MAC1 gene encodes a 416 amino acid long polypeptide with a predicted molecular mass of 46.5 kDa (Jungmann et al., 1993). Analysis of the ScMaclp sequence reveals that it is a typical yeast zinc-finger transcription factor that contains a number of interesting structural features. The N-terminal region is basic and contains the minimal DNA-binding domain, which maps to a fragment containing the first 159 residues (Jensen et al., 1998). A pair of zinc binding modules within the DNA binding domain are thought to bind two Zn(II) molecules. This N-terminal region of Maclp also contains a ‘copper fist’ motif, CX_{2}CX_{8}CXH, which is highly conserved among the copper sensing transactivators ScAce1p, CgAmt1p, and SpCuflp. A pair of cysteine rich motifs with the consensus sequence CXC_{4}CX_{2}CX_{2}H are also found in the C-terminal portion of ScMac1p and have been named REPI (position 264-279) and REP2 (position 322-337). These C-terminal repeats are present in a number of other copper sensing transcription factors such as ScAce1p (Jungmann et al., 1993). The C-terminal cysteine rich motifs are important for the regulation of Maclp, and are each able to co-ordinate the binding of 4 Cu(I) atoms to produce a polycopper cluster (Brown et al., 2002; Jensen et al., 1998).
The phenotypes displayed by ScmaclΔ mutants highlight the importance of ScMac1p in cellular copper and iron homeostasis. The ScmaclΔ mutant is respiratory deficient, displays defective ferric/cupric reductase activity, and is hypersensitive to H₂O₂, cadmium, lead, and zinc (Jungmann et al., 1993). All of these phenotypes can be rescued by the addition of copper or iron to the growth media except the respiratory deficiency, which is only rescued by copper. The mac1Δ mutant also has defects in the regulation of CTR1, CTR3, FRE1, and FRE7 (Hassett & Kosman et al., 1995; Jungmann et al., 1993). A dominant allele of MAC1 has also been isolated that contains a H279Q substitution in REPI, due to a single T→A transversion (Jungmann et al., 1993). The MAC1up1 mutant is hypersensitive to copper, oxidative stress, and has elevated levels of ferric/cupric reductase activity that do not respond to changes in iron and copper levels (Hassett & Kosman, 1995; Jungmann et al., 1993). These phenotypes are the result of the constitutive expression of the Mac1p target genes in the MAC1up1 mutant (Georgatsou et al., 1997; Georgatsou et al., 1999; Hassett & Kosman, 1995; Jungmann et al., 1993).

The analysis of gene expression changes in ScmaclΔ and ScMAC1up1 mutants has proved to be a useful tool for the identification of the ScMAC1 regulon (Gross et al., 2000). Microarray studies have identified 6 genes (CTR1, CTR3, FRE1, FRE7, YFR055w, and YJL217w) in S. cerevisiae that are specifically regulated by ScMac1p (DeFreitas et al., 2003; Gross et al., 2000). The functions and importance of the transporters Ctr1p (Dancis et al., 1994), Ctr3p (Knight et al., 1996), and the ferric/cupric reductase Fre1p (Dancis et al., 1990) in high affinity copper uptake are well known. The cellular role of FRE7 is still unclear, although a role in cellular copper trafficking has been proposed (Hassett & Kosman, 1995). Sequence analysis suggests that YFR055w encodes a putative cystathionine β-lyase, and this may mean that cells raise cysteine levels in response to copper starvation (Gross et al., 2000). However, this hypothesis has not been tested and the importance of Yfr055p is still unclear. The Yjl217p protein sequence shows similarity to a family of conserved hypothetical bacterial proteins of unknown function.

One area of ScMac1p function that has been extensively studied is its response to changes in copper levels. In contrast to ScAft1p the transcriptional activity of
ScMac1p is not regulated through the localisation of the protein (Serpe et al., 1999; Yamaguchi-Iwai et al., 2002). The ScMac1p protein is permanently located in the nucleus and analysis of the amino acid sequence reveals the presence of a NLS sequence in the N- and C-terminal regions of the protein (Serpe et al., 1999) Instead the transcriptional activity of ScMac1p is tightly regulated by at least two mechanisms that allow ScMac1p to respond to both physiological and toxic concentrations of copper. When cells are exposed to moderate levels of copper, ScMac1p transcriptional activity is inhibited by an intermolecular interaction between the DNA-binding and transactivation domains (Jensen & Winge, 1998; Joshi et al., 1999; Serpe et al., 1999). If the copper concentration in media increases above 10 μM ScMac1p is rapidly degraded in a copper specific manner that does not require the synthesis of new protein (Zhu et al., 1998). It has also been suggested that the phosphorylation state of Mac1p may play a role in its regulation (Heredia et al., 2001).

A copper mediated intermolecular interaction within ScMac1p was first suggested because both the DNA-binding and transactivation domains are required for the copper induced inhibition of either activity (Graden & Winge, 1997; Jensen et al., 1998). A yeast two-hybrid approach was then used to demonstrate a direct interaction between the DNA-binding and transactivation domains of Mac1p (Jensen & Winge, 1998). This led to the hypothesis that the binding of Cu ions to the C-terminal cysteine rich motifs of ScMac1p could stimulate the formation of an intramolecular interaction between the DNA-binding and transactivation domains. This would cause it to adopt a 'silent' conformation that masks the DNA-binding and transactivation domains.

There is currently a wealth of evidence to support this model of ScMac1p inhibition, and regions that are important for the intramolecular interaction have been identified. The REPI motif appears to be the major copper sensing domain, with mutation of any of the conserved cysteine or histidine residues within this region producing phenotypes similar to the ScMAC"up1" allele (Jensen & Winge, 1998; Keller et al., 2000; Yamaguchi-Iwai et al., 1997; Zhu et al., 1998). The constitutive phenotype associated with this dominant allele is therefore probably due to its inability to form intramolecular interactions in response to copper. The role of REPII in the copper dependent modulation of Mac1p activity is less well understood. Mutations in the
conserved amino acids in REPII do not affect the copper responsiveness of ScMac1p and its role is therefore believed to be relatively minor (Voutsina et al., 2001). It is thought that REPII is the major transactivation domain of Mac1p, as when studied independently it is a much stronger transactivator than REPI. However, another study has shown that in the context of the whole protein REPII is a relatively weak transactivator (Keller et al., 2000). They propose the alternative theory that REPII modulates the efficiency of DNA-binding. Evidence for this includes the observation that Mac1p DNA-binding is more efficient in the absence of REPII. The phosphorylation state of Mac1p is also important in the formation of the intramolecular interaction (Heredia et al., 2001). The activation domain of Mac1p is post-translationally phosphorylated, and unphosphorylated Mac1p is unable to bind DNA or form intramolecular interactions. However, the phosphorylation state of Mac1p does not respond to changes in copper availability, which makes its significance in vivo unclear.

In response to copper limitation ScMac1p binds to cis-acting copper responsive elements (CuREs) within the promoters of the target genes CTR, CTR3, FRE1, FRE7, YFR055w, and YJL217w (Gross et al., 2000). These CuREs have the consensus sequence 5'-TTTGC(T/G)C(A/G)-3', and are similar to the DNA regulatory elements recognised by ScAce1p, which also contain an A/T rich region located upstream of a related core sequence (Labbe et al., 1997; Yamaguchi-Iwai et al., 1997). The CuREs are essential for the ScMac1p mediated transcription of genes, and ScMac1p is known to bind directly to these elements within the promoters of CTR1, CTR3, and FRE1 (Labbe et al., 1997; Yamaguchi-Iwai et al., 1997). Analysis of Mac1p binding and comparisons with other copper responsive transcription factors suggests that Mac1p binds in a modular fashion (Jamison McDaniels et al., 1999). The ScMac1p sequence contains a RGRP motif that is known to be involved in the binding of a number of well characterised proteins to the minor groove of A/T rich DNA sequences (Aravind & Landsman, 1998; McDaniels et al., 1999). Experimental evidence suggests that the motif carries out a similar function in ScMac1p by recognising and binding to A/T rich sequence at the 5' end of the CuREs (Jamison McDaniels et al., 1999). A second unknown region within the DNA-binding domain of ScMac1p is responsible for binding to the major groove in the GCTCA section of the CuRE.
While Mac1p is able to bind to individual CuRE elements the presence of at least two is necessary to give significant levels of \textit{CTR1} expression (Jensen \textit{et al.}, 1998). The CuREs can be arranged as either direct or inverted repeats, and the spacing between the sequences appears to have little impact on the level of Mac1p transcriptional activation of genes. The CuREs function synergistically, with the addition of extra CuREs increasing \textit{CTR1} expression to a greater extent than can be explained simply by the additive binding of Mac1p. Except for \textit{YFR055w} and \textit{YJL217w} all of the known Mac1p target gene promoters contain at least two CuRE repeats (DeFreitas \textit{et al.}, 2003; Gross \textit{et al.}, 2000). Both \textit{YFR055w} and \textit{YJL217w} pose an interesting question as they contain a single CuRE within their promoters, although a number of similar sequences are also present. It is currently not known whether the single conserved CuRE sequences within their promoters are sufficient for Mac1p mediated transcriptional activation, or if the closely related sequences are also recognised by Mac1p.

Due to the requirement for at least two CuRE elements to produce sufficient levels of copper responsive \textit{CTR1} transcription, and the presence of multiple elements within most of its target gene promoters, it is currently thought that ScMac1p functions as a homodimer (Joshi \textit{et al.}, 1999; Serpe \textit{et al.}, 1999). This theory is supported by EMSA assays showing that Mac1p binary and ternary complexes are present at the \textit{CTR1} promoter (Serpe \textit{et al.}, 1999). Interestingly, the formation of (Mac1p)$_2$-DNA complexes is affected by the CuRE sequences. The \textit{CTR1} promoter contains two CuREs that differ in the presence of either a TA or TT at the 5' end. Mutagenesis of the CuREs reveals that ScMac1p binds more strongly to sequences containing a 5'-TA, which support the formation of (Mac1p)$_2$-DNA complexes. If both \textit{CTR1} CuREs contain a 5'-TA then the proportion of (Mac1p)$_2$-DNA complexes increases, and conversely if both have a 5'-TT then ternary complexes are less abundant when compared to the wild-type promoter. The expression of reporter genes fused to the \textit{CTR1} promoter also increases with the addition of extra 5'-TA containing CuREs. It has therefore been suggested that the (Mac1p)$_2$-DNA complexes are the active complexes in facilitating transcription from the target genes (Joshi \textit{et al.}, 1999).

Further evidence for the formation of ScMac1p homodimers was provided by the discovery of a Mac1p-Mac1p intermolecular interaction using a yeast two-hybrid
based approach (Joshi et al., 1999). The protein interacting domain was mapped to a minimal fragment containing residues 388-406, which are predicted to form a helix (Serpe et al., 1999). This intermolecular interaction is copper independent but is negatively regulated by the N-terminal DNA-binding domain. The ability to form the Mac1p-Mac1p interaction is essential as mutations within the dimerisation domain abolish the ability of Mac1p to activate the transcription of genes in vivo. This supports the theory that Mac1p homodimers are the active complexes that induce the transcription of genes.

The current model extrapolated from the data involving Mac1p-DNA binding suggests that under low copper conditions Mac1p is able to bind to CuREs within target gene promoters (Joshi et al., 1999; Serpe et al., 1999). This occurs more efficiently at CuREs containing a 5'-AT and recruitment of a second Mac1p molecule is facilitated and stabilised by a Mac1p-Mac1p interaction. When copper is freely available Mac1p is inhibited from binding to DNA by the formation of an intramolecular interaction that also prevents the formation of Mac1p-Mac1p intermolecular interactions.

**Post-transcriptional Regulation of Copper Uptake**

In addition to being regulated by Mac1p at the transcriptional level high affinity copper uptake in *S. cerevisiae* is regulated post-translationally (reviewed in van Ho, 2002). Both Ctrl1p and Mac1p are regulated post-transcriptionally through two distinct pathways (Ooi et al., 1996; Zhu et al., 1998). The first involves the internalisation of ScCtrl1p into endosome-like vesicles in response to moderate levels of copper (Ooi et al., 1996). When the levels of copper are raised ScCtrl1p and ScMac1p are degraded in a copper specific fashion (Ooi et al., 1996; Zhu et al., 1998). The endocytosis of Ctrl1p occurs when 0.1-1μM copper is added to *S. cerevisiae* cultures grown in copper limited conditions (Ooi et al., 1996). The mechanism behind this process, and the fraction of Ctrl1p that is internalised, is currently unknown. It is also unclear whether the internalised Ctrl1p is degraded or recycled to the cell surface. A number of possible functions for the endocytosis of Ctrl1p have been proposed. These include the suggestion that the internalisation of Ctrl1p may provide a less efficient method of copper acquisition, preventing the influx of large quantities of
copper. Alternatively, the endocytosis of Ctr1p may modulate the uptake of copper by altering the amount of transporter at the surface.

The second regulatory mechanism involves the degradation of Ctr1p and Mac1p when copper levels exceed 10μM (Ooi et al., 1996; Zhu et al., 1996). They are therefore degraded when the concentration of copper exceeds the functional range of the high affinity copper transporters Ctr1p and Ctr3p (Dancis et al., 1994; Knight et al., 1996). However, it is interesting to note that Ctr3p does not undergo copper responsive degradation (Pena et al., 2000). Degradation is copper specific although the mechanism involved is still unknown. The degradation of Mac1p is rapid, copper specific, and does not require the synthesis of new proteins (Zhu et al., 1998). The REPI domain of ScMac1p is essential for this process because the products of the ScMac1<sup>up</sup> allele are resistant to copper responsive degradation (Yonkovich et al., 2002). This suggests that the formation of intramolecular interactions is important for the targeting of Mac1p. The insensitivity of the protein produced by the ScMac1<sup>up</sup> allele to copper responsive degradation partially explains its ability to constitutively activate the expression of its target genes.

Relatively little is also known about the copper specific degradation of ScCtr1p. In copper limiting conditions, ScCtr1p is stable for at least 2-3 hours but disappears from the cell surface within 30-60 minutes when 10 μM copper is added to media (Ooi et al., 1996). The degradation of ScCtr1p does not require endocytosis or vacuolar degradation, suggesting this process occurs at the plasma membrane. A putative copper binding motif, CX₅CX₃CX₂H, located in the cytosolic domain is essential for degradation, raising the possibility that the protease may recognise or interact with this region. Interestingly, ScMac1p is also essential for the copper responsive degradation of ScCtr1p (Yonkovich et al., 2002). However, due to the localisation of ScMac1p it is unlikely to directly degrade ScCtr1p, and is therefore thought to activate the protease responsible for this process.

1.10 Background to the Project

The central aim of this project was to identify and characterise ferric reductases in the opportunistic pathogen <i>C. albicans</i>. Previous work in our laboratory demonstrated
that *C. albicans* is able to reduce iron and copper at the cell surface (Morrissey *et al.*, 1996). The cell surface reductase activity associated with *C. albicans* is negatively regulated by iron and copper in common with *S. cerevisiae*. Two ferric reductases have subsequently been isolated from *C. albicans* in our laboratory (Hammacott *et al.*, 2001). Both CaFre1p and CaFre2p were identified through their ability to complement an *S. cerevisiae* strain with severely reduced cell surface reductase activity. Initial releases of the *C. albicans* genome sequence also contained a number of putative ferric reductases. One of the aims of this approach was to examine the genome sequence data for the presence of further potential ferric reductases. A number of the targets identified using this approach were then examined experimentally in an attempt to understand their role within the cell.

Due to requirement of copper for high affinity iron uptake our laboratory has also examined the process of high affinity copper uptake and its regulation in *C. albicans* (Marvin *et al.*, 2003; Marvin *et al.*, 2004). We have identified a copper transporter, CaCtrlp (Marvin *et al.*, 2003), whose deletion causes severe defects in high affinity iron uptake (Marvin *et al.*, 2004). The expression of *CaCTR1* increases in response to copper limitation and is dependent on the transcription factor CaMac1p (Marvin *et al.*, 2004). A *S. cerevisiae* homologue of CaMac1p is also known to regulate the expression of two ferric reductases in response to copper limitation (Georgatsou & Alexandraki, 1999; Jungmann *et al.*, 1993; Martins *et al.*, 1998; Yamaguchi-Iwai *et al.*, 1997). The role of CaMac1p in the expression of the *C. albicans* ferric reductases was therefore examined during this study.

### 1.11 Project Aims

The aims of this project were:

- To identify genes encoding putative ferric reductases in *C. albicans* present in the genome sequence.
- Analyse the effect of iron or copper limitation on the expression of the putative ferric reductases.
- The construction of *C. albicans* strains carrying deletions of some of the ferric reductases identified.
• Analyse ferric reductase for mutant phenotypes associated with defects in iron and copper uptake and metabolism
• Identify factors that are involved in regulating the expression of the putative ferric reductases in response to iron and copper limitation
Chapter 2
Materials and Methods

2.1 Strains Used During This Study

Bacterial Strains
The *E. coli* strain DH5α (φ80lacZΔM15, recA1, endA1, gyrA96, thi-1, hsdR17 (K, mK+), supE44, relA1, deoR, Δ(lacZYA-argF)U169) was used for all of the bacterial work throughout this study.

C. albicans Strains
The *C. albicans* strains used during the course of this study are shown in Table 2.1.

2.2 Bacterial Media and Growth Conditions

Luria-Bertani Media (LB)
Media consisted of 1% Bacto-tryptone (Oxoid), 0.5% yeast extract (Oxoid), 0.5% sodium chloride, and was adjusted pH 7.2 with sodium hydroxide. Solid media was made by the addition of 2% Bacto-agar (Oxoid). For the selection of antibiotic resistant colonies ampicillin was added to a final concentration of 100 μg.mL⁻¹.

Growth Conditions
All *E. coli* strains were routinely cultured at 37°C with liquid cultures undergoing continuous agitation at 200 rpm.

Measurement of Cell Titre
The cell density of liquid cultures was determined by measuring the optical density at a wavelength of 600 nm using a spectrophotometer. The density was determined using the assumption that an OD₆₀₀ of 1 corresponds to ~8x10⁸ cells.ml⁻¹.
Table 2.1 *C. albicans* strains used during this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SC5314</td>
<td>Clinical isolate</td>
<td>Gillum <em>et al.</em>, 1984</td>
</tr>
<tr>
<td>CAF-2</td>
<td>ura3Δ::λimm434/URA3</td>
<td>Fonzi &amp; Irwin, 1993</td>
</tr>
<tr>
<td>CAI-4</td>
<td>ura3Δ::λimm434/ura3Δ::λimm434</td>
<td>Fonzi &amp; Irwin, 1993</td>
</tr>
<tr>
<td>DAY185</td>
<td>As CAI4 but HIS1::his1::hisG/his1::hisG; ARG4::URA3::arg4::hisG</td>
<td>Davis <em>et al.</em>, 2000</td>
</tr>
<tr>
<td>BWP17</td>
<td>As CAI4 but arg4::hisG/arg4::hisG; his1Δ::hisG/his1Δ::hisG</td>
<td>Wolson <em>et al.</em>, 1999</td>
</tr>
<tr>
<td>JHC1.2</td>
<td>As CAI4 but fre1Δ::hisG/fre1Δ::hisG-URA3-hisG</td>
<td>Hammacott PhD Thesis, 2000</td>
</tr>
<tr>
<td>RM2.1 &amp; 2</td>
<td>As CAI4 but fre2Δ::hisG-URA3-hisG/FRE2</td>
<td>This Study</td>
</tr>
<tr>
<td>RM2.3 &amp; 4</td>
<td>As CAI4 but fre2Δ::hisG/FRE2</td>
<td>This Study</td>
</tr>
<tr>
<td>RM2.5 &amp; 6</td>
<td>As CAI4 but fre2Δ::hisG/fre2Δ::hisG-URA3-hisG</td>
<td>This Study</td>
</tr>
<tr>
<td>RM5.1 &amp; 2</td>
<td>As CAI4 but fre5Δ::hisG-URA3-hisG/FRE5</td>
<td>This Study</td>
</tr>
<tr>
<td>RM5.3 &amp; 4</td>
<td>As CAI4 but fre5Δ::hisG/FRE5</td>
<td>This Study</td>
</tr>
<tr>
<td>RM5.5 &amp; 6</td>
<td>As CAI4 but fre5Δ::hisG/fre5Δ::hisG-URA3-hisG</td>
<td>This Study</td>
</tr>
<tr>
<td>RM10.1&amp;2</td>
<td>As CAI4 but fre10Δ::hisG-URA3-hisG/FRE10</td>
<td>This Study</td>
</tr>
<tr>
<td>RM10.3&amp;4</td>
<td>As CAI4 but fre10Δ::hisG/FRE10</td>
<td>This Study</td>
</tr>
<tr>
<td>RM10.5&amp;6</td>
<td>As CAI4 but fre10Δ::hisG/fre10Δ::hisG-URA3-hisG</td>
<td>This Study</td>
</tr>
<tr>
<td>RMA1&amp;2</td>
<td>As CAI4 but aftΔ::hisG-URA3-hisG/AFT</td>
<td>This Study</td>
</tr>
<tr>
<td>RMA3&amp;4</td>
<td>As CAI4 but aftΔ::hisG</td>
<td>This Study</td>
</tr>
<tr>
<td>RMA7</td>
<td>As CAI4 but aftΔ::hisG-URA3-hisG/aftΔ::hisG</td>
<td>This Study</td>
</tr>
<tr>
<td>MEM-m2</td>
<td>As BWP17 but mac1Δ::URA3/mac1Δ::ARG4; his1::hisG/his1::hisG::HIS1</td>
<td>Marvin <em>et al.</em>, 2004</td>
</tr>
<tr>
<td>MEM-c3</td>
<td>As BWP17 but ctr1Δ::URA3/ctr1Δ::ARG4; his1::hisG/his1::hisG::HIS1</td>
<td>Marvin <em>et al.</em>, 2003</td>
</tr>
<tr>
<td>RM10.7</td>
<td>As CAI4 but fre10Δ::hisG/fre10Δ::hisG-URA3-hisG; Clp10-FRE10</td>
<td>This study</td>
</tr>
<tr>
<td>RMLac</td>
<td>As CAI4 but plac-FRE10promoter</td>
<td>This study</td>
</tr>
</tbody>
</table>
2.3 Yeast media and Growth Conditions

Yeast Peptone Glucose Media (YPD)
Media consisted of 1 % (w/v) yeast extract (Oxoid), 2 % (w/v) Bactopeptone (Oxoid) 2 % (v/v) glucose and 50µM uridine, with the addition of 2 % Bacto-agar (Oxoid) for solid media.

Synthetic Glucose Media (SD)
Media consisted of 0.67 % (w/v) yeast nitrogen base without amino acids (Bio101) and 2 % (v/v) glucose. Solid media was created by the addition of 2 % Bacto-agar (Oxoid). Where applicable the media was supplemented with the appropriate amino acids at the concentrations shown in Table 2.2 (Sherman et al., 1986). For the counter-selection of CaURA3 SD media was supplemented with 50 µM uridine and 0.1 % 5-FOA.

Minimal Defined Media (MD)
Minimal defined media is based on Wickerhams nitrogen base recipe (Wickerham et al., 1951) with modifications by made by Eide and co-workers (Eide et al., 1992). The media consists of 10 % (v/v) salt and trace solution, 0.1 % (v/v) vitamin solution, 7 mM calcium chloride, 20 mM tri-sodium citrate pH 4.2, 2 % (v/v) glucose, and 1.25 % (w/v) Bacto-agar (Oxoid) in the case of solid media. Composition of salt and trace solution and vitamin solution are detailed in Tables 2.3 and 2.4 respectively.

Growth Conditions
All yeast strains were routinely cultured at 30°C, with liquid cultures being continuously agitated at 200 rpm.

Measurement of Cell Density
The cell density of liquid cultures was determined using a haemocytometer with a modified Thoma ruling, or by measuring the optical density of cultures in a spectrophotometer at a wavelength of 600 nm. The relationship between optical density and cell numbers was determined for each strain by measuring cell numbers with a counting chamber, as mentioned above.
Table 2.2 Amino acid supplements

<table>
<thead>
<tr>
<th>Component</th>
<th>Stock Concentration</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>adenine hemisulphate</td>
<td>2 mg.ml⁻¹</td>
<td>20 μg.ml⁻¹</td>
</tr>
<tr>
<td>histidine</td>
<td>8 mg.ml⁻¹</td>
<td>20 μg.ml⁻¹</td>
</tr>
<tr>
<td>leucine</td>
<td>12 mg.ml⁻¹</td>
<td>30 μg.ml⁻¹</td>
</tr>
<tr>
<td>uridine</td>
<td>5 mg.ml⁻¹</td>
<td>50 μg.ml⁻¹</td>
</tr>
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</table>

Table 2.3 Salt and trace solution

<table>
<thead>
<tr>
<th>Component</th>
<th>Stock concentration</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>ammonium sulphate</td>
<td>75.7 mM</td>
<td>7.57 mM</td>
</tr>
<tr>
<td>potassium dihydrogen orthophosphate</td>
<td>50.2 mM</td>
<td>5.02 mM</td>
</tr>
<tr>
<td>di-potassium hydrogen orthophosphate</td>
<td>9.2 mM</td>
<td>0.92 mM</td>
</tr>
<tr>
<td>magnesium sulphate</td>
<td>20.3 mM</td>
<td>2.03 mM</td>
</tr>
<tr>
<td>sodium chloride</td>
<td>17.1 mM</td>
<td>1.71 mM</td>
</tr>
<tr>
<td>boric acid</td>
<td>1.62 μM</td>
<td>162 nM</td>
</tr>
<tr>
<td>potassium iodide</td>
<td>0.6 μM</td>
<td>60 nM</td>
</tr>
<tr>
<td>zinc sulphate</td>
<td>2.44 μM</td>
<td>244 nM</td>
</tr>
</tbody>
</table>

Table 2.4 Vitamin solution

<table>
<thead>
<tr>
<th>Component</th>
<th>Stock concentration</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>d-biotin</td>
<td>8.19 μM</td>
<td>8.19 nM</td>
</tr>
<tr>
<td>thiamine hydrochloride</td>
<td>1.19 mM</td>
<td>1.19 μM</td>
</tr>
<tr>
<td>pyridoxine hydrochloride</td>
<td>1.95 mM</td>
<td>1.95 μM</td>
</tr>
<tr>
<td>myo-inositol</td>
<td>11 mM</td>
<td>11 μM</td>
</tr>
<tr>
<td>d-pantothenic acid calcium salt</td>
<td>0.84 mM</td>
<td>0.84 μM</td>
</tr>
</tbody>
</table>
2.4 Transformation of *E. coli* and *C. albicans*

**Calcium Chloride Transformation of Escherichia coli** (Mandel & Higa, 1970)

Cultures were grown to a density of 0.5-0.6 (OD$_{600}$) and harvested by centrifugation at 4000 rpm for 5 minutes at 4°C. Cells were washed with 0.5 volumes of ice-cold 100 mM calcium chloride and suspended in 0.5 volumes of ice-cold calcium chloride. Following incubation on ice for 30 minutes, the cells were harvested and suspended in 0.05 culture volumes of ice-cold calcium chloride. The competent cells were then transformed immediately or stored for later use in 20 % glycerol at -80°C.

To transform competent cells a 200 µl aliquot was incubated with the transforming DNA on ice for 30 minutes. The transformation samples were then heat shocked at 42°C for 2 minutes, and 1 ml of LB media added immediately afterwards. The cells were then incubated at 37°C for 90 minutes, harvested by centrifugation at 13000 rpm for 1 minute, and suspended in 100 µl of fresh LB. Cells were then plated on the appropriate selective media and incubated overnight at 37°C.

**Lithium Acetate Transformation of Candida albicans** (Wilson et al., 1999)

Initially 50 ml of YPD was inoculated with the relevant *C.albicans* strain and incubated overnight. The culture was then harvested, washed twice with distilled water, and used to inoculate 50 ml of fresh YPD at a concentration of 2x10$^6$ cell.ml$^{-1}$. Cells were grown for at least three complete doublings, harvested and washed twice with 25 ml LiAc/Te (100 mM Lithium acetate pH 7.5, 1xTE pH 7.5), and suspended in 500 µl of fresh buffer. The competent yeast cells were then divided into 50 µl aliquots that were mixed with 50 µg of denatured salmon sperm and 1-10 µg of transforming DNA. Following a 30 minute incubation at room temperature 700 µl of PLATE solution (100 mM lithium acetate, pH 7.5; 1xTE, pH 7.5; 40 % polyethylene glycol 3350) was added to each tube added and mixed thoroughly by inversion. The transformation reactions were then incubated overnight at room temperature and heat shocked at 42°C for 1 hour the following morning. Cells were then harvested by centrifugation at 5000 rpm for 2 minutes, washed with 1 ml of water, and resuspended in 100 µl distilled water. The transformation samples were then plated onto selective SD media and incubated at 30°C for 3-10 days.
2.5 Plasmids Used and the Preparation of Nucleic Acids

Plasmids and Vectors
A list of the plasmids and vector used during this study is presented in Table 2.5. Diagrams of the plasmids showing their relevant features are also provided in Figures 2.1-2.5.

Preparation of Plasmid DNA from Escherichia coli
Plasmid DNA was extracted from *E. coli* using Plasmid Mini or Midi kits supplied by Qiagen. The DNA was extracted and purified by following the manufacturers' instructions supplied. This method utilises an alkaline lysis method (Birnboim & Doly, 1979) and ion exchange chromatography to purify plasmid DNA.

Small Scale Preparation of C. albicans Genomic DNA
Overnight cultures grown in 10 ml of YPD were harvested and suspended in 200μl of DNA breaking buffer (2 % (w/v) Triton X-100; 1 % (w/v) SDS; 100 mM NaCl; 10 mM Tris-HCl, pH 8.0; 1 mM EDTA, pH 8.0). Approximately 200 μl of acid washed glass beads were then added to each sample along with 200 μl of phenol:chloroform:IAA. The tubes were then vortexed vigorously for 7 minutes and 200 μl of TE (pH 8.0) added. Samples were then centrifuged at 13000 rpm for 10 minutes and the aqueous phase transferred to a fresh tube containing 500 μl chloroform:IAA. The samples were then vortexed briefly, centrifuged at 13000 rpm for 10 minutes, and the aqueous phase transferred to a fresh tube. The DNA was then precipitated by the addition of 800 μl of ice-cold 100 % ethanol and incubation at -20°C for at least 1 hour. The precipitated DNA was pelleted by centrifugation at 13000 rpm for 20 minutes, suspended in 400 μl of TE (pH 8.0) containing 75 μg.ml⁻¹ RNaseA, and incubated at 37°C for 1 hour to degrade contaminating RNA. The DNA was then precipitated again by the addition of 800 μl 100% ethanol and 40 μl 3M sodium acetate (pH 5.2), and the samples incubated at -20°C for at least 1 hour. The precipitated DNA was then pelleted by centrifugation at 13000 rpm for 20 minutes, washed with 1 ml 70 % ethanol, and air-dried before being suspended in 50 μl distilled water.
<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pUC18</td>
<td>ori; LacZ&lt;sup&gt;+&lt;/sup&gt;; amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Yanisch-Perron et al., 1985</td>
</tr>
<tr>
<td>pMB-7</td>
<td>hisG-URA3-hisG cassette in pUC18</td>
<td>Fonzi &amp; Irwin, 1993</td>
</tr>
<tr>
<td>Plac-poly</td>
<td>ori; amp&lt;sup&gt;R&lt;/sup&gt;, CaRPS10, CaURA3; LacZ</td>
<td>Brown et al., unpublished</td>
</tr>
<tr>
<td>Clp10</td>
<td>ori; amp&lt;sup&gt;R&lt;/sup&gt;; CaRPS10; CaURA3</td>
<td>Murad et al., 2000</td>
</tr>
<tr>
<td>pRM2.1</td>
<td>pMB-7 plus CaFRE2 5' flank</td>
<td>This study</td>
</tr>
<tr>
<td>pRDFRE2</td>
<td>pMB-7 with CaFRE2 disruption cassette</td>
<td>This study</td>
</tr>
<tr>
<td>pRD5.1</td>
<td>pMB-7 with CaFRE5 5' flank</td>
<td>This study</td>
</tr>
<tr>
<td>pRDFRE5</td>
<td>pMB-7 with CaFRE5 disruption cassette</td>
<td>This study</td>
</tr>
<tr>
<td>pRD10.1</td>
<td>pUC18 with CaFRE10 5' flank</td>
<td>This study</td>
</tr>
<tr>
<td>pRD10.2</td>
<td>pUC18 with CaFRE10 5' and 3' flank</td>
<td>This study</td>
</tr>
<tr>
<td>pRDFRE10</td>
<td>pUC18 with CaFRE10 disruption cassette</td>
<td>This study</td>
</tr>
<tr>
<td>pRDA.1</td>
<td>pMB-7 with CaAFT 5' flank</td>
<td>This study</td>
</tr>
<tr>
<td>pRDAFT</td>
<td>pMB-7 with CaAFT disruption cassette</td>
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<tr>
<td>pRRFRE10</td>
<td>Clp-10 with CaFRE10 ORF</td>
<td>This study</td>
</tr>
<tr>
<td>pREFRE10</td>
<td>Plac-poly with CaFRE10 promoter</td>
<td>This study</td>
</tr>
</tbody>
</table>
Figure 2.1 Plasmids containing C. albicans reporter genes. (a) The plasmid plac-poly contains the LacZ reporter gene derived from Streptococcus thermophilus. It also contains the bla gene encoding ampicillin resistance for selection and maintenance in E. coli. The CaURA3 gene was used as an auxotrophic marker for selection in C. albicans. The RPS10 gene was used for targeted integration into the C. albicans genome. (b) Plasmid pRE-FRE10 was created by inserting the CaFRE10 promoter into the Xmal/SphI sites of plac-poly.
Figure 2.2 Plasmids used in the disruption of *C. albicans* genes. (a) The plasmid pMB-7 was used in the creation of cassettes for the disruption of *C. albicans* genes (Fonzi & Irwin, 1993). The *bla* gene encodes ampicillin resistance for selection and maintenance in *E. coli*. The *ori* is the origin of replication derived from pBR322. pMB-7 also contains the disruption module used for the replacement of *C. albicans* genes. This module includes the auxotrophic marker *CaURA3* flanked by two *hisG* repeats derived from *Salmonella typhimurium*. Recombination between these repeats allows the removal of *CaURA3* following counter-selection with 5-FOA. (b) Plasmid pRDAFT was constructed by inserting the 5' and 3' flanking regions of *CaAFT* into pMB-7 at the SphI/PstI and BglII/KpnI sites respectively. This was used for the disruption of *CaAFT*. 
Figure 2.3 *C. albicans* Disruption Plasmids. Plasmids containing modules for the disruption of *CaFRE2* and *CaFRE5* were constructed by inserting regions flanking the 5' and 3' regions of the genes of interest into pMB-7 (Figure 2a). The plasmids contain an origin of replication (ori) and a gene encoding for ampicillin resistance (bla). (a) pRDFRE2 was created by inserting the 5' and 3' regions flanking *CaFRE2* into the KpnI/BglII and SphI/PstI sites of pMB-7 respectively. (b) pRDFRE5 was created by inserting the 5' and 3' regions flanking *CaFRE5* into the SphI/PstI and KpnI/BglII sites of pMB-7 respectively.
Figure 2.4 Plasmids for the disruption of CaFRE10. The cassette for the disruption of CaFRE10 was constructed in the cloning vector pUC18 (a). The pUC18 vector contains an origin of replication (ori) and a gene encoding ampicillin resistance (bla) for selection and maintenance in E. coli. It also contains the LacZ alpha peptide and the lac promoter allowing blue white selection. The CaFRE10 5' flanking sequence was inserted into the SphI/PstI sites of pUC18 followed by the insertion of the 3' region at the KpnI/SalI sites. A KpnI/PstI fragment containing the disruption module from pMB-7 (Fonzi & Irwin, 1993) was then inserted into the relevant sites between the CaFRE10 flanking sequences to create the final disruption construct pRD-FRE10 (b). Therefore pRD-FRE10 contains regions flanking CaFRE10 and the disruption module from pMB-7 comprising of the auxotrophic marker CaURA3 flanked by repeated hisG sequences from Salmonella typhimurium.
Figure 2.5 Plasmids used in the reintegration of C. albicans genes. (a) The plasmid Clpl10 was used for the reintegration of genes into C. albicans strains at the RPS10 loci (Murad et al., 2000). The plasmid contains the ampR gene that encodes for ampicillin resistance for selection and maintenance in E. coli. The CaURA3 was used as an auxotrophic marker for selection in C. albicans. (b) Plasmid pRM-FRE10 was constructed by inserting the CaFRE10 ORF and flanking sequences into the Xhol site of Clp10.
Isolation of total RNA from C. albicans (Schmitt et al., 1990)

Cells were harvested from exponentially growing cultures, washed with 5 ml of distilled water, and re-suspended in 400 µl of freshly prepared AE buffer (50 mM sodium acetate, pH 5.3; 10 mM EDTA). 80 µl of 10 % (w/v) SDS was added to the cells, which were vortexed for 30 seconds prior to the addition of 480 µl of phenol equilibrated with AE buffer. Samples were vortexed briefly and incubated at 65°C for 5 minutes, before being frozen in a dry ice/ethanol bath for 5 minutes. The freeze-thaw process was repeated 3 times, with a final incubation at 65°C for 5 minutes. Samples were then centrifuged at 13000 rpm for 5 minutes and the aqueous phase removed to a fresh tube. An equal volume of phenol/chloroform was added to the samples and the aqueous phase separated by centrifugation as before. The extraction of the aqueous phase using phenol/chloroform was repeated until proteinaceous matter was no longer observed at the interface. The RNA was then precipitated by the addition of 2 volumes of ice-cold absolute ethanol and 1/10 volume sodium acetate, pH 5.2. Samples were incubated overnight at -20°C to optimise the precipitation and the RNA then pelleted by centrifugation at 13000 rpm for 20 minutes at 4°C. The supernatant was removed and the pelleted RNA washed with 1 ml of 70 % ethanol before being air-dried and re-suspended in 100 µl DEPC-treated water.

2.6 Manipulation of Nucleic Acids

Measurement of Nucleic Acid Concentration and Purity

The concentration and purity of nucleic acids was determined by measuring the absorbance of samples at 260 nm and 280 nm in a UV spectrophotometer. Absorbance values were converted to concentrations using the following constants.

\[
1 \text{ OD}_{260} \text{ unit } = 50 \mu g \text{ of double stranded DNA}
\]
\[
1 \text{ OD}_{260} \text{ unit } = 33 \mu g \text{ single stranded DNA}
\]
\[
1 \text{ OD}_{260} \text{ unit } = 40 \mu g \text{ RNA}
\]

Pure DNA = ratio \(\text{OD}_{260}:\text{OD}_{280} \geq 1.8\)

Restriction Analysis of DNA

Restriction endonucleases were obtained from New England Biolabs Ltd. DNA was digested accorded to the manufacturers instructions using the buffers supplied.
Routine digestion of plasmid DNA was carried out using 1 µg DNA, which was incubated with 1 unit of enzyme for 3 hours in a total volume of 20 µl. Yeast genomic DNA was digested with 1 unit of enzyme overnight in a total volume of 20 µl, with the addition of a further unit of enzyme in the morning for 3 hours.

**Agarose Gel Electrophoresis**

DNA was separated and visualised from gels consisting of agarose dissolved and run in 1x Tris acetate electrophoresis buffer (TAE). DNA samples were mixed with 6x loading buffer (15 % Ficoll type 400; 0.25 % orange, bromophenol blue, or xylene cyanol and bromophenol blue), loaded on the gel, and separated by passing an electrical field through the 1x TAE buffer. After electrophoresis gels were stained with 0.5 µg.ml⁻¹ ethidium bromide in 1x TAE buffer for approximately 10 minutes prior to visualisation using a UV transilluminator.

**Recovery of DNA from Agarose Gels**

Following gel electrophoresis the band containing the DNA of interest was excised using a scalpel. The DNA was then extracted and purified from the gel slice using a QIAquick gel extraction kit (Qiagen) according to the manufacturer’s instructions.

**Ligation of DNA**

Prior to ligation vector and insert DNA was quantified by visualising samples by gel electrophoresis. The phosphate groups were then removed from vector molecules by incubating 1 µg of vector DNA with 1 unit of shrimp alkaline phosphatase (Promega) for 1 hour at 37°C in a total volume of 20 µl of water supplemented with the supplied buffer. The samples were then incubated at 65°C for 30 minutes to inactivate the phosphatase.

Ligations of vector and insert DNA were generally performed at molar ratios of 1:1 and 1:3 (Dugaiczyk et al., 1975). The reactions were carried out in a total volume of 20 µl with the vector, insert, and water being incubated at 65°C for 10 minutes and then chilled on ice for 5 minutes. Following this 1 unit of T4 DNA ligase (New
England Biolabs) and its supplied buffer were added to the reactions, which were incubated overnight at 16°C.

**Denaturing Gel Electrophoresis of RNA**

RNA samples were separated on formaldehyde-denaturing gels. Gels were made from 1.5% (w/v) agarose dissolved in 1x MOPS (20mM MOPS, pH 7.0, 5mM Sodium acetate, 0.1mM EDTA) and 5% formaldehyde. Prior to loading RNA samples were denatured by the addition of 2 μl 5x MOPS, 10 μl deionised formamide, and 3.5 μl of 40% formaldehyde and heating at 65°C for 10 minutes. Samples were then chilled on ice for 5 minutes prior to the addition of 2.5 μl 6x loading dye and 1 μl 5 mg.m1⁻¹ ethidium bromide. The samples were then loaded onto the denaturing gel, run in 1x MOPS for 3 hours at 8.7 volts.cm⁻¹, and visualised on a UV transilluminator.

**2.7 Southern and Northern Blotting and Hybridisation**

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

DNA was transferred from agarose gels to a nylon membrane using a method derived from that of Southern (1975), with the addition of a depurination step to improve the transfer of high molecular weight molecules (Wahl et al., 1979).

Following the visualisation of DNA agarose gels were washed in depurination solution (0.25 M hydrochloric acid) for 10 minutes with constant agitation. The gel was then rinsed in distilled water and washed in denaturing solution (0.5 M sodium hydroxide; 1 M sodium chloride) for 30 minutes with constant agitation. After rinsing with distilled water the gel was incubated for 30 minutes in neutralising solution (0.5 M Tris-HCl, pH 7.4; 3M sodium chloride) with constant agitation. The gel was then rinsed in distilled water before being placed on a glass plate covered with Whatmann 3MM paper acting as a wick over a reservoir of 20x SSC. A nylon filter, Hybond N, and then 2 pieces of 3MM soaked in 6x SSC were then placed over the gel, followed by a stack of paper towels. A glass plate and weight were then placed on top of the towels to aid transfer, which was allowed to proceed overnight. The following day the membrane was removed, allowed to dry, and the DNA fixed by exposure to UV light in a UV cross-linker (Amersham Life Sciences) at 254 nm with a total energy of 70x10³ micro-joules.cm⁻².
Northern Transfer
Following visualisation the denaturing gel was placed on a glass plate covered with Whatmann 3MM paper acting as a wick for a reservoir of 10x SSC. A piece of nylon membrane followed by 2 pieces of 3MM paper soaked in 3x SSC were then placed over the gel, followed by a stack of paper towels. A glass plate and weight were then placed on top of the paper towels and transfer allowed to proceed overnight. The filter was then removed, air-dried, and the RNA fixed in a UV cross-linker at 254 nm with a total energy of $70 \times 10^3$ micro-joules.cm$^{-2}$.

Slot Blotting
RNA was also transferred to nylon membranes using a PR600 slot blot vacuum filtration manifold from Amersham Biosciences. Samples containing 5 μg of total RNA were diluted to a total volume of 50 μl with 1 x TE buffer pH 8.0. The RNA was then denatured by the addition of 35 μl of 20 x SSC and 20 μl of 37 % formaldehyde, heated at 65°C for 15 minutes, and stored on ice until use. To prepare the vacuum manifold for blotting 2 sheets of 3MM filter paper were placed on the apparatus followed by the nylon membrane and the apparatus was then sealed. All filters and membranes were pre-soaked in filtered 20 x SSC prior to assembly of the apparatus. After assembling the apparatus a vacuum was applied and the wells washed with 500 μl of filtered 20 x SSC. The denatured RNA was then loaded into the wells, which were washed with a further 500 μl of filtered 20 x SSC following the complete passage of the samples. After the final wash the membrane was removed from the apparatus while still under vacuum. Membranes were allowed to air-dry before the RNA was fixed in a UV cross-linker at 254 nm with a total energy of $70 \times 10^3$ μjoules.cm$^{-2}$.

Radioactive Labelling of Probes
Radio-labelled probes for filter hybridisation were prepared by the incorporation of α-$^{32}$P-dCTP using random hexamer priming (Feinberg & Vogelstein., 1983). The DNA fragment to be labelled (~25 ng) was made up to 16 μl with distilled water and denatured by boiling for 10 minutes, followed by snap cooling on ice. The DNA was then labelled by the addition of 5 μl oligo-labelling buffer (OLB; see below), 1 μl 10
mg.ml⁻¹ BSA, 1 µl Klenow and 2.5 µl α-³²P-dCTP. The labelling reaction was incubated at 37°C for 1 hour before being fractionated to remove unincorporated nucleotides. The probe was fractionated using NICK columns (Amersham Life Sciences) according to the manufacturers’ instruction. The fractionated probe was denatured by boiling for 10 minutes, snap-cooled on ice, and then hybridised with the filter.

**OLB** consisted of solutions A, B, and C in the ratio 2:3:3

**Solution A:** 100 µl solution O (1.25 M Tris-HCl, pH 8.0; 0.125 M magnesium chloride), 18 µl 2-mercapto-ethanol, 5 µl each dATP, dTTP, and dGTP.

**Solution B:** 2M HEPES, pH 6.6

**Solution C:** Random Hexadeoxynucleotides(90 OD units.ml⁻¹)

**Filter Hybridisation**

Filters were pre-hybridised for at least 1 hour in 25 ml Church-Gilbert buffer (0.5 M sodium phosphate, pH 7.4; 7 % (w/v) SDS; 1 mM EDTA, pH 8.0) at 65°C in bottles with constant rotation. The denatured radioactively labelled probe was then added and hybridised to the filter overnight at 65°C. Following hybridisation the filter was washed repeatedly with wash buffer (3x SSC; 0.1 % SDS) at 65°C until radioactive material was no longer detected in the discarded wash solution. Filters were then removed, wrapped in Saran wrap, and placed in autoradiography cassettes with X-ray film. The X-ray film was exposed at -80°C prior to developing.

**2.8 DNA Sequencing and Polymerase Chain Reaction (PCR)**

**DNA Sequencing**

Sequencing reactions were performed in a total volume of 20 µl and comprised 8 µl of termintor pre-mix (BigDye® v3.1 Cycle Sequencing Kit, Applied Biosystems), ~200 ng of template DNA, and 3.2 pmol of the relevant sequencing primer. The sequencing reaction was overlaid with ~20 µl of light mineral oil and thermal cycling performed in a Hybaid Omni-E thermal cycler. A typical sequencing reaction thermal cycling program involved an initial denaturation step at 96°C for 30 seconds followed by 30 cycles of denaturation at 96°C for 30 seconds, annealing at 50°C for 30 seconds, and extension at 60°C for 4 minutes. Following PCR the reaction products
Table 2.6 Primers Used for the Construction of Probes for Northern Blotting

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence 5' 3'</th>
<th>Target Site</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaACT1F</td>
<td>GGTAGACCAAAGACATCAAGG</td>
<td>CaACT1 +61 to +80</td>
</tr>
<tr>
<td>CaACT1R</td>
<td>GAACCACCAATCCAGACAGAG</td>
<td>CaACT1 +979 to +966</td>
</tr>
<tr>
<td>CaFRE1F</td>
<td>GATTCTTTTCTCGGTTTCTGG</td>
<td>CaFRE1 +2457 to +2477</td>
</tr>
<tr>
<td>CaFRE1R</td>
<td>GGCCATAATCAAGTGATACAAG</td>
<td>CaFRE1 +2297 to +2275</td>
</tr>
<tr>
<td>CaFRE2F</td>
<td>CAATGACTATTCGCTCAAAG</td>
<td>CaFRE2 +2218 to +2238</td>
</tr>
<tr>
<td>CaFRE2R</td>
<td>TTGCTGGGGATTGTTAGTTAC</td>
<td>CaFRE2 +2322 to +2301</td>
</tr>
<tr>
<td>CaFRE5F</td>
<td>TCCTTGATTATGGAGCAGG</td>
<td>CaFRE5 +469 to +488</td>
</tr>
<tr>
<td>CaFRE5R</td>
<td>ATAAATGCATGACCACCGGG</td>
<td>CaFRE5 +1379 to +1360</td>
</tr>
<tr>
<td>CaFRE10F</td>
<td>ATAAACGGTAAAGGTATTGCC</td>
<td>CaFRE10 +985 to +1005</td>
</tr>
<tr>
<td>CaFRE10R</td>
<td>GTTTGAGCAGGTTGTTAGGCC</td>
<td>CaFRE10 +1529 to +1508</td>
</tr>
<tr>
<td>CaFRE12F</td>
<td>GCCTACATGAAGTACAAGC</td>
<td>CaFRE12 +61 to +79</td>
</tr>
<tr>
<td>CaFRE12R</td>
<td>GGACCACGTGAATGACTG</td>
<td>CaFRE12 +607 to +590</td>
</tr>
<tr>
<td>CaSMF2F</td>
<td>CAGGTTTAGTGGTCAGTG</td>
<td>CaSMF2 +77 to +94</td>
</tr>
<tr>
<td>CaSMF2R</td>
<td>CATCTCGTGGTGCTCTTC</td>
<td>CaSMF2 +766 to +783</td>
</tr>
<tr>
<td>CaSMF12F</td>
<td>CCACAACTGGGAGTTTCTAC</td>
<td>CaSMF12 +65 to +83</td>
</tr>
<tr>
<td>CaSMF12R</td>
<td>GACCCATGATAGAAGTGGTGC</td>
<td>CaSMF12 +800 to +781</td>
</tr>
<tr>
<td>CaMRS4F</td>
<td>GAGGCATTACCTGATGATCG</td>
<td>CaMRS4 +52 to +70</td>
</tr>
<tr>
<td>CaMRS4R</td>
<td>GCTATAACCACAGCAATC</td>
<td>CaMRS4 +728 to +711</td>
</tr>
<tr>
<td>CalSU1F</td>
<td>CTCAACAATCAAGAGTTTCTT</td>
<td>CalSU1 +44 to +64</td>
</tr>
<tr>
<td>CalSU1R</td>
<td>CTTGAAACAGTTGAAGCAGC</td>
<td>CalSU1 +521 to +502</td>
</tr>
</tbody>
</table>
## Table 2.7 Primers Used for the Construction of Disruption Cassettes

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence 5' → 3'</th>
<th>Target Site</th>
<th>Features</th>
</tr>
</thead>
<tbody>
<tr>
<td>DIS2B</td>
<td>GATCAGATCTGATTTAGGAAACTCTTCTTGTTG</td>
<td>CaFRE2 -1 to -24</td>
<td>BglII site</td>
</tr>
<tr>
<td>DIS2K</td>
<td>GATCGGTACCCGCACTACTACTGGTGAAATC</td>
<td>CaFRE2 -963 to -943</td>
<td>KpnI site</td>
</tr>
<tr>
<td>DIS2P</td>
<td>GATCCTGCAGCTTGCTGAGGATTGAAGTTAC</td>
<td>CaFRE2 +2217 to +2238</td>
<td>PstI site</td>
</tr>
<tr>
<td>DIS2S</td>
<td>GATGCATGCTAGCAATTTCCGGAC</td>
<td>CaFRE2 +3211 to +3196</td>
<td>SphI site</td>
</tr>
<tr>
<td>DIS5K</td>
<td>TTTCTGTACCAATTTCTACTT</td>
<td>CaFRE5 -886 to -861</td>
<td>KpnI site</td>
</tr>
<tr>
<td>DIS5B</td>
<td>GGTTAACCCCTTGATAGATCTG</td>
<td>CaFRE5 -24 to -42</td>
<td>BglII site</td>
</tr>
<tr>
<td>DIS5P</td>
<td>GATCCTGCAGGGAATGGCTGTGTATCTTTT</td>
<td>CaFRE5 +2190 to +2210</td>
<td>PstI site</td>
</tr>
<tr>
<td>DIS5S</td>
<td>CTGCATGCAATTGAAATGATCTGATGGAC</td>
<td>CaFRE5 +3056 to +3030</td>
<td>SphI site</td>
</tr>
<tr>
<td>DIS10S</td>
<td>GGTTAAAGAGATAGCATGCAGCCATCGG</td>
<td>CaFRE10 -2873 to -2845</td>
<td>SphI site</td>
</tr>
<tr>
<td>DIS10P</td>
<td>CTGGGAGGAGGGCAGCTAGG</td>
<td>CaFRE10 -140 to -167</td>
<td>PstI site</td>
</tr>
<tr>
<td>DIS10K</td>
<td>AATATGCTGACATTTCTTGATCTGC</td>
<td>CaFRE10 +2461 to +2488</td>
<td>KpnI site</td>
</tr>
<tr>
<td>DIS10S</td>
<td>GGTTGAGCTCTCCTTAGTAGTGGTCC</td>
<td>CaFRE10 +4445 to +4420</td>
<td>SacI site</td>
</tr>
<tr>
<td>DISAS</td>
<td>GATGAGATCTGATTTAGGAAACTCTTCTTGTTG</td>
<td>CaAFT -987 to -958</td>
<td>SphI site</td>
</tr>
<tr>
<td>DISAP</td>
<td>GATCCTGCAGCAACACAATACTGCACTG</td>
<td>CaAFT -20 to -40</td>
<td>PstI site</td>
</tr>
<tr>
<td>DISAB</td>
<td>GATGAGATCTGAGCTGTTGGAATACCAAG</td>
<td>CaAFT +2383 to 2403</td>
<td>BglII site</td>
</tr>
<tr>
<td>DISAK</td>
<td>CATTATACGGTACCAGAAGAAG</td>
<td>CaAFT +3356 to +3419</td>
<td>KpnI site</td>
</tr>
</tbody>
</table>
Table 2.8 Primers Used for the Creation and Confirmation of Constructs

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence 5' → 3'</th>
<th>Target Site</th>
<th>Features</th>
</tr>
</thead>
<tbody>
<tr>
<td>FRE10FORF</td>
<td>GATCCTCGAGCAAACGTCTATTAACCAGTAC</td>
<td><em>CaFRE10</em> -969 to -950</td>
<td>XhoI site</td>
</tr>
<tr>
<td>FRE10RORF</td>
<td>GATCCTCGAGGGAACAGTTCACATAAGAAGAG</td>
<td><em>CaFRE10</em> +2555 to +2534</td>
<td>XhoI site</td>
</tr>
<tr>
<td>FRE10Fprom</td>
<td>GATCGCATGGCCGCTTATAACCAGTACATGC</td>
<td><em>CaFRE10</em> -965 to -946</td>
<td>SphI site</td>
</tr>
<tr>
<td>FRE10Rprom</td>
<td>GATCCCCGAGGATAGCTACCATATTGTATCTATATTC</td>
<td><em>CaFRE10</em> +12 to -15</td>
<td>XmaI site</td>
</tr>
<tr>
<td>RP10R</td>
<td>CGTATTCACTTAATCCCACAC</td>
<td><em>RPS10</em> +941 to +920</td>
<td>-</td>
</tr>
<tr>
<td>LacZF</td>
<td>CGAGGCTTCAATTCTGAAC</td>
<td><em>LacZ</em> +2081 to 2101</td>
<td>-</td>
</tr>
<tr>
<td>LacZR</td>
<td>CATGTAGCATCACGATTCTTGC</td>
<td><em>LacZ</em> +2951 to +2930</td>
<td>-</td>
</tr>
</tbody>
</table>
were purified using the Dye-Ex v 2.0 kit (Qiagen) following the manufacturers instructions. The purified reaction was then dried under vacuum and submitted to the University of Leicester’s Protein and Nucleic Acid Chemistry Laboratory (PNACL) for analysis using an ABI model 373A sequencer. Synthetic oligonucleotide primers used for sequencing and PCR were also supplied by PNACL and are shown in Figures 2.6 to 2.8.

**Polymerase Chain Reaction**

DNA fragments were amplified by PCR performed using a ThermoHybaid Omni-E thermal cycler. A typical 50 µl PCR reactions comprised 100 ng template DNA, 50 pmole of each primer, 5 units of Taq polymerase, and 4.5 µl of 11.1x PCR buffer (see below). The remainder of the reaction volume was made up with distilled water and overlaid with mineral oil to prevent evaporation. A typical reaction profile consisted of 40 cycles with a denaturation step (95°C for 1 minute), an annealing step (50-60°C for 1 minute), and an extension step at 72°C (1 minute per kb DNA amplified).

**11.1x PCR buffer**: 450 mM Tris-HCl, pH 8.8; 110 mM ammonium sulphate; 45 mM magnesium chloride; 67 mM ß-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.

**2.9 Measurement of Ferric/Cupric Reductase Activity and Iron Uptake**

**Quantitative Ferric Reductase Assay**

Starter cultures of the appropriate strains were grown in 10 ml YPD for approximately 8 hours and were used to inoculate 10 ml of MD media at a density of 3.6 x 10⁴ cells.ml⁻¹. After overnight growth cultures were harvested, washed three times with 5ml of distilled water, and used to inoculate 10 ml of the appropriate media at a cell density of 2x10⁵ cells.ml⁻¹. Cultures were then grown for a further 5 hours before being harvested, washed twice with 5 ml of ice-cold distilled water, and resuspended in 1ml of ice-cold assay buffer (50 mM sodium citrate, pH 6.5; 5 % (v/v) glucose). To assay for ferric reductase activity 50 µl of cells was transferred to a fresh tube and
incubated at 30°C for 10 minutes. The assay was then started by the addition of 800 
μl reductase buffer (50 mM sodium citrate, pH 6.5; 5 % (v/v) glucose; 1 mM ferric 
chloride; 1 mM BPS) to the samples, including a control reaction containing no cells. 
The samples were then incubated at 30°C for 5 minutes and the cells harvested by 
centrifugation at 13000 rpm for 2 minutes. The supernatant was then transferred to a 
cuvette and the absorbance at 520 nm measured.

The amount of iron reduced by cells was determined by constructing a standard curve 
to calculate the relationship between OD_{520} and the concentration of Fe^{2+} ions. The 
standard curve was determined by setting up a number of reactions containing 
reductase buffer and a known concentration of ferrous chloride. The reactions were 
processed as above and the results used to draw a standard curve.

**Measurement of Iron Uptake**

The rate of iron uptake in mutants was determined by measuring $^{55}$FeCl$_3$ accumulation 
within the cell using a method adapted from that previously reported (Dancis et al., 
1990). Cells were grown overnight in YPD media and harvested, washed, and 
inoculated into 10 ml of fresh YPD at a final density of 1 x 10^6 cell.ml$^{-1}$. Cultures 
were then grown to a final density of 1 x 10^7 cell.ml$^{-1}$ and harvested, washed twice 
with 20ml of cold water, and suspended in 1 ml of ice cold assay buffer (5 % glucose, 
10 mM sodium citrate pH 6.5). Cells were then diluted in 1ml of cold assay buffer to 
a final density of 1x10^7 cells.ml$^{-1}$ and incubated at 30°C for 15 minutes. Following 
this incubation $^{55}$FeCl$_3$ was added to a final concentration of 1 μM and cells incubated 
at 30°C for 30 minutes in a shaking waterbath. The cells were then harvested onto 
glass filters using a vacuum manifold and washed twice with 10 ml ice cold 0.25 M 
EDTA (pH 6.5) and twice with 10 ml ice cold water. Filters were then air dried and 
placed in scintillation vials with 5 ml of scintillation fluid. The filters were allowed to 
re-hydrate for 30 minutes and the level of $^{55}$FeCl$_3$ incorporated in the cells determined 
by counting β emissions in a scintillation counter.

**2.10 Quantitative β-Galactosidase Assays**

Cultures were initially grown in 5 ml of media under the desired conditions and then 
harvested, washed twice with 5 ml ice-cold distilled water, and resuspended in 5 ml
ice-cold Z-buffer (60 mM Na$_2$PO$_4$; 40 mM Na$_2$HPO$_4$; 10 mM KCl, 50 mM β-mercaptoethanol). The cell density was then determined by measuring the absorbance at 600nm. The cells were then permeabilised by treating 800 μl of cells with 60 μl chloroform and 40 μl 0.1 % (w/v) SDS and vortexing the mixture for 15 seconds. The permeabilised cells were then equilibrated at 30°C for 15 minutes prior to the addition of 200 μl ONPG (4 mg.ml$^{-1}$). The cells were then incubated at 30°C until a pale yellow colour developed and the reaction was stopped by the addition of 400 μl of 1M sodium carbonate. Cells were then harvested by centrifugation at 13000 rpm for 5 minutes, the supernatant transferred to a cuvette, and the absorbance at 420 and 550 nm measured. The activity of the sample was then calculated using the equation shown below.

\[
\text{Activity} = \frac{\text{OD}_{420} - (\text{assay volume} \times \text{OD}_{550})}{\text{OD}_{600} \times \text{volume assayed} \times \text{time}}
\]
Chapter 3
Identification and Sequence Analysis of Putative Ferric Reductases in the *C. albicans* Genome Sequence

3.1 Introduction

In our laboratory we have previously shown that *C. albicans* possesses a cell surface reductase activity that increases in iron or copper limiting conditions (Morrissey *et al.*, 1996). We have also identified two putative ferric reductases, *CaFRE1* and *CaFRE2*, which are able to complement the defects in cell surface reductase activity associated with *ScfrelAfre2A* mutants (Hammacott *et al.*, 1999). A further ferric reductase encoded by *CaFRE10* has subsequently been identified that is also able to complement the mutant phenotypes of a *CafrelAfre2A* mutant when under the control of an inducible promoter (Knight *et al.*, 2002). One of the main aims of this study was to attempt to identify and characterise further ferric reductases in *C. albicans*. A bioinformatics based approach was initially used to take advantage of the resources available since the completion of the *C. albicans* genome sequencing project. This analysis was repeated periodically as the assembly and annotation of the *C. albicans* genome sequence has improved considerably during the course of this study. The putative protein products identified were also analysed using a variety of bioinformatics software packages to gain information regarding their function within the cell. Once again this was repeated periodically to take advantages in improvements in bioinformatics software.

*C. albicans* Genome Sequence

The identification and analysis of genes in *C. albicans* has been greatly aided by the recent completion of the genome sequencing project and the publication of the diploid genome assembly (Braun *et al.*, 2004; Jones *et al.*, 2004). The assembly of the *C. albicans* genome sequence has been complicated by the high degree of heterozygosity between alleles on homologous chromosomes. When *C. albicans* alleles are compared polymorphisms are present at an average frequency of every 237 bases (Jones *et al.*, 2004) compared to every 1000bp in humans (Risch & Merikangas, 1996). The majority of these polymorphisms are base substitutions and it has been suggested that these mutations may contribute to genetic diversity in *C. albicans*.
(Jones et al., 2004). The heterozygosity observed in *C. albicans* may be due to its asexual nature, which means that gene exchange is essentially absent increasing the level of heterozygosity between alleles. When organisms reproduce sexually the level of heterozygosity is limited by recombination and segregation (Welch & Meselson, 2000). In contrast an elevated level of heterozygosity has been demonstrated in bdelloid rotifers, which lack a sexual cycle (Welch & Meselson, 2000).

The assembly of the *C. albicans* genome sequence was also complicated by the presence of a number of gene families that appear to have expanded. Therefore a complex diploid genome assembly was required to compensate for these characteristics of the genome. The most comprehensive analysis of the *C. albicans* genome sequence has identified a total of 6354 ORFs within the 14.88 Mb genome (Braun et al., 2004; Jones et al., 2004). However, it is recognised that this is an initial estimate as there are still gaps present in the genome assembly and the heterogeneity of the genome means that some genes will undoubtedly prove to be duplicates.

### 3.2 Identification of Putative Ferric Reductases in the *C. albicans* Genome Sequence

Analysis of the *C. albicans* genome sequence reveals that it contains a total of 17 ORFs encoding putative ferric reductases with significant homology to ScFre1p and CaFre1p (Table 3.1). As shown in Figure 3.1 the eukaryotic ferric reductases are multiple membrane spanning proteins containing a number of highly conserved motifs that have been implicated in NADPH, FAD, and haem binding (Finegold et al., 1998; Segal & Abo, 1993). The putative *C. albicans* proteins were therefore analysed for the presence of these features. Sequence alignments generated using ClustalW (Figure 3.2) showed that the *C. albicans* proteins contain conserved histidine residues spaced 13 or 14 residues apart. This arrangement has been implicated in haem binding in *S. cerevisiae* (Finegold et al., 1998). The proteins also contain a conserved NADPH binding motif based around a core with the consensus sequence HPFT (Segal & Abo, 1993). In addition all of the proteins contain a glycine rich domain and most also have a glycine-cysteine couplet that are both implicated in FAD binding in other proteins (Segal & Abo, 1993).
Table 3.1. Putative ferric reductase encoding ORFs within the *C. albicans* genome sequence. Genes encoding putative ferric reductases were identified within the *C. albicans* genome sequence through their homology to ScFre1p, CaFre1p, and CaFre10p using Blast searches (Altschul et al., 1997). The putative ORFs were also examined for the presence of NADPH, FAD, and haem binding domains that are characteristic features of eukaryotic ferric reductases (Finegold et al., 1996). The ORFs all encode proteins with multiple transmembrane domains and many have N-terminal signal sequences as determined by the software package Phobius (Käll et al., 2004). All of the proteins appear to localise to the plasma membrane (PM) as determined using WoLF PSORT (http://wolfpsort.seq.cbrc.jp/).
<table>
<thead>
<tr>
<th>ORF</th>
<th>Gene Name(s)</th>
<th>Size (amino acids)</th>
<th>Transmembrane Domains</th>
<th>Signal Peptide</th>
<th>FAD binding domain</th>
<th>NADPH binding domain</th>
<th>Bis-heme binding motif</th>
<th>N-terminus Orientation (In/Out)</th>
<th>Localisation</th>
</tr>
</thead>
<tbody>
<tr>
<td>orf19.1263</td>
<td>FRE1/CFL1</td>
<td>760</td>
<td>7</td>
<td>1-18</td>
<td>HPFTFTTTES</td>
<td>GPY, CGHP</td>
<td>Y</td>
<td>O</td>
<td>PM</td>
</tr>
<tr>
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<td>Y</td>
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<td>1-18</td>
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<td>GPY, CGHP</td>
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<td>-</td>
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<td>GPY, CGHP</td>
<td>Y</td>
<td>I</td>
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<td>FRE5/CFL4</td>
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<td>HPFTFFVES</td>
<td>GPY, CGHP</td>
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<td>GPY, CGHP</td>
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<td>1-18</td>
<td>HPFTIVDS</td>
<td>GPYS, CGAP</td>
<td>Y</td>
<td>O</td>
<td>PM</td>
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<td>PM</td>
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<td>-</td>
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<td>Y</td>
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<td>Y</td>
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Figure 3.1 Comparison of features associated with eukaryotic ferric reductases. The protein sequences of ferric reductases from *C. albicans*, *Arabidopsis thaliana*, *S. cerevisiae*, *Aspergillus fumigatus*, and *S. pombe* were aligned using Clustal W (Chenna et al., 1993) and examined for the presence of several common features. The protein sequences were also analysed using Phobius for the presence of transmembrane domains and N-terminal signal (Käll et al., 2004). The cartoon shows a representation of the results obtained, with all of the proteins containing multiple transmembrane domains and motifs that are associated with the binding of FAD, NADPH, and haem (Finegold et al., 1999; Segal & Abo, 1993).
Figure 3.2. Conserved motifs in the putative \textit{C. albicans} ferric reductases. The amino acid sequences of the putative \textit{C. albicans} ferric reductases were aligned using ClustalW (Channa \textit{et al.}, 2003) located at the European Bioinformatics Institute (www.ebi.ac.uk) and displayed using Boxshade 3.2 (www.ch.embnet.org). As shown the proteins contain conserved motifs that have previously been characterised as FAD, NADPH, and haem binding sites (Finegold \textit{et al.}, 1996; Segal & Abo, 1993). Residues that are identical to the consensus sequence are shaded in black, and those that are similar are shaded in grey. Residues that do not agree with the consensus are not shaded and gaps in the alignment are displayed as dashes.
Distribution of Ferric Reductases in C. albicans and Other Fungi

As described earlier the C. albicans genome sequence contains more putative ferric reductases than either S. cerevisiae or S. pombe, which contain 9 and 2 respectively (Georgatsou et al., 1999; Roman et al., 1993). Interestingly, this expansion of iron uptake genes extends to the iron transporters as C. albicans has 4 putative iron permeases and at least 4, and possibly 6, multicopper oxidases (Knight et al., 2002; Ramanan & Wang, 2002). In comparison S. cerevisiae and S. pombe each contain 2 oxidases and permeases with one complex functioning at the cell surface and the other at the vacuole (Stearman et al., 1996; Urbanowski et al., 1999). The publication of the C. albicans diploid genome sequence also noted this expansion and has shown that a number of other gene families including lipases, aspartyl proteinases, and agglutinins are also much larger than in S. cerevisiae (Braun et al., 2004). It has been suggested that these expanded gene families may be an adaptation to the wide variety of environmental conditions encountered within the human body and allow it to colonise a number of niches within the host (Braun et al., 2004).

The genome sequences of other fungi were also examined for the presence of putative ferric reductases. The results are shown in Table 3.2 and the evolutionary relationship of the various species is demonstrated in Figure 3.3. This analysis showed that the number of putative ferric reductases varies widely, with C. albicans having the most. However, this may be due to the fact that many of the genome sequences are in the early stages of assembly and few experimental studies have been performed on ferric reductases in most of these species. The number of fungi whose genomes have been sequenced and made publicly available is also relatively small. Another problem encountered when examining the genomes of different fungi is that the number of ferric reductase encoding genes can vary when more than one strain has been sequenced as with A. fumigatus and C. neoformans. In A. fumigatus the number of ferric reductases varies between 1 and 9 and while this could be due to strain variation it could also be a result of differences in the approaches taken during the sequencing, assembly, and annotation of the genomes by independent groups. This is a distinct possibility as the number of potential ferric reductases in the C. albicans genome has increased as the different assemblies of the genome sequence data have been released.
<table>
<thead>
<tr>
<th>Species</th>
<th>No. Putative Ferric Reductases</th>
</tr>
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<tbody>
<tr>
<td>Saccharomyces cerevisiae</td>
<td>9</td>
</tr>
<tr>
<td>Ashbya gossypii</td>
<td>4</td>
</tr>
<tr>
<td>Kluyveromyces lactis</td>
<td>7</td>
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<td>Debaryomyces hansenii</td>
<td>7</td>
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<td>Candida albicans</td>
<td>19</td>
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<tr>
<td>Candida glabrata</td>
<td>3</td>
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<tr>
<td>Magnaporthe grisea</td>
<td>9</td>
</tr>
<tr>
<td>Neurospora crassa</td>
<td>10</td>
</tr>
<tr>
<td>Aspergillus fumigatus (1)</td>
<td>18</td>
</tr>
<tr>
<td>Aspergillus fumigatus (2)</td>
<td>1</td>
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<tr>
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<td>1</td>
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<td>6</td>
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<tr>
<td>Ustilago maydis</td>
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</tr>
</tbody>
</table>

Table 3.2. Distribution of genes encoding putative ferric reductases among fungi.  
Putative ferric reductase genes were identified using Blast searches against CaFre1p and utilising the taxonomy report feature (Altschul et al., 1997). The proteins encoded by the putative genes were then checked for the presence of features that are consistent with their role as ferric reductases.
Figure 3.3 Evolutionary relationship of fungi. The phylogenetic tree demonstrates the evolutionary relationship between a number of fungal species as determined from 18S rDNA sequences. Diagram obtained from the Pathogenic Fungal Database (//timm.main.teikyo-u.ac.jp/pfdb/cover/taxonomic_list_eng.html).
Analysis of C. albicans Putative Ferric Reductase Protein Sequences

After identifying the putative C. albicans ferric reductases their protein sequences were analysed using a variety of bioinformatics tools to provide more evidence for their role as ferric reductases, and to help determine a role for the individual proteins. Many of the results obtained from this analysis are summarised in Table 3.1. The protein sequences were first aligned and analysed using the program MatGat to determine the level of sequence identity and similarity between the putative proteins and the results are shown in Table 3.3 (Campanella et al., 2003). The proteins were also aligned using ClustalW and displayed using the program Boxshade to visualise any similarities between the proteins (Chenna et al., 2003).

This analysis suggests that the C. albicans ferric reductases can be roughly divided into 4 groups depending on their similarity to other ferric reductases in C. albicans and S. cerevisiae. The first group contains CaFre1-1lp and CaFre31p, which share at least 30% identity and 47% similarity at the amino acid level. Of particular note are CaFre1p and CaFre2p that share 75.9% identity and 86.3% similarity. This high degree of homology and the observation that the genes are adjacent in the genome suggests that they may be the result of a duplication event. These proteins are also of note as previous work in our laboratory identified CaFRE1 and CaFRE2 in a screen for genes that could complement the phenotypes associated with a S. cerevisiae fre1Δfre2Δ mutant (Hammacott et al., 2000). Complementation with these genes restored wild-type levels of ferric reductase activity in low iron conditions, although Northern blot analysis demonstrated that the genes were not regulated in an iron responsive fashion in S. cerevisiae (Hamamcott et al., 2001; Hammacott, PhD thesis 2000).

When compared with the S. cerevisiae reductases the putative reductases in this first group are all most closely related to ScFre1-6p. However, the level of similarity between the putative reductases and ScFre1-6p is relatively consistent so it is difficult to classify any of the C. albicans proteins as being direct homologues of individual S. cerevisiae proteins. The similarity within this group of C. albicans proteins can also
### Table 3.3. Analysis of homology among putative ferric reductases in \textit{C. albicans}.

The putative ferric reductase protein sequences from \textit{C. albicans} were aligned and the percentage identity and similarity calculated using MatGat (Campanella \textit{et al.}, 2003).
be seen in the NADPH, FAD, and haem binding motifs, which are particularly well conserved. With the exception of Frp1-2p they are also the only putative *C. albicans* reductases to contain a cysteine-glycine couplet implicated in NADPH binding (Segal & Abo, 1993).

Two of the other putative ferric reductases, CaFrp1p and CaFrp2p, have distinct differences to the other putative reductases and comprise a second group. While they share 36% identity and 62.2% similar they only share low levels of homology to the other putative reductases, suggesting that the two proteins have similar functions within the cell. In contrast to the other reductases these two proteins are also more closely related to ferric reductases within *S. pombe* than *S. cerevisiae*.

The third possible group of putative ferric reductases includes the protein products of *CaFRE13* and *CaFRE14*. They are the only two that share significant levels of homology with each other (23.5% identity and 41.2% similarity). Comparisons with *S. cerevisiae* reveal that these proteins are most closely related to the relatively uncharacterised ferric reductases Ygl160p and Ylr047p. In common with Ygl160p and Ylr047p the NADPH, FAD, and haem binding motifs are absent or poorly conserved compared to the other reductases.

The remaining ferric reductase in *C. albicans* is encoded by *CaFRE12*, whose protein product displays relatively low levels of homology to all of the other putative proteins. The NADPH, FAD, and haem binding domains are also relatively poorly conserved. Comparisons with *S. cerevisiae* show that the *CaFRE12* protein product is most similar to ScFre7p (31.6% identity and 55.6% similarity). In comparison the other reductases in *C. albicans* share less than 20% identity and 40% similarity with ScFre7p. Compared to the other proteins ScFre7p and CaFre12p are also relatively short and lack an N-terminal signal sequences and cysteine-glycine couplet. This suggests that the *CaFRE12* may encode a functional homologue of ScFre7.

As expected, analysis of the putative ferric reductases protein sequences reveals that they are multiple membrane spanning proteins with between 5 and 8 transmembrane domains as predicted using the software package Proteus (Käll *et al.*, 2004). In common with *S. cerevisiae* the majority of the *C. albicans* ferric reductases contain
cleavable N-terminal signal peptides when analysed using Phobius and SignalP 3.0 (Bendtson et al., 2004a; Käll et al., 2004). The proteins were also analysed for transmembrane domains using other software packages including PSORTII, TMHMM, TMMOD, and DAS. However, these software packages gave inconsistent results due to the difficulties in discriminating between the transmembrane domains and N-terminal signal sequences in the proteins. This problem has been recognised previously (Ikeda et al., 2001) and Phobius is reported to perform much better because it combines programs for the recognition of transmembrane domain and signal peptides (Käll et al., 2004).

Analysis of the positions of the transmembrane domains in comparison to those of the conserved motifs reveals that the haem binding motifs are located within putative transmembrane domains. In contrast the NADPH and FAD domains are located in the C-terminal tail of all of the putative ferric reductases except CaFre14p where they are located before the final transmembrane domain, as shown in Figure 3.4. When this observation is combined with the predictions for membrane topology it provides some potentially useful insights. The topology predictions using Phobius suggest that the N-terminal regions of all of the proteins except CaFre4p and CaFrp1p are located extracellularly. Therefore, the C-terminal tail of all of the proteins except CaFre11p and CaFre12p are located intracellularly (Figure 3.4). This may provide some insights into the function of the reductases as in CaFre11p and CaFre12p the NADPH and FAD binding domains would be on the wrong side of the membrane if these proteins were involved in the extracellular reduction of iron. These reductases could therefore play a role in the intracellular trafficking of iron because their position in the membranes of cellular organelles would place the FAD and NADPH binding domains in the cytoplasm. This would allow the reduction of iron within organelles such as the vacuole for its export via an oxidase/permease transporter similar to the ScFth1p/ScFet5p complex (Urbanowski & Piper, 1999). In S. cerevisiae localisation experiments suggest that ScFre5p and ScFre6p are involved in the reduction of iron within the mitochondria (Sickmann et al., 2003) and vacuole (Huh et al., 2003) respectively. However, when analysed in the same fashion as the C. albicans proteins,
Figure 3.4 Predicted Membrane Topology of Putative Ferric Reductases. The sequences of the putative ferric reductases were analysed using Phobius (Kall et al., 2004) to predict the presence of transmembrane domains, and the topology of the N-terminus. The majority of the ferric reductases have a topology similar to CaFre1p, with the C-terminal tail being located intracellularly. The exceptions are CaFre11p, CaFre14p, and CaFre12p. In the majority of the proteins the binding domains for NADPH (•), FAD (•), and haem (•) are located in a similar position to CaFre1p.
the C-terminal tail of ScFre1-7p is always located intracellularly. To determine whether the topology predictions are accurate and relevant will therefore require experimental analysis of the proteins in question.

The protein sequences of the putative reductases were also analysed using WoLF PSORT (wolfsort.seq.cbrc.jp/) and pTARGET (Guda & Subramanium, 2005) in an attempt to gain information regarding their potential localisation within the cell. These programs predict that all of the putative ferric reductases localise to the plasma membrane (Table 3.1). When the *S. cerevisiae* ferric reductase protein sequences are analysed in a similar fashion they are also predicted to localise to the plasma membrane, even though experimental evidence demonstrates that Fre5p and Fre6p are located intracellularly. One explanation for this discrepancy may be that the proteins contain multiple membrane spanning domains, and often N-terminal signal peptides, and are therefore automatically classified as plasma membrane proteins.

**Analysis of the Promoter Sequences of Putative Ferric Reductase Encoding Genes**

In *S. cerevisiae* the expression pattern of the reductases provides some clues regarding their function. While *ScFRE1* is regulated in response to iron and copper starvation *ScFRE2-6* are only regulated by iron and *ScFRE7* by copper. The metal responsive regulation of these genes is mediated by the transcription factors Aft1p, Aft2p, and Mac1p (Blaiseau *et al.*, 2001; Georgatsou *et al.*, 1999; Martins *et al.*, 1998;) and the DNA recognition sequences in the promoters of their target genes have been determined (Blaiseau *et al.*, 2001; Labbe *et al.*, 1997; Yamaguchi-Iwai *et al.*, 1996). Previous work in our laboratory has identified a ScMac1p homologue in *C. albicans* that is required for the copper responsive regulation of the high affinity copper transporter *CaCTR1* (Marvin *et al.*, 2003, Marvin *et al.*, 2004). The *CaCTR1* promoter contains three motifs that are identical to copper responsive elements, CuREs, in the promoters of ScMac1p target genes, which suggests that the Mac1p recognition sequences are highly conserved in the two organisms. Further evidence for the function of these putative CuREs comes from the observation that *CaCTR1* is regulated in a copper responsive manner in *S. cerevisiae* (Marvin *et al.*, 2003).
The promoters of the putative ferric reductases were first searched for the presence of CuREs within the first 1000bp. The promoters of the putative reductases CaFRE3 (-530 and -801 relative to the ATG), CaFRE6 (-666), CaFRE10 (-427), and CaFRE12 (-132 and 176) contain at least one direct or inverted repeat of the CuRE sequence TTTGCTC within their promoters. However, in *S. cerevisiae* at least two CuREs are thought to be required for the efficient transcription of Mac1p target genes (Joshi et al., 1999; Serpe et al., 1999). This suggests that CaFRE3 and CaFRE12 are more likely to be direct targets for Mac1p mediated transcriptional regulation. The potential regulation of CaFRE12 by Mac1p in a copper responsive manner provides further support for its role as a ScFre7p homologue.

The promoters of the putative ferric reductase encoding genes were also examined for the presence of putative Aftlp and Aft2p binding sites. In *S. cerevisiae* Aftlp is thought to recognise and bind most strongly to the consensus sequence (T/C)(G/A)CACCC(G/A) within the promoters of its target genes (Yamaguchi-Iwai et al., 1996). The consensus binding sequence for Aft2p is still unclear but it has been demonstrated that it binds preferentially to the shorter (G/A)CACCC sequence (Courel et al., 2005). However, sequence analysis of a number of putative Aft1/2p target genes identified through micro-array studies suggests that they are able to bind to a number of alternative sequences that are based around an essential CACCC core (Courel et al., 2005). The relative flexibility of the Aft1/2p recognition sites was also noted in another study that compared data from the binding of transcription factors to their target genes in *S. cerevisiae* and other closely related yeast species (Harbison et al., 2004).

Due to the possibility of different Aft1p and Aft2p binding motifs the promoters of the putative ferric reductases in *C. albicans* were examined for the presence of all of the currently proposed recognition sequences. Only the promoter of CaFRE4 (-790 from the ATG) contains a perfectly conserved inverted copy of the sequence (T/G)(G/A)CACCC(G/A). In contrast, the shorter (G/A)CACCC motif is present in the promoters of CaFRE2 (-767), CaFRE3 (-355), CaFRE4 (-789), and CaFRP1 (-518). The core CACCC motif is present in CaFRE2 (-794), CaFRE3 (-354), CaFRE5 (-142), CaFRE7 (-238), CaFRE8 (-127), CaFRP1 (-518 and -608), and CaFRE12 (-429). In conclusion, there are a number of potential Aft1/2p binding sites within the
promoters of these genes, although unlike Mac1p the issue is complicated by the possibility of multiple regulatory sequences for the binding of Aft1/2p.

### 3.3 Identification of Aft1p and Aft2p Homologues in the C. albicans Genome

As there are potential Aft1p and Aft2p binding sequences within the promoters of the majority of the putative ferric reductases the *C. albicans* genome database was examined for the presence of homologues of these iron responsive transcription factors. A Blast search of the *C. albicans* genome identified one ORF, *orf19.2272*, with a predicted protein product that shares significant levels of homology to Aft1p and Aft2p. The *orf19.2272* protein product shows 17.2 % identity and 34.5 % similarity to Aft1p and 15.6 % identity and 28.1 % similarity to Aft2p. While this level of homology is quite low it must be noted that in *S. cerevisiae* the paralogous ScAft transcription factors themselves are only 26.2 % identical and 37.7 % similar. When aligned using ClustalW the main areas of homology between the three proteins are located in the N-terminal region, which has previously been observed when Aft1p and Aft2p were compared (Blaiseau *et al.*, 2001).

To provide further evidence for the identification of *orf19.2272* as a homologue of Aft1/2p their protein sequences were analysed using a range of bioinformatics software and the results are summarised in Figure 3.5. Consistent with its role as a potential transcription factor the putative CaAftp does not contain any putative transmembrane domains or N-terminal signal peptides as determined using Phobius and PSORTII. The analysis using PSORTII also suggests that the putative CaAftp localises to the nucleus and contains a number of potential nuclear localisation signals within the N-terminal region. This prediction was confirmed using PredictNLS (www.cubic.bioc.columbia.edu) that predicts the presence of an NLS located at residues 257-273 with the sequence KRKNMKANTMKSKKLK (Cokol *et al.*, 2000). A similar situation has been noted in Aft1p where experimental analysis has confirmed computer predictions that it contains two NLS sequences that are each independently able to facilitate its import into the nucleus in low iron conditions (Ueta *et al.*, 2003).
Fig 3.5 Comparison of ScAftlp, ScAft2p, and CaAftp protein features. The Aft2p and CaAftp were analysed for the presence of features previously identified in Aft1p (Ueta et al., 2003; Yamaguchi-Iwai et al., 1994; Yamaguchi-Iwai et al., 2002). These include a glutamine rich region, histidine rich regions, nuclear localisation (NLS) and export sequences (NES), and four cysteine residues. The cartoon shows the results of this analysis, with each of the proteins being drawn to scale.
In addition to the NLS sequences ScAftlp contains a nuclear export sequence (NES) that has been characterised experimentally (Yamaguchi-Iwai et al., 2002). The putative CaAftlp does not contain a conserved sequence similar to the NES in ScAftlp when the two proteins are aligned. When analysed using the software package NetNES (www.cbs.dtu.dk/), which examines proteins for sequences that conform to those associated with a wide variety of NES, the putative CaAftp also appears to lack a NES sequence (la Cour et al., 2004). However, the same software package fails to identify the experimentally characterised NES in ScAftlp. While Aft2p is predicted to localise to the nucleus it does not contain any putative NLS or NES sequences. However, its localisation has not been tested experimentally, although Aft2p has been identified in the S. cerevisiae mitochondrial proteome (Sickmann et al., 2003). This raises some interesting questions as it is thought that the signal for the activation of transcription in response to low iron conditions originates from the mitochondria (Chen et al., 2004; Rutherford et al., 2005).

The putative CaAftp was also examined for features that are present in ScAftlp such as histidine rich regions, a C-terminal glutamine rich region, and four cysteine residues within a N-terminal basic region (Yamaguchi-Iwai et al., 1995). While Aft2p contains the conserved cysteine residues within a basic region the glutamine and histidine rich regions are absent (Blaiseau et al., 2001). To help determine whether any particular residues were abnormally distributed in the putative CaAftp the predicted amino acid sequence was inspected visually and analysed using SAPS (Statistical Analysis of Protein Sequences), which examines multiple aspects of protein composition (Brendel et al., 1992). The putative CaAftp contains approximately 2 % histidine residues, within the normal range for yeast proteins, which are distributed evenly throughout the protein. In contrast there are an abnormally high proportion of glutamine residues, 9.1%, which are mainly concentrated in the central portion of the protein. This region spanning residues 300-656 has a glutamine content of 17.9% with three clusters containing particularly high glutamine content at residues 300-387 (22.7 %), 465-539 (36 %), and 624-656 (39.4 %). Therefore, while the putative CaAftp encodes a protein with a glutamine rich region similar to Aft1p it is located more centrally.
**Distribution of Fungal Aftp Homologues**

Currently an iron responsive transcriptional activator has only been identified in *S. cerevisiae* so in addition to *C. albicans* the genomes sequences of other fungi were examined to determine the distribution of Aft1/2p homologues. Blast searches of the currently available fungal genomes using the Aft1p and Aft2p protein sequences reveals the presence of potential homologues in *Ashbya gossypii*, *C. albicans*, *Candida glabrata*, *Debaryomyces hansenii*, and *Kluyveromyces lactis*. These proteins also all share a number of the previously discussed features that are in common with Aft1p and Aft2p.

### 3.4 Discussion

The completion of the *C. albicans* genome sequence has provided a powerful tool for the analysis of genes in this pathogenic fungus. The genome sequence contains a total of 17 complete ORFs encoding putative ferric reductases with significant homology to experimentally characterised proteins in *S. cerevisiae* and *C. albicans*. All of them have multiple membrane spanning regions and are predicted to be present in the plasma membrane. When compared to *S. cerevisiae* there are twice as many ferric reductases in *C. albicans* and this may reflect the need to adapt to numerous complex environments encountered within the human host. The genomes of a number of other fungi were also examined and they all contain at least one ferric reductase, although the number present varies greatly.

The analysis of the putative ferric reductases using a variety of bioinformatics tools has provided support for the role of these proteins in the reduction of iron. All of the proteins contain motifs implicated in the binding of NADPH, FAD and haem that are indicative of all of the eukaryotic ferric reductases currently examined. Comparisons between the proteins and their *S. cerevisiae* counterparts suggest that they can be roughly divided into four groups with the majority being most similar to ScFre1-6p. The *C. albicans* proteins encoded by *CaFRE13* and *CaFRE14* also appear to be potential homologues of the poorly characterised *S. cerevisiae* proteins Ylr047p and Ygl160p. A homologue of the copper responsive ScFre7p encoded by *CaFRE12* also appears to be present. Interestingly the final two reductases in *C. albicans*, CaFrp1-2p, appear to most similar to the reductases that have been encountered in *S. pombe*. This
is extremely interesting because while SpFrp2p is uncharacterised SpFrp1p is thought to be a direct homologue of ScFre1p, with identical functions within the cell (Roman et al., 1993).

Analysis of the promoters of the putative ferric reductases has provided some clues to the function of the putative reductases. The promoters of CaFRE3, CaFRE6, CaFRE10, and CaFRE12 contain sequences that are identical to the ScMac1p binding sites in the promoters of its target genes (Labbe et al., 1997). In S. cerevisiae the binding of ScMac1p to CuREs is thought to be required for significant levels of transcriptional activation to occur (Joshi et al., 1999; Serpe et al., 1999). In C. albicans only the promoters of CaFRE3 and CaFRE12 contain more than one CuRE suggesting they are the only ferric reductases that are truly regulated by CaMac1p in a copper responsive fashion. The presence of Mac1p binding sites in the promoter of CaFRE12 provides further support for its role as a homologue of ScFre7p and the conservation of the CuREs between the two species. Work is currently underway in our laboratory to determine whether or not the CuREs are functional in these genes. Reporter gene constructs containing the promoters of CaCTR1 and CaFRE12 are being analysed to determine the affect of mutations in the CuREs to determine whether this leads to a loss of copper responsive regulation.

Analysis of the putative ferric reductase promoters for putative Aft1p and Aft2p binding sites proved more difficult as the binding sites for these transcription factors are much more poorly defined. As only one binding site is required for Aft1p or Aft2p mediated transcriptions it is also much more difficult to determine if any observed sites are functional or artefacts. Only the promoter of CaFre4p contains the full version of the previously proposed ScAft1p binding site but many others contain the shorter version that has been proposed for ScAft2p. However, the alternative Aft2p binding sequence (G/C)CACCC is quite short and the probability of this sequence occurring randomly is therefore relatively high. In conclusion while the promoters of many of the putative ferric reductases contain a number of putative binding sites for ScAft1/2p like transcription factors it is impossible to determine whether they are functional simply using sequence analysis.
As there are putative Aft1/2p binding sites within the promoters of the putative ferric reductases the genome sequence was searched for the presence of a homologous iron responsive transcription factor. A single ORF was identified in the C. albicans genome sequence with low but significant homology to both ScAft1p and ScAft2p. The putative CaAftp is most similar to ScAft1p at the amino acid level and also contains a glutamine rich domain and a putative NLS sequence that are absent from ScAft2p. However, in common with ScAft2p there is an absence of histidine rich regions located at the N and C-termini of the protein. Therefore the putative CaAftp shares several features with the S. cerevisiae proteins suggesting that it may be a true homologue. However, it is hard to draw a firm conclusion regarding the role of putative CaAftp simply from the protein sequence protein because ScAft1p and ScAft2p share a relatively low level of homology and contain different features within their protein sequences. This is due to the paralogous nature of the two transcription factors which has allowed them to diverge and fulfill different roles within the cell. As C. albicans did not undergo the whole genome duplication event that produced many of the paralogous genes in S. cerevisiae (Wolfe et al., 1997) the single homologue would therefore be expected to share relatively low levels of homology in non-essential regions of the protein.

In conclusion the bioinformatics approach taken during this study suggests that C. albicans has 14 further ferric reductases in addition to the three that have been identified experimentally (Hammacott et al., 2000; Knight et al., 2002). Due to the presence of potential iron responsive elements within the promoters of a number of the ferric reductases the genome sequence was examined for the presence of homologues of the iron responsive transcription factors ScAft1p and ScAft2p. A single ORF was identified whose putative protein product shared homology to both ScAft1p and ScAft2p, although comparisons of features within the protein sequences suggest it is most closely related to ScAft1p. This use of bioinformatics to identify genes highlights the importance of the completion of the C. albicans genome sequence in the study of this fungal pathogen. However, while the sequence data and computer analysis of the putative proteins supports the respective role of the identified proteins it does not guarantee their function. Therefore to confirm their role in iron uptake expression and functional analysis was performed for a number of the putative reductases and the putative CaAFT.
Chapter 4
Functional Analysis of Putative Ferric Reductase Encoding Genes in C. albicans

4.1 Introduction

The findings presented in the previous chapter demonstrate that the C. albicans genome sequence contains 17 putative ferric reductases. The work presented in this chapter attempts to confirm the role of some of these reductases in iron and copper transport. To begin analysing the ORFs the effect of iron or copper limitation upon their expression was examined. If the reductases were involved in the reduction of iron or copper they would presumably be regulated by the level of these two metals. The reductases CaFRE2, CaFRE5, and CaFRE10 were also selected for further study by constructing null mutants using a ‘URA-blasting’ based approach (Fonzi et al., 2003). The deletion of ferric reductases in other organisms produces a range of phenotypes including defects in the reduction and uptake of iron and copper (Dancis et al., 1990; Hassett & Kosman, 1995; Roman et al., 1993) and the inability to utilise respiratory carbon sources such as glycerol (DeFreitas et al., 2004).

4.2 Expression of Ferric Reductases in C. albicans

Northern Blot Analysis of Ferric Reductase Gene Expression

The expression of a number of the putative ferric reductases was examined to determine whether they were expressed (Figure 4.1). The response of transcript levels to copper or iron limitation was also examined. Expression of the reductases was examined in both SC5314 and CAI-4 as previous studies have shown that the deletion of CaURA3 in CAI-4 resulted in the loss of function of an adjacent gene, CaIRO1 (Chibana et al., 2005; Garcia et al., 2001). This is of importance in this study as CaIRO1 is capable of partially complementing some of the mutant phenotypes associated with Scaft1p mutants. However, they also report that CaIro1p is not of functional homologue of ScAft1p, and the reason behind this complementation is unknown. The expression of the ferric reductases was therefore examined in both CAI-4 and SC5314 to determine whether the loss of CaIRO1 has an affect on the expression of the putative ferric reductases.
Figure 4.1 Expression of putative ferric reductases in \textit{C. albicans}. Cells were grown to mid exponential phase in MD media containing either BPS or BCS and then harvested, washed twice, and the RNA extracted. Iron restrictive media containing 100 μM BPS, 100 μM CuCl$_2$, and either 0 μM or 100 μM FeCl$_3$. Copper restrictive media containing 100 μM BCS and 100 μM FeCl$_3$ was supplemented with either 0 μM or 100 μM CuCl$_2$. Samples of RNA were transferred to nylon membranes using a dot blotter, fixed, and hybridised with radio-labelled probes. The probes used were internal fragments of the genes \textit{CaACT1}, \textit{CaFRE5}, \textit{CaFRE7}, \textit{CaFRE10}, and fragments from the 5’ UTR of \textit{CaFRE1} and \textit{CaFRE2}. 

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Transcripts were detected for CaFRE1, CaFRE2, CaFRE5, CaFRE10, and CaFRE12. The transcripts for CaFRE10 and CaFRE12 were the most abundant, with blots requiring an exposure time of 2-3 days compared to at least a week for those produced by the other genes. The CaFRE10 transcript levels increased 2-3 fold in response to iron limitation, but were unaffected by the concentration of copper in the media. In S. cerevisiae the expression of ScFRE2-3 also increases in response to iron but not copper limitation (Georgatsou et al., 1999; Martins et al., 1998).

In contrast to CaFRE10 the levels of CaFRE12 transcript increased in response to copper limitation in the media. Due to the considerable increase in the abundance of the CaFRE12 transcript the rise in expression is difficult to quantify with any accuracy. The pattern of CaFRE12 expression was identical to that of the S. cerevisiae ferric reductase ScFRE7, whose expression is only altered in response to copper, and not iron limitation (Georgatsou et al., 1999; Martins et al., 1998). The expression of the other ferric reductases examined during the course of this study did not alter significantly in response to iron or copper limitation. The expression of all of the ferric reductases examined was also not affected by the lack of CalRO3 in CAI-4, suggesting that its loss has little impact upon the ferric reductase.

Use of the Reporter Gene β-Galactosidase to Study the Expression of the Putative Ferric Reductases

An alternative method to northern analysis for the study of gene expression involves the use of reporter genes. However, many of the readily available systems were not suitable for use in this study as they contained multiple copies of a leucine codon that is translated as serine in C. albicans (Santos et al., 1997). A number of reporter systems have been modified for use in C. albicans including those using GFP (Barelle et al., 2004), luciferase (Srinkinganta et al., 1996), and β-galactosidase (Uhl & Johnson, 2001). During this study it was decided to examine the suitability of the β-galactosidase reporter gene for examining ferric reductase gene expression. The LacZ reporter gene used was originally isolated from Streptococcus thermophilus and does not contain any of the problematical leucine codons (Uhl & Johnson, 2001). The expression of CaFRE10 was examined to validate the use of this system for examining the ferric reductases. This particular gene was chosen as Northern analysis
suggested it was the most highly expressed of any of the ferric reductases, maximising the chance of detecting its expression using the reporter gene. The CaFRE10 promoter was therefore cloned adjacent to the *S. thermophilus* LacZ gene in the plasmid plac-poly (Figure 2.1) and integrated into the genome.

To create the reporter construct the CaFRE10 promoter (-990 to +12 relative to the start codon) was amplified by PCR using the primers FRE10Fprom and FRE10Rprom (Table 2.8). These primers incorporated restriction recognition sequences for the restriction enzymes *Sph*I and *Xma*I. The amplified fragment was digested with *Sph*I and *Xma*I and cloned into the corresponding restriction sites in plac-poly, creating the plasmid pREFRE10. The reporter construct was linearised by digestion with *Stu*I and transformed into CAI-4 and uridine auxotrophs selected. As a control linearised plac-poly was also transformed into CAI-4.

The activity of the CaFRE10 promoter was analysed after growth in both iron and copper limited media. Following growth in the relevant conditions β-galactosidase activity was determined using a permeabilised cell assay as described in the materials and methods. These assays showed that the level of β-galactosidase activity in CAI-4 containing plac-FRE10 increased by ~25% in response to iron limitation while copper limitation had no effect (Figure 4.2). This increase in expression in response to iron, but not copper, limitation is consistent with the results obtained by Northern analysis.

The results of this analysis demonstrated that the *S. thermophilus* LacZ gene was a suitable reporter gene for the study of ferric reductase expression. This approach is therefore being used in our laboratory to study the promoters of CaFRE12, CaCTR1 and CaMAC1. The aim of these studies is to determine whether CuREs within the promoters of these genes are required for regulation in response to copper limitation. In all of these reporter constructs the β-galactosidase activity increases in response to copper limitation, in agreement with results obtained by Northern analysis.
Figure 4.2 Examination of CaFRE10 expression using the β-Galactosidase reporter gene. The strain CAI-4 containing either Clp10 or pREFRE10 was grown to mid-exponential in iron or copper restrictive media and harvested, washed twice, and the levels of β-galactosidase activity determined using a permeabilised cell assay. Iron restrictive MD media contained 100 μM BPS, 100 μM CuCl₂, and 0 or 100 μM FeCl₃. Copper restrictive media contained 100 μM BCS, 100 μM FeCl₃, and 0 or 100 μM CuCl₂. Values are expressed as the percentage of RMLac β-galactosidase activity when grown in iron restrictive conditions supplemented with 100 μM iron. Values are expressed as mean values of three independent experiments and error bars represent standard deviation.
4.3 Construction of Putative Ferric Reductase Mutants

One of the most informative methods for analysing the function of genes is to study the effect of their deletion. A \textit{CafrelA} mutant had previously been generated in our laboratory (Hammacott, PhD Thesis, 2000). During this study \textit{CaFRE2}, \textit{CaFRE5}, and \textit{CaFRE10} genes were chosen for disruption. A number of criteria were used in the selection of these three genes, including the availability of complete sequence data for the entire ORF and surrounding region. This was important because the genome sequence database was in an early stage of assembly when the target genes were selected with complete sequence data available for only 7 putative ferric reductases. While the genome sequence contained a number of other fragmented sequences it was impossible to determine whether or not they were real ORFs and to retrieve sufficient sequence data to plan Southern blot strategies to confirm the disruptions. The targets were also chosen on the basis of their expression. Northern blot analysis performed during this study confirmed that each of these genes was expressed. The \textit{CaFRE10} gene was chosen as a target because it was the most highly expressed, and showed the greatest increase in expression in response to iron limitation. One of the reasons for the selection of \textit{CaFRE2} was that it had previously been identified along with \textit{CaFRE1} in a screen for \textit{C. albicans} genes that could complement the defects in cell surface reductase activity associated with a \textit{ScfrelAfre2A} mutant (Hammacott, PhD thesis 2000). \textit{CaFRE5} was selected for disruption as initial analysis with bioinformatics software packages suggested it contained a mitochondrial targeting sequence.

\textit{Disruption Strategy}

One of the most widely used methods for the disruption of genes in \textit{C. albicans} is known as ‘URA-blasting’ and involves the sequential disruption of both wild-type alleles with a recyclable cassette containing the \textit{CaURA3} gene as a counter-selectable auxotrophic marker (Fonzi et al., 1993). A diagram outlining this process is shown in Figure 4.3. A disruption cassette is initially created by inserting regions of DNA adjacent to the ORF of interest either side of the ‘URA-blasting’ disruption module. This module is contained on a plasmid and comprises the \textit{CaURA3} gene flanked by repetitive sequences from the \textit{hisG} gene in \textit{Salmonella typhimurium}. After cloning the disruption cassette is separated from the parental plasmid by digestion and
Figure 4.3 ‘Ura-blasting’. *C. albicans* is transformed with a disruption cassette consisting of a *hisG/URA3/ hisG* module flanked by regions of DNA homologous to the 5’ and 3’ regions of the gene of interest. Homologous recombination results in the replacement of a copy of the gene of interest with the disruption cassette (i). Selection on 5-FOA containing media results in loss of the entire *URA3* gene following recombination between the *hisG* sequences (ii). The heterozygote is then transformed with the disruption cassette (iii) and homologous recombination results in the deletion of the second copy of the gene of interest.
transformed into the *C. albicans* strain CAI-4, which lacks both copies of the *CaURA3* gene. In a successful disruption event the flanking sequences on the cassette will undergo homologous recombination and replace a wild-type copy of the allele of interest with the disruption cassette. As *C. albicans* is diploid the remaining wild-type allele must be replaced, and the cassette can be reused by counter-selecting for the loss of *CaURA3* from the heterozygote. To select for the loss of *CaURA3* the heterozygous mutant is plated on media containing 5-FOA, which is lethal to cells with a fully functioning uracil biosynthetic pathway. This selects for the loss of *CaURA3* by homologous recombination between the two repetitive *hisG* sequences. After *CaURA3* excision the disruption cassette can be used to replace the remaining wild-type allele.

**Disruption of CaFRE2**

A cassette was constructed for the disruption of *CaFRE2* by amplifying and cloning regions of DNA adjacent to the ORF either side of a disruption module in the plasmid pMB7. The region 5' of the *CaFRE2* ORF (-1 to -943) was first amplified using the primers DIS2B and DIS2K (Table 2.7) that incorporated recognition sites for the restriction enzymes *BgIII* and *KpnI*. The fragment was then digested with *BglII* and *KpnI* and cloned into the corresponding sites in pMB7 to generate the plasmid pRM2.1. To amplify the 3' flanking region (+2217 to +3213) the primers DIS2S and DIS2P (Table 2.7) were used, which incorporated *SphI* and *PstI* recognition sequences. The disruption cassette was then finished by digesting the fragment with *SphI* and *KpnI* and cloning it into the corresponding sites in pRM2.1.

The *CaFRE2* disruption cassette was isolated for transformation into CAI-4 by digestion of pRDFRE2 with *SphI* and *KpnI*. It was then cleaned and concentrated using a Qiagen Minielute PCR purification kit and aliquots of the cassette transformed into CAI-4 with integrants being identified by uridine prototrophy. A total of 9 transformants were obtained and their genotype determined by Southern analysis. Genomic DNA digested with *EcoRI* was hybridised with a radio-labelled probe generated by PCR using the primers DIS2P and DIS2S. This combination of enzyme digestion and probe allow all possible genotypes generated during the disruption process to be distinguished (Figure 4.4a,b). In total 4 of these transformants displayed the correct genotype (data not shown) and two were chosen for further study (RM2.1
Figure 4.4 Southern blot analysis of potential Cafre2ΔΔ mutants. DNA isolated from potential Cafre2ΔΔ mutants generated during the course of this study was analysed by Southern analysis to determine the genotypes of the strains. The DNA was digested with EcoRI, which allows all of the possible genotypes created to be distinguished. The region of DNA surrounding CaFRE2 is shown (a) and EcoRI restriction sites are shown in red. The positions that the primers used to create the disruption cassette bind to are also shown along with the region to which the radio-labelled probe used binds. The predicted sizes of bands to which the probe binds following digestion of DNA with EcoRI is also shown (b). Results of the Southern analysis (c) of strains CAI-4 (lane 1), and potential Cafre2ΔΔ mutants (Lane 2 to 11). Lanes 6 and 11 have the correct genotype (CAI-4 fre2Δ::hisG-URA3-hisG/fre2Δ::hisG) and were named RM2.5 and RM2.6.
(a) 

(i) CaFRE2

![Diagram of CaFRE2 with gene locations and probes.

(ii) Cafre2Δ::hisG-URA3-hisG

![Diagram of Cafre2Δ::hisG-URA3-hisG with gene locations and probes.

(iii) Cafre2Δ::hisG

![Diagram of Cafre2Δ::hisG with gene locations and probes.

(b) 

<table>
<thead>
<tr>
<th>EcoRI</th>
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<tr>
<td>CaFRE2</td>
<td>1111</td>
</tr>
<tr>
<td>Cafre2Δ::hisG-URA3-hisG</td>
<td>3444</td>
</tr>
<tr>
<td>Cafre2Δ::hisG</td>
<td>5077</td>
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(c) 

![Image of gel electrophoresis with lanes and bands corresponding to gene sizes.

Lane 1 2 3 4 5 6 7 8 9 10 11

5077bp →

3444bp →

1111bp →
& RM2.2). To counter-select for the *CaURA3* gene strains were then plated on media containing FOA, and their genotypes determined by Southern analysis (data not shown). A uridine auxotroph with the correct genotype from each of the heterozygous strains was selected for further analysis and named RM2.3 and RM2.4.

The remaining *CaFRE2* wild-type allele was disrupted by transforming RM2.3 & 4 with the disruption cassette. A total of 25 uridine prototrophs were selected from each of the transformed strains and their genotypes determined (Figure 2.4). The radio-labelled probe used hybridises to a single 1111 bp band in *EcoRI* digested CAI-4 genomic DNA (Figure 4.4c). In the majority of the transformants a band of 1111 bp was still detected, showing that they still possessed a wild-type *CaFRE2* allele. In contrast, in two of the transformants the radio-labelled probe binds to two bands of 3444 bp and 5077 bp in size respectively in *EcoRI* digested DNA. This was consistent with the banding pattern expected if both alleles of *CaFRE2* were disrupted. These two strains were therefore selected for further study and named RM2.5 and RM2.6.

**Disruption of CaFRE5**

The *CaFRE5* disruption cassette was made by amplifying regions of DNA adjacent to the *CaFRE5* ORF and cloning them either side of a disruption module in the plasmid pMB7. The 5' flank (-24 to -886) of *CaFRE5* was amplified with the primers DIS5K and DIS5B (Table 2.7), which incorporated restriction sites for the restriction enzymes *KpnI* and *BgII* at either end of the fragment. This amplified fragment was digested with *KpnI* and *BgII* and cloned into the corresponding enzyme sites in pMB7 to produce the plasmid pRD5.1. The 3' flanking region (+2190 to +3056) of *CaFRE5* was amplified using the primers DIS5P and DIS5S (Table 2.7). These primers incorporated restriction enzyme sites for *PstI* and *SphI*, which were used to clone the fragment into the corresponding sites in pRD5.1 to produce the final disruption plasmid pRDFRE5.

The *CaFRE5* disruption cassette was separated from pRDFRE5 by digestion with *SphI* and *KpnI*, and the reaction purified and concentrated using a Qiagen Minielute PCR purification Kit. The cassette was transformed into CAI-4 and a total of 15 uridine prototrophs were obtained. Genomic DNA was isolated from these colonies and their genotype determined by Southern analysis after digestion with *BgII* or *KpnI*.
Figure 4.5 Southern blot analysis of Cafre5AA mutants. Following the disruption of the first and second CaFRE5 alleles Southern blot analysis was used to determine the genotype of the strains generated. Genomic DNA was isolated and digested with either BglII or KpnI, which allows all of the possible genotypes created to be distinguished. The region of DNA surrounding CaFRE5 is shown (a), and recognition sites for BglII (red) and KpnI (blue) shown. The binding sites of the primers used to generate the disruption cassette are also shown (filled arrows) along with the region where the radio-labelled probe used binds. The sizes of the predicted hybridising bands for each of the possible genotypes is also shown (b). Results of the Southern analysis are shown (c) for strains CAI-4 (Lane 1); RM5.1 & 2 (Lane 2 & 3), RM5.3 & 4 (Lane 4 & 5), RM5.5 & 6 (Lane 6 & 7).
using a probe generated using the primers DIS5P and DIS5S (Figure 4.5). The enzymes and probe were chosen as they allow the three possible genotypes generated during the disruption process to be distinguished. Southern blot analysis revealed that the disruption cassette had correctly replaced a single copy of the wild-type allele in all 13 of the transformants. Two of these colonies, RM5.1 and RM5.2, were selected for further study and plated onto media containing 5-FOA to select for the loss of \textit{CaURA3}. The complete loss of the \textit{CaURA3} gene was confirmed by Southern analysis and a single isolate derived from each of the heterozygous strains chosen for further study (RM5.3 and RM5.4).

To disrupt the remaining copy of \textit{CaFRE5} RM5.3 and RM5.4 were transformed with the disruption cassette and uridine prototrophs selected. Multiple transformations were performed until a total of 50 colonies were obtained from each of the strains. The transformants were analysed by Southern analysis to confirm their genotype (Figure 4.5). This confirmed that 2 homozygous null mutants had been generated from each of the heterozygous strains. A colony from each of the transformed strains was then selected for further study and named RM5.5 and RM5.6.

\textbf{Disruption of \textit{CaFRE10}}

The \textit{CaFRE10} disruption cassette was created by cloning fragments of DNA adjacent to the ORF either side of a disruption module in the plasmid pMB7. The 5' flanking region of \textit{CaFRE10} (-2873 to -140 relative to the start codon) was amplified using the primers DIS10S and DIS10P (Table 2.7) that incorporate sites for the restriction enzymes \textit{SphI} and \textit{PstI}. The amplified fragment was digested with \textit{SphI} and \textit{PstI} and cloned into the corresponding sites in the plasmid pUC18 to generate the plasmid pRD10.1. The 3' flanking region of \textit{CaFRE10} (+2461 to +4445) was then amplified using the primers DIS10K and DIS10S (Table 2.7) that incorporated restriction sites for the restriction enzymes \textit{SacI} and \textit{KpnI} to generate the plasmid pRD10.2. The \textit{hisG-CaURA3-hisG} disruption module was then excised from pMB-7 by digestion with \textit{PstI} and \textit{KpnI} and cloned into the corresponding sites in pRD10.2. The completed disruption plasmid was named pRDFRE10.

The \textit{CaFRE10} disruption cassette was separated from pRDFRE10 by digestion with \textit{SphI} and \textit{SacI}, and the reaction cleaned and concentrated using a Qiagen Minielute
Figure 4.6 Southern blot analysis of Cafre10ΔΔ mutants. Following the disruption of the first and second CaFRE10 alleles, Southern analysis was performed to determine the genotypes of the strains generated. Genomic DNA was isolated and digested with XmnI, which allows all of the possible genotypes created to be distinguished. The region surrounding CaFRE10 is shown (a) along with the positions of XmnI restriction sites in red. Binding sites for the primers used to create the disruption cassette are also illustrated (filled arrows) along with the region where the radio-labelled probe hybridises. The sizes of the bands to which the radio-labelled probe hybridises is also shown (b). Results of the Southern blot analysis are shown (c) for strains SC5314 (Lane1), CAI-4 (Lane2), RM10.1 & 2 (lane 3 & 4), RM10.4 & 5 (Lanes 5 & 6), and RM10.5 & 6 (Lanes 7 & 8).
(a) CaFRE10

(i) CaFRE10

(ii) Cafre10::hisG-URA3-hisG

(iii) Cafre10::hisG

(b) | XmnI |
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<td>CaFRE10</td>
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<td>Cafre10::hisG-URA3-hisG</td>
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<td>Cafre10::hisG</td>
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(c) Lane

10290bp -> 6771bp -> 4835bp
PCR purification column. This was transformed into CAI-4 and a total of 6 uridine prototrophs were obtained. Genomic DNA was isolated from each of the transformants and their genotype determined by Southern analysis following digestion with \textit{XmnI} (Figure 4.6). When probed with a fragment of DNA spanning the 5' flank of \textit{CaFRE10} this allows all of the possible genotypes generated during the disruption process to be distinguished. In all of the transformants the disruption cassette had correctly replaced a single copy of the wild-type allele with RM10.1 and RM10.2 being selected for further study. To select for the loss of the \textit{CaURA3} gene in RM10.1 and RM10.2 they were plated on media containing 5-FOA. Uridine auxotrophs were then examined by Southern analysis for the complete loss of the \textit{CaURA3} gene. A uridine auxotroph derived from each of the heterozygotes was selected for further study and named RM10.3 & RM10.4.

The homozygous null mutant was created by transforming RM10.3 and RM10.4 with the disruption cassette a second time. A total of 10 uridine prototrophs were produced following the transformation of RM10.3 and 16 from RM10.4. Southern blot analysis of these strains revealed that a single strain from each of the transformations had both alleles of \textit{CaFRE10} disrupted (Figure 4.6). These \textit{Cafrel0ΔΔ} mutants were named RM10.5 and RM10.6.

4.3 Phenotypic Analysis of Ferric Reductase Mutants

\textit{Effect of Iron and Copper Limitation on the Growth of the Ferric Reductase Mutants}

In yeast the reduction of iron is essential prior to its transport via the high affinity permease/oxidase complex (Dancis \textit{et al.}, 1990). This means that defects in the reduction of iron at the cell surface would be expected to decrease the ability of cells to transport ferric iron across the cell membrane. In \textit{S. cerevisiae} \textit{ScfrelA} mutants display decreased levels of ferric iron, uptake resulting in reduced growth in iron limited conditions (Dancis \textit{et al.}, 1990). The subsequent deletion of \textit{ScFRE2} in the \textit{ScfrelA} mutant further reduces ferric iron uptake and growth in iron limiting conditions (Georgatsou \textit{et al.}, 1994). The \textit{C. albicans} mutants were therefore tested for their ability to grow in iron and copper restrictive conditions.
Figure 4.7 Effect of iron or copper limitation on the growth of ferric reductase mutants. Cells were grown to mid exponential phase in MD medium containing 100 μM BPS and BCS, washed twice, and suspended at a final concentration of 1x10^7 cells.ml^-1. Serial dilutions were performed and samples transferred to the appropriate growth media “using a hedgehog”. The ability of cells to grow on MD media supplemented with either (a) 100 μM BPS or (b) 100 μM BCS was tested. To determine whether growth defects were due to iron or copper limitation media that was supplemented with either 100 μM FeCl₃, 100 μM CuCl₂, or both metals was also used. The pattern of strains spotted onto the media, and the dilution series used, is illustrated in (c).
The effect of iron and copper limitation on the \textit{C. albicans} ferric reductase mutants was tested by comparing their ability to grow in MD media supplemented with the chelators BPS or BCS (Figure 4.7). When the growth of the mutants was compared to the reference strains there were no differences in either iron or copper limiting conditions. It was noted that the growth of all of the strains was slower in metal deplete conditions, with the addition of the limiting metal increasing the rate of growth. The only difference between the strains tested was that while the growth of the \textit{Cafre10ΔΔ} mutant was indistinguishable from the reference strains its colouration differed under a variety of conditions. When the MD-BPS and MD-BCS media was supplemented with copper all of the strains except the \textit{Cafre10ΔΔ} mutant had a dark brown colouration. A similar colour difference was also observed in MD-BPS media supplemented with iron where the \textit{Cafre10ΔΔ} mutant was pale pink compared to dark red. The colour changes observed in the colonies may have been due to the accumulation of extracellular reduced metal. Both the chelators used in this study bind reduced forms of the specific metals producing a colour change. Therefore, in MD-BCS media supplemented with copper the cells reduce extracellular copper and its binding to BCS produces a brown colouration. This colour change is the basis of assays used to measure rates of cell surface iron and copper reduction. The colour difference observed in the \textit{Cafre10ΔΔ} mutant therefore suggests that its ability to reduce extracellular iron and copper could be impaired.

\textbf{Measurement of Cell Surface Associated Ferric Reductase Activity in the Ferric Reductase Mutants}

One of the most useful assays for determining the involvement of proteins in the reduction of iron and copper is the measurement of the cell associated ferric and cupric reductase activity. In \textit{S. cerevisiae} the levels of ferric reductase activity increase in response to iron or copper limitation. The majority of this increase is abolished in \textit{ScfrelA Δ} mutants and is even lower in \textit{ScfrelAfre2Δ} mutants (Dancis \textit{et al.}, 1990; Georgatsou \textit{et al.}, 1994). Previous work in our laboratory has demonstrated that \textit{C. albicans} also possesses a cell associated ferric reductase activity that is regulated in response to iron and copper (Morrissey \textit{et al.}, 1996). The putative ferric reductase mutants were therefore assayed to determine the level of cell associated ferric reductase activity under a variety of conditions (Figure 4.8).
Figure 4.8 Cell surface ferric and cupric reductase activity in ferric reductase mutants. Cultures were grown to mid-exponential phase in iron or copper restrictive media and harvested, washed twice, and the cell surface ferric (a) and cupric (b) reductase activity determined. Iron restrictive media contained 100 μM BPS, 100 μM CuCl₂, and 0 or 100 μM FeCl₃. Copper restrictive media contained 100 μM BCS, 100 μM FeCl₃, and 0 or 100 μM CuCl₂. Values are expressed as a percentage of the reductase activity of CAF-2 in BPS with 100 μM iron. The mean values of at least three independent experiments are shown and error bars represent standard deviation. The strains tested were CAF-2, JHC1.2 \((\text{CafrelΔΔ})\), RM2.5 \((\text{Cafre5ΔΔ})\), RM5.5 \((\text{Cafre5ΔΔ})\), and RM10.5 \((\text{Cafre10ΔΔ})\).
As previously observed (Morrissey et al., 1996), the levels of ferric reductase activity increased in response to iron and copper limitation in the reference strain CAF-2. The increase in activity was greater in response to iron rather than copper limitation. The Cafrel\(\Delta\Delta\) and Cafre5\(\Delta\Delta\) mutants had comparable levels of cell associated ferric reductase activity to the reference strain. Their response to iron and copper limitation was also indistinguishable from the reference strain. In contrast, basal levels of ferric reductase activity decreased by \(\sim 75\%\) in the Cafre10\(\Delta\Delta\) mutant. The increase in activity in response to iron and copper limitation was also severely decreased. The Cafre2\(\Delta\Delta\) mutant also showed a small decrease in the response of ferric reductase activity to iron limitation. When these phenotypes are compared to \(S.\ cer\) evisiae, the large decrease in ferric reductase activity noted in the Cafre10\(\Delta\Delta\) mutant was reminiscent of that observed in Scfre1\(\Delta\Delta\) mutants (Dancis et al., 1990). The smaller increase in response to iron limiting conditions observed in the Cafre2\(\Delta\Delta\) mutant was similar to that associated with the deletion of ScFRE2 (Georgatsou et al., 1994).

As the Cafre10\(\Delta\Delta\) mutant showed such a large decrease in cell surface ferric reductase activity the levels of cupric reductase activity were also determined (Figure 4.8). In the reference strain CAF-2 the level of cupric reductase activity increased in response to both iron and copper limitation. In contrast the levels of cupric reductase activity in the Cafre10\(\Delta\Delta\) mutant were reduced by half and fail to increase in response to iron limitation. However, the levels of cupric reductase activity did increase slightly in response to copper limitation in the Cafre10\(\Delta\Delta\).

**Ferric Iron Uptake in Ferric Reductase Mutants**

As the reduction of iron is required prior to its transport via the Fet3p/Ftr1p complex in \(S.\ cer\) evisiae, defects in cell surface ferric reductase activity reduce the ability of the cell to transport iron into the cell (Dancis et al., 1990). The ability of cells to transport iron can be determined by incubating them with \(^{55}\text{FeCl}_3\) and measuring its accumulation within the cell. The rate of \(^{55}\text{Fe}\) accumulation in the putative ferric reductase mutants was therefore measured and compared to the reference strain CAF-2 (Figure 4.9). All of the putative ferric reductase mutants except the Cafre10\(\Delta\Delta\) mutant had comparable rates of iron uptake to the reference strain CAF-2. In contrast, the rate of iron uptake was reduced by \(77\%\) in the Cafre10\(\Delta\Delta\) mutant. This suggests
Figure 4. 9 Ability of ferric reductase mutants to acquire ferric iron. Cells were grown to mid exponential phase in YPD and harvested, washed twice, and suspended in assay buffer. The cells were then incubated with $^{55}$FeCl$_3$ and cells harvested after 30 minutes (a). The amount of FeCl$_3$ within the cells was then determined by scintillation counting and the rate of uptake calculated. The values shown represent the mean of two independent experiments and error bars represent the standard deviation. (b) Alternatively, cells were harvested at 30 minute intervals and the amount of iron uptake determined. The results of a representative experiment are shown.
that the reduction of iron by CaFre10p is an important step prior to its transport across the plasma membrane. However, the Cafre10ΔΔ mutant was still able to transport a significant amount of iron into the cell, which may explain its ability to grow in low iron conditions. The activity of the remaining 16 reductases may be responsible for the residual iron transport. Alternatively, other iron transport systems that do not require the reduction of iron such as that using CaArn1p may also be responsible for the residual transport (Ardon et al., 2001).

**Sensitivity of Putative Ferric Reductase Mutants to Oxidative Stress**

One of the phenotypes associated with some defects in iron and copper uptake and metabolism is an increased sensitivity to oxidative stress. In *S. cerevisiae* both Scafl1ΔΔ and Scmac1ΔΔ mutants have an increased sensitivity to oxidative stress generated by the presence of hydrogen peroxide in the media (Casas et al., 1997; Jungmann et al., 1993). The toxicity of hydrogen peroxide is caused by the result of oxidation of exposed [4Fe-4S] clusters in enzymes such as aconitase by superoxide (DeFreitas et al., 2000; Liochev & Fridovich, 1994). Oxidation of these Fe-S clusters causes the release of a single iron molecule, which inactivates the enzymes. The released iron is also available to catalyse the Fenton reaction, causing further damage through the generation of hydroxyl radicals (Halliwell & Gutteridge, 1984).

The response of the putative ferric reductase mutants to oxidative stress was tested by comparing their growth on YPD medium supplemented with 0.008% hydrogen peroxide (Figure 4.10). This concentration of hydrogen peroxide was chosen as it had previously shown to inhibit the growth of a Scmac1ΔΔ mutant (Jungmann et al., 1993). When compared to the reference strains SC5314 and CAF-2 the growth of the ferric reductase mutants was indistinguishable. All of the strains grew fully on the media tested with the addition of iron, copper, or both metals having no affect upon growth. The growth of the ferric reductase mutants was also tested on media containing higher concentrations of hydrogen peroxide. However, it was found that when the concentration was raised above 0.008% it severely inhibited or abolished growth of all of the strains. The results of these experiments therefore showed that the ferric reductase mutants did not have an increased sensitivity to oxidative stress.
Figure 4.10. Effect of carbon source and hydrogen peroxide on the growth of C. albicans ferric reductase mutants. Cells were grown to mid exponential phase in MD media containing 100 μM BPS and BCS, washed twice, and suspended at a final concentration of 1x10^7 cell ml⁻¹. Serial dilutions were then performed and the cells transferred to the appropriate growth media “using a hedgehog”. The ability of the strains to utilise a variety of carbon sources was tested using YP medium supplemented with (a) 2 % glucose or (b) 2 % glycerol. The growth of strains on YPD medium supplemented with 0.008 % H₂O₂ (c) was also examined to test the sensitivity of strains to oxidative stress. To determine whether growth defects were due to iron or copper limitation media supplemented with 100 μM FeCl₃, 100 μM CuCl₂, or both metals was also used. The order of strains and serial dilutions used is shown (d).
(a) YPD

(b) YPG

(c) YPD + 0.008% H$_2$O$_2$

(d)

(i) SC5314
(ii) CAF-2
(iii) JHC1.2 (Cafre1.4)
(iv) RM5.5 (Cafre5.4)
(v) RM10.5 (Cafre10.4)
(vi) RM2.5 (Cafre2.4)
Examination of the Putative Ferric Reductase Mutants for Respiratory Defects

Due to the essential nature of iron and copper in a variety of metabolic enzymes their shortage can lead to growth defects. These include the inability of some iron and copper uptake mutants to utilise respiratory carbon sources (Blaiseau et al., 2001; DeFreitas et al., 2000; Jungmann et al., 1993). Respiratory growth is thought to be affected in mutants because iron and copper are essential co-factors in a number of enzymes involved in the tricarboxylic acid cycle and the respiratory chain (DeFreitas et al. 2003). Examples of these iron requiring enzymes include succinate dehydrogenase, aconitase, and cytochromes, including cytochrome c oxidase which also requires copper. Indeed fifteen percent of all proteins involved in respiratory metabolism require iron for their correct functioning (DeFreitas et al., 2003). Shortages in iron and copper uptake would therefore be expected to disrupt this process within the cell. While this theory is commonly held there is some contradictory evidence to suggest that while CaaflAA mutants are respiratory deficient their levels of cytochrome c oxidase activity are unaffected (Blaiseau et al., 2001).

To test the respiratory competency of the putative ferric reductase mutants their ability to grow on media containing glycerol or glucose as the sole carbon sources was compared (Figure 4.10). All of the strains showed comparable patterns of growth on the respective media, demonstrating that the putative ferric reductase mutants are respiratory competent. However, while the Cafre10ΔΔ mutant did not show any growth defects the colouration of the colonies differed from the other strains when the media was supplemented with copper. When all of the strains were grown on media without additional copper they were pure white in colour. In contrast all of the strains except the Cafre10ΔΔ mutant, which remains white, have a brown colouration when the media is supplemented with copper. The reason behind this colour change is unclear but is copper specific and may simply be due to an increased accumulation of copper in the cells when the metal is in abundance.
4.4 Reintegration of *CaFRE10*

To confirm that the phenotypes associated with the *CaFrelOAA* mutant were due to the disruption of the ferric reductase a wild-type allele was reintroduced. A fragment of the *CaFRE10* ORF with 1000bp of promoter and 100bp of 5' sequence was therefore cloned into the vector Clp10 and integrated into the genome. The *CaFRE10* ORF and additional sequences were amplified using the primers FRE10FORF and FRE10RORF (Table 2.8) that incorporated recognition sites for the restriction enzyme Xhol at either end of the fragment. Following digestion with Xhol the fragment was cloned into Clp10 at the corresponding restriction site to form the plasmid pRRFRE10. To confirm the integration and orientation of the *CaFRE10* fragment the vector was digested with *BgIII* and *HindIII*. To integrate the vector into the genome pRRFRE10 was linearised by digestion with *Stul* and transformed into CAI-4 and the *CafrelOAA* mutant. Uridine prototrophs were selected and the correct integration of the vector confirmed by PCR.

The ability of the *CaFRE10* ORF to complement the *CaFrelOAA* mutant was examined by measuring its impact on cell surface reductase activity (Figure 4.11). Analysis of the strains showed that the deletion of a single *CaFRE10* allele decreased ferric reductase activity by approximately half. The majority of the remaining activity is then lost following the deletion of the remaining allele. When a single copy of *CaFRE10* was reintroduced it only partially restored ferric reductase activity when compared to the heterozygote. However, there is a definite increase in activity when compared to the *CaFrelOAA* mutant with a larger increase in ferric reductase activity in response to low iron conditions being observed. This suggests that the deletion of the *CaFRE10* allele is solely responsible for the phenotypes associated with the *CafrelOAA* mutant.

4.5 Discussion

The aim of the work presented during this chapter was to experimentally characterise some of the putative ferric reductases identified through analysis of the *C. albicans* genome sequence. Transcripts have been detected for *CaFRE1, CaFRE2, CaFRE5, CaFRE10, and CaFRE12* demonstrating that these predicted ORFs are expressed. When their expression was examined in response to iron and copper limitation it was
Figure 4.11 The affect of CaFRE10 reintegration upon the ferric reductase defect of Cafre10ΔΔ mutants. The ferric reductase activity associated with strains CAF-2, RM10.3 (Cafre10Δ/CaFRE10), RM10.5 (Cafre10ΔΔ), RM10.7 (Cafre10ΔΔ with pRRFRE10 inserted), and RM10.7 with Clp10 reintegrated into the genome. Cells were grown in MD media supplemented with 100 μM BPS, 100 μM CuCl₂, and 0 or 100 μM FeCl₃ and harvested at mid-exponential phase. The ferric reductase activity of the strains was then determined. Values are expressed as a percentage of the ferric reductase activity associated with CAF-2 grown in MD-BPS with 100 μM FeCl₃. The values represent the mean of three independent experiments with error bars representing the standard deviation.
noted that CaFRE10 and CaFRE12 are the most highly expressed. The levels of CaFRE12 transcript increase greatly in response to copper limitation but are unaffected by the concentration of copper in the media. This pattern of expression is identical to ScFRE7 and provides further evidence for CaFRE12 being a functional homologue of this S. cerevisiae reductase. The copper responsive expression also suggests that the two CuREs identified in the CaFRE12 promoter by sequence analysis are functional. In contrast to CaFRE12 the levels of CaFRE10 transcript only increase in response to iron limitation in the media. This pattern of expression is identical to that of the S. cerevisiae genes ScFRE2-6 (Georgatsou et al., 1999; Martins et al., 1998). The expression of CaFRE10 therefore suggests its role is primarily in the reduction of iron prior to its transport.

A reporter construct containing the CaFRE10 promoter fused to β-galactosidase showed a similar response to iron limitation to that observed using Northern analysis. However, while the CaFRE10 transcript increases 2-3 fold in response to iron limitation the level of β-galactosidase activity produced by the reporter construct only increased by ~25%. There are a number of possible explanations for this discrepancy in the level of iron responsive regulation. For example, the CaFRE10 promoter region may lack additional regulatory elements that are located further downstream. This is possible as the intergenic space separating CaFRE10 and the adjacent gene COX11 is unusually large at 3503 bp in length. Alternatively, the activity of the CaFRE10 promoter could have been affected by the fact that it is not integrated at its native genomic location. Because only partial rescue is observed it is also possible that the decrease in ferric reductase activity observed in the Cafrel0AA mutant was the result of events other than the deletion of CaFRE10.

To further investigate the function of the putative ferric reductase CaFRE2, CaFRE5, and CaFRE10 were disrupted and the mutants analysed for a variety of phenotypes. A Cafrel1AA mutant that had previously been created in our laboratory was also tested to determine any phenotypic differences. When compared to wild-type and reference strains the Cafrel1AA and Cafre5AA mutants show no phenotypic changes. However, this does not necessarily mean that they are not ferric reductases involved in iron and copper uptake and metabolism. While S. cerevisiae contains multiple ferric reductases
only $ScfrelA$ and $Scfrel2A$ mutants have significant changes in ferric reductase activity and iron uptake (Dancis et al., 1990; Georgatsou et al., 1994). As these two reductases are responsible for the majority of ferric iron reduction and uptake they mask any changes occurring due to the deletion of the other reductases. Small changes in ferric reductase activity are also difficult to spot as the assay used to measure the activity is quite variable.

The $Cafre2A^\Delta$ mutant has identical growth phenotypes to the wild-type strain but displays defects in cell surface ferric reductase activity when compared to the wild-type strains. The small drop in activity noted is similar to that in $Scfrel2A$ mutants (Georgatsou et al., 1994) but does not appear to interfere with the rate of iron uptake. As there is still a high level of ferric reductase activity in the mutant it is possible that iron uptake is unaffected as the transport of iron into the cell is still likely to be the limiting step in uptake rather than the availability of reduced iron. Therefore while ScFre2p is involved in the reduction of iron at the cell surface it is not essential for the transport of ferric iron.

In growth tests the $Cafre10A^\Delta$ performs identically to the wild-type strains although it has significant defects in the reduction and transport of ferric iron. The levels of ferric reductase activity are reduced in the $Cafre10A^\Delta$ mutant and respond poorly to iron and copper limitation. This decrease in the reduction of ferric iron has important implications for iron transport as the rate of ferric iron uptake is also reduced by 77%. These phenotypes are consistent with those observed in $ScfrelA$ mutants (Dancis et al., 1990) suggesting that they have a similar role. However, in contrast to $ScfrelA$ mutants the $Cafre10A^\Delta$ mutant is still able to grow in iron limiting conditions. It is possible that the residual levels of iron uptake in the $Cafre10A^\Delta$ mutant are sufficient to support growth in the iron limited conditions tested. $C.\ albicans$ may also contain significant levels of stored iron that in combination with the residual levels of iron transport are capable of supporting growth in iron limited conditions for a significant period of time. Alternatively other non-reductive iron uptake pathways such as the uptake of siderophores via CaArn1p (Ardon et al., 2001) may be able to support growth in iron limiting conditions.
To confirm whether the phenotypes associated with the deletion of CaFRE10 were solely due to the deletion of the putative ferric reductase a wild-type allele was reintroduced using the vector Clp10. The effect of the reintroduced allele on the ferric reductase activity of the C Fate10ΔΔ mutant was then assayed. The CaFRE10 allele is able to partially rescue the defects in ferric reductase activity associated with the C Fate10ΔΔ mutant when compared to the heterozygous mutant. The reason for the lack of complete rescue is probably that the reintroduced CaFRE10 allele is not expressed properly. Evidence to support this theory comes from the fact that when the same region of promoter was fused to a reporter gene and assayed for activity the rise in reporter gene activity in response to iron limitation was less than expected. Therefore, as discussed earlier either the region of promoter used in both constructs is too short or the integration of the constructs at the RPS10 locus affects expression.

In conclusion the experimental analysis of the putative ferric reductases suggests a number of possible roles for the putative reductases examined. The expression of CaFRE12 lends further weight to the argument that it encodes a homologue of ScFre7p that performs a similar cellular function. The precise nature of this function is unknown but a role for ScFRE7 in intracellular copper trafficking has been proposed (Hassett & Kosman, 1995). The results obtained from the analysis of CaFRE10 are more confusing and suggest a number of possibilities. While the expression of CaFRE10 is identical to that of ScFRE2-6 the defects in the reduction and uptake of iron suggest that it performs a similar function to ScFre1p. When the growth phenotypes associated with the C Fate10ΔΔ mutant are compared to S. cerevisiae they are similar to those associated with the deletion of ScFRE2-6. Therefore, while ScFre1p and CaFre10p are the major cell surface ferric reductases in their respective organisms they each have distinctive properties. Whether this is a result of differences in the protein functions or differences in the iron uptake systems of the two organisms is still unclear.
Chapter 5
Investigation of the Role of CaAFT and CaMAC1 in the Regulation of the C. albicans Ferric Reductases

5.1 Introduction
In S. cerevisiae transcription of the ferric reductases is activated in response to iron and copper deprivation through the activity of the transcription factors Aft1p, Aft2p, and Mac1p (Georgatsou et al., 1999; Martins et al., 1998). Other components of the high affinity iron and copper uptake systems are similarly regulated by iron and copper availability. This tight regulation of the iron and copper uptake systems is necessary to prevent the influx and accumulation of high levels of iron and copper (Rutherford & Bird, 2004), which are able to catalyse the formation of free radicals within the cell (Stohs & Bagchi, 1993).

In S. cerevisiae the transcription factor ScAft1p regulates the expression of the most highly iron responsive genes (Courel et al., 2005; Rutherford et al., 2003). These genes include those encoding components of the high affinity iron uptake system such as the ferric reductases, the permease/oxidase complex, and the siderophore transporters (Courel et al., 2005; Rutherford et al., 2003). In contrast Aft2p predominantly regulates genes whose protein products are involved in intracellular iron trafficking and metabolism, including the vacuolar metal transporter SMF3 (Courel et al., 2005; Rutherford et al., 2003). However, the majority of iron responsive genes in S. cerevisiae are transcriptionally regulated by a combination of ScAft1p and ScAft2p. The copper responsive transcription factor ScMac1p regulates the expression of genes encoding components of the high affinity copper uptake pathway such as ScCTR1 and ScCTR3 (Labbe et al., 1997; Yamaguchi-Iwai et al., 1997). Interestingly, ScFREL is regulated by ScMac1p, ScAft1p, and ScAft2p in response to both iron and copper limitation (Hassett & Kosman et al., 1995; Yamaguchi-Iwai et al., 1995; Yamaguchi-Iwai et al., 1997; Rutherford et al., 2003). This is presumably due to the involvement of ScFrelp in the reduction of both iron and copper prior to its transport into the cell (Hassett & Kosman et al., 1995).
The aim of the work presented in this chapter was to identify the transcription factors involved in the iron and copper responsive regulation of the putative ferric reductases in *C. albicans*. Previous work in our laboratory has identified a *C. albicans* homologue of the copper responsive transcription factor ScMac1p, which is required for the copper responsive regulation of *CaCTR1* (Marvin *et al.*, 2004). A *Camac1ΔΔ* mutant strain also displays phenotypes that are consistent with defects in iron and copper uptake and metabolism (Marvin *et al.*, 2004). A potential homologue of ScAft1p and ScAft2p was identified in Chapter 3 using a bioinformatics based approach. The involvement of CaMac1p and CaAftp in the expression of the putative ferric reductases was therefore examined.

### 5.2 Disruption of Putative CaAFT

In order to investigate the function of the putative *CaAFT* gene, a null mutant was generated using a ‘URA-blasting’ based approach to delete both wild-type alleles (Fonzi & Irwin, 1993). The deletion of both copies of *CaAFT* was confirmed by Southern blotting, and the resulting mutant analysed for a number of phenotypes associated with defects in iron and copper uptake and metabolism.

**Construction of Putative CaAFT Disruption Cassette**

To create the disruption cassette for *CaAFT* sequences flanking the ORF were amplified and cloned either side of the ‘URA-blasting’ cassette in the plasmid pMB7 (Gow *et al.*, 1999). Initially, a region containing bases -20 to -987 relative to the putative *CaAFT* start codon was amplified using the primers DISAS and DISAP (Table 2.7), which incorporated restriction sites for the enzymes *SphI* and *PstI*. The fragment produced was digested with *SphI* and *PstI* and cloned into the corresponding restriction sites in pMB7 to produce the plasmid pRDA.1. The region of DNA 3’ to the putative *CaAFT* containing bases +2383 to +3419 was then amplified using the primers DIAB and DIAK (Table 2.8) that incorporated recognition sites for the restriction enzymes *BglII* and *KpnI*. The PCR product produced was then cloned into the *BglII* and *KpnI* restriction sites in pRDA.1 to create the plasmid pRDAFT, containing the *CaAFT* disruption cassette.
The putative *CaAFT* disruption cassette was separated from pRDAFT by digestion with the restriction enzymes *Sph*I and *Kpn*I and purified using a Qiagen Minielute PCR Purification Kit. The disruption cassette was then transformed into CAI-4 and cells selected for uridine prototrophy. Multiple transformations yielded a total of 16 colonies that were analysed by Southern blotting. Genomic DNA was isolated and digested with *Bgl*II or *Pst*I, which allow all of the possible genotypes throughout the disruption process to be distinguished (Figure 5.1). When the genomic DNA was digested with *Pst*I or *Bgl*II the radio-labelled probe hybridised to two bands of 6412bp and 2175bp or 7794bp and 6198bp respectively in 15 of the colonies. Therefore the DNA from each of the colonies produces a band that corresponds to the wild-type allele and a single disrupted allele. The digestion and hybridisation of the DNA from the remaining colony produce a single band of 6412bp or 7794bp, which corresponds to the expected size of the wild-type. Two of the correct colonies were then selected for further study and named RMA1 and RMA2.

In order to allow the disruption of the remaining *CaAFT* allele RMA1 and RMA2 were selected for uridine auxotrophy on plates containing 5-FOA and two colonies, RMA3 and RMA4, examined for the complete loss of the *CaURA3* gene by Southern blotting. In RMA4 the 6198bp band produced by digestion with *Bgl*II is replaced by one of 5857bp, confirming the complete loss of the *CaURA3* allele by homologous recombination. The strain RMA3 produces a single band at 7794bp, suggesting that this strain contained two copies of the wild-type putative *CaAFT* allele. Therefore, only RMA4 was transformed with the disruption cassette to replace the second *CaAFT* allele. Due to the low transformation efficiency of *C. albicans* multiple transformations were required to generate a total of 100 colonies, which were analysed to determine their genotype. The larger number of colonies required for this second round of screening is caused by the fact that the disruption cassette often targets the first disrupted allele. A total of 3 homozygous null mutants were identified by Southern blotting following digestion with *Pst*I, and were named RMA5 to RMA7. The radiolabelled probe bound to two DNA fragments of 5857bp and 2175bp in size within the digested DNA from these strains. The remaining wild-type allele therefore appeared to have been replaced by the disruption cassette. However, subsequent analysis with *Bgl*II showed that while strains RMA5 and RMA6 lack a copy of the wild-type allele the band that replaces it is larger than expected. In contrast, the
Figure 5.1 Southern blot of *Caaft*ΔΔ mutants. Following disruption of the first and second *CaAFT* alleles strains Southern blot analysis was used to confirm the genotype of strains. Genomic DNA was isolated and digested with either *PstI* or *BgII*, which allows all of the possible genotypes created to be distinguished. The genomic region surrounding *CaAFT* is shown (a) along with the positions where the restriction enzymes cut, the region to which the radiolabelled probe used hybridises, and the positions of the primers used to create the disruption cassette (filled arrows). The predicted sizes of hybridising bands are also shown (b). Results for the Southern blot analysis are shown (c) for SC5314 (Lane 1), CAI-4 (Lane 2), RMA1 & 2 (Lane 3 & 4), RMA3 & 4 (Lane 5 & 6), and RMA5 to RMA7 (Lanes 7 to 9).
5875bp fragment generated by digestion of RMA7 genomic DNA with BgIII is consistent with the replacement of the remaining wild-type allele with the disruption cassette. The strain RMA7 (CaαftΔΔ) was therefore chosen for subsequent analysis for mutant phenotypes and expression changes.

5.3 Phenotypic Analysis of Camac1ΔΔ and CaαftΔΔ Mutants

As Caαftlp and CaMac1lp are potential regulators of the putative ferric reductases strains carrying deletions of these genes were examined for phenotypes associated with defects in iron and copper uptake and metabolism. In our laboratory we have previously generated a Camac1ΔΔ mutant strain (Marvin et al., 2004), and a putative CaαftΔΔ mutant was created during this study. These mutants were therefore tested for a number of phenotypes associated with defects in iron and copper responsive regulation in S. cerevisiae. As controls the mutants were compared to wild-type strains and a Cactr1ΔΔ mutant, which has been extensively characterised in our laboratory (Marvin et al., 2003; Marvin et al., 2004). The Cactr1ΔΔ mutant shows extensive defects in iron and copper uptake including reduced iron uptake, poor growth on copper and iron limited media, poor growth on non-fermentable carbon sources, and an increased sensitivity to oxidative stress (Marvin et al., 2003; Marvin et al., 2004).

In S. cerevisiae the deletion of ScAFT1 produces a range of phenotypes including defects in the reduction and uptake of ferrous iron, poor growth in iron limiting conditions, poor growth on non-fermentable carbon sources, and an increased sensitivity to oxidative stress (Yamaguchi-Iwai et al., 1995; Yamaguchi-Iwai et al., 1996). The deletion of ScMAC1 produces identical defects in addition to poor growth in copper limited conditions (Jungmann et al., 1993).

**Effect of Iron and Copper Limitation on the Growth of the Camac1ΔΔ and CaαftΔΔ Mutants**

To test for defects in iron and copper uptake the growth of the Camac1ΔΔ and Caαft1ΔΔ mutants was examined on metal limiting media (Figure 5.2). When the growth of all of the strains was compared it was noted that they all grew more slowly on MD-BPS and MD-BCS media without the addition of iron and copper. The growth
Figure 5.2 Effect of iron and copper depletion upon the growth of the CaaftΔΔ and Camac1ΔΔ mutants. Cells were grown in MD media supplemented with 100 μM BPS and BCS and harvested at mid exponential phase, washed, and diluted to a cell density of 1x10⁷ cell ml⁻¹. Serial dilutions were then performed and the strains transferred to media “using a hedgehog”. The strains were tested on MD media supplemented with either 100 μM BPS or 100 μM BCS. To determine whether any growth defects were due to iron or copper depletion strains were also spotted on media supplemented with either 100μM FeCl₃, 100mM CuCl₂, or both metals.
of the \textit{Caaf} mutant was indistinguishable from the control strains in both copper and iron limiting conditions. This suggests that the deletion of \textit{CaAFT} does not cause major defects in iron and copper uptake and metabolism. In contrast the \textit{Camac} mutant and \textit{Cactr} mutant grew extremely poorly on MD-BPS and MD-BCS media. Growth of these mutants was restored by the addition of copper, but not iron, to the growth media. Copper was therefore the growth limiting factor in these mutants in the presence of BPS or BCS. Growth of \textit{Cactr} and \textit{Camac} mutants is probably observed in media containing MD-BPS as the chelator is capable of binding both iron and copper. The failure of iron to complement the growth defects of the \textit{Cactr} and \textit{Camac} mutants demonstrated they are not simply a result of defects in iron uptake due to insufficient copper entering the cell.

\textbf{Measurement of Cell Surface Ferric and Cupric Reductase Activity in the \textit{Camac} and \textit{Caaf} Mutants}

If CaMac1p and CaAf1p are involved in the regulation of the putative ferric reductases their deletion would be expected to affect the cell surface ferric/cupric reductase activity. When cell surface ferric reductase activity was measured in the \textit{Caaf} mutant and wild-type, and its response to iron and copper limitation compared, the levels obtained were indistinguishable (Figure 5.3). In contrast ferric reductase activity was consistently elevated in the \textit{Camac} mutant in both metal replete and limited conditions. One explanation for this increase is that the deletion of \textit{Camac} results in the inappropriate expression of iron regulated genes such as the ferric reductases. Support for this theory comes from \textit{S. cerevisiae} microarray studies showing that the deletion of \textit{ScMACl} results in the activation of \textit{ScAft1p} and \textit{Aft2p} regulated genes in response to copper limitation (DeFreitas \textit{et al.}, 2004).

When the cupric reductase activity of the strains was compared it was evident that the \textit{Caaf} mutant is once again indistinguishable from the wild-type. In contrast the \textit{Camac} mutant had defective levels of cupric reductase activity when grown in copper limited media. Interestingly, the level of cupric reductase activity in the \textit{Camac} mutant does not decrease in response to the addition of copper to the growth media. This may suggest that even when copper is abundant there is insufficient copper entering the cell to fulfil all of the cellular needs.
Figure 5.3 Measurement of cell surface ferric and cupric reductase activity in *CaafitaA* and *CamaclAA* mutants. Cultures were grown to mid-exponential phase in iron or copper restrictive media and harvested, washed twice, and the cell surface ferric (a) and cupric (b) reductase activity determined for the strains SC5314, MEM-m2 (*CamaclAA*), and RMA7 (*CaafitaA*). Iron restrictive MD media contained 100 μM BPS, 100 μM CuCl₂, and 0 or 100 μM FeCl₃. Copper restrictive MD media contained 100 μM BCS, 100 μM FeCl₃, and 0 or 100 μM CuCl₂. Values are expressed as a percentage of the ferric reductase activity of CAF-2 in BPS + Fe conditions. The mean values for three (ferric) or two (cupric) independent experiments are shown. Errors bars represent standard deviation.
**Ferric Iron Uptake in the CmaclΔΔ and CaaftΔΔ Mutants**

In *S. cerevisiae*, *ScmaclΔΔ* and *ScaftΔΔ* mutants display defects in the uptake of ferric iron. The deletion of *ScAftlp* affects iron uptake due to the failure of the mutant to up-regulate genes encoding components of the high affinity iron uptake pathway, including *ScFET3* and *ScFTR1*, in response to iron limitation (Yamaguchi-Iwai et al., 1995; Yamaguchi-Iwai *et al.*, 1996). The deletion of *ScMaclp* inhibits iron uptake because of the lack of ScCtrlp mediated copper uptake (Dancis *et al.*, 1994b; Jungmann *et al.*, 1993). This effects iron uptake as there is insufficient copper entering the cell to supply the multicopper oxidase ScFet3p, reducing the rate of high affinity iron uptake (Askwith *et al.*, 1994; Dancis *et al.*, 1994b). The *C. albicans* mutants were therefore examined to determine their ability to transport $^{55}$FeCl$_3$, as previously described in Chapter 4. The deletion of the putative *CaftΔΔ* had no impact on the rate of iron uptake in *C. albicans* under the conditions tested (Figure 5.4). In contrast iron uptake in the *Camac1ΔΔ* mutant is severely reduced when compared to the reference strain DAY185. The deletion of *MAC1* in both *S. cerevisiae* and *C. albicans* therefore results in similar defects in high affinity iron uptake.

In an attempt to more accurately quantify the decrease in iron uptake observed in the *Camac1ΔΔ* mutant the rate of $^{55}$FeCl$_3$ uptake was determined by measuring iron uptake over a period of time (Figure 5.4). In common with the earlier results ferric iron uptake was reduced in the *Camac1ΔΔ* mutant during the first 30-60 minutes of the assay. However, following this initial lag the rate of iron uptake increases until it is comparable to that of the control strain. The relevance of this result will be discussed later.

**Sensitivity of Camac1ΔΔ and CaaftΔΔ Mutants to Oxidative Stress**

To determine whether the *CaaftΔΔ* and *Camac1ΔΔ* mutants displayed defects in their ability to cope with oxidative stress their growth was examined on YPD media supplemented with 0.008 % hydrogen peroxide (Figure 5.5). The growth of the control strains and the *CaaftΔΔ* mutant was unimpaired by hydrogen peroxide, and was unaffected by the addition of iron, copper, or both metals to the growth medium. As shown previously the *Cactr1ΔΔ* mutant has an increased sensitivity to hydrogen...
Figure 5.4 Ferric iron uptake in *Caaft*ΔΔ and *Camac*ΔΔ mutants. The strains DAY185, CAF-2, MEM-m2 (*Camacl*ΔΔ), and RMA7 (*Caaft*ΔΔ) were grown to mid exponential phase in YPD and harvested, washed twice, and suspended in assay buffer. (a) Cells were then incubated with $^{55}$FeCl$_3$ at 30°C and the amount of $^{55}$FeCl$_3$ within the cells determined after 30 minutes by scintillation counting. The mean rates of iron uptake calculated from for two independent experiments are shown, and error bars represent the standard deviation. (b) Alternatively, cells were harvested at 30 minute intervals and the amount of $^{55}$FeCl$_3$ iron within the cells calculated. The results of a single representative experiment are shown.
Figure 5.5 Effect of carbon source and hydrogen peroxide on the growth of *Camac1*ΔΔ and *Caafl*ΔΔ mutants. Cells were grown in MD media supplemented with 50 μM BPS and BCS and harvested at a mid exponential phase. Cells were then washed twice and diluted to a cell density of 1 x 10⁷ cell ml⁻¹. Serial dilutions of the cells were then spotted onto the appropriate media “using a hedgehog”. Cells were spotted onto YP medium containing either (a) 2 % glucose (YPD) or (b) 2 % glycerol (YPG) as a carbon source. Cells were also tested for their sensitivity to oxidative stress using (c) YPD medium containing 0.008 % H₂O₂. To determine whether growth defects were due to iron and copper deprivation growth was also tested on media supplemented with 100 μM FeCl₃, 100 μM CuCl₂, or 100 μM of both metals. The strains and serial dilutions used are illustrated in (d).
(a)

YPD | YPD + Fe | YPD + Cu | YPD + Fe + Cu

(i) SC5314
(ii) CAF-2
(iii) DAY185
(iv) MEM-c3 (ctr1ΔΔ)
(v) MEM-m2 (mac1ΔΔ)
(vi) RMA7 (aft1ΔΔ)
(vii) RMA7 (aft1ΔΔ)

(b)

YPG | YPG + Fe | YPG + Cu | YPG + Fe + Cu

(i) SC5314
(ii) CAF-2
(iii) DAY185
(iv) MEM-c3 (ctr1ΔΔ)
(v) MEM-m2 (mac1ΔΔ)
(vi) RMA7 (aft1ΔΔ)
(vii) RMA7 (aft1ΔΔ)

(c)

H₂O₂ | H₂O₂ + Fe | H₂O₂ + Cu | H₂O₂ + Fe + Cu

(i) SC5314
(ii) CAF-2
(iii) DAY185
(iv) MEM-c3 (ctr1ΔΔ)
(v) MEM-m2 (mac1ΔΔ)
(vi) RMA7 (aft1ΔΔ)
(vii) RMA7 (aft1ΔΔ)

(d)

1×10⁷ → 5×10⁴

(i) SC5314
(ii) CAF-2
(iii) DAY185
(iv) MEM-c3 (ctr1ΔΔ)
(v) MEM-m2 (mac1ΔΔ)
(vi) RMA7 (aft1ΔΔ)
(vii) RMA7 (aft1ΔΔ)
peroxide that can be complemented by the addition of copper, but not iron, to the 
growth media (Marvin et al., 2003). The Camac1ΔΔ mutant was also sensitive to 
hydrogen peroxide and this defect was complemented by the addition of copper to the 
media. The addition of copper presumably restores growth as the low affinity copper 
uptake systems will be active under these conditions and is capable of transporting 
sufficient copper to restore cellular functions. The presence of excess copper in the 
media may also be beneficial because copper, or copper complexed with molecules 
other than SOD, is capable of scavenging superoxide in in vitro assays (Lin & 
Culotta, 1995; Tamai et al., 1993).

Examination of the Camac1ΔΔ and Caaf1ΔΔ Mutants for Respiratory 
Defects
As mentioned earlier Scaft1ΔΔ and Scmac1ΔΔ mutants are respiratory deficient and 
are therefore unable to efficiently utilise carbon sources such as ethanol and glycerol 
(Blaiseau et al., 2001; DeFreitas et al., 2000; Jungmann et al., 1993). Previous studies 
in our laboratory have shown that CactrlΔΔ and Camac1ΔΔ mutants are also unable 
to utilise respiratory carbon sources (Marvin et al., 2003; Marvin et al., 2004). The 
addition of copper to the growth media is capable of restoring respiratory competency 
to the C. albicans mutants. To determine whether the Caaf1ΔΔ mutant had similar 
defects it was grown on media containing glucose (YPD) or glycerol (YPG) as the 
sole carbon source (Figure 5.5). The growth of the Caaf1ΔΔ on YPG and YPD was 
indistinguishable from the controls, suggesting that it is fully respiratory competent. 
As reported previously, the Camac1ΔΔ and CactrlΔΔ mutants were respiratory 
deficient, and their ability to utilise glycerol was restored by the addition of excess 
copper to the growth medium (Marvin et al., 2003; Marvin et al., 2004). Interestingly, 
the growth of CactrlΔΔ and Camac1ΔΔ was also impaired on YPD media and can be 
restored to wild-type levels by supplementation with copper. This may simply be due 
to the fact that YP may only contain low levels of copper (Marcus Marvin, personal 
communication).

Effect of Alkaline pH on the Growth of Camac1ΔΔ and Caaf1ΔΔ mutants
A number of reports have highlighted the importance of iron and copper uptake in the 
survival of S. cerevisiae and C. albicans in alkaline environments (Bensen et al.,
As the environmental pH increases iron and copper become less soluble, making their transport more difficult. One solution to this problem that has been observed in *S. cerevisiae* and *C. albicans* is to increase the expression of components of the high affinity iron and copper uptake systems in response to alkaline pH (Bensen *et al.*, 2004; Lamb *et al.*, 2001; Serrano *et al.*, 2004). This process is mediated by the pH sensing transcriptional activator Rim101p in both organisms (Bensen *et al.*, 2004; Lamb *et al.*, 2001).

To ascertain the affect of pH on the growth of the *C. albicans* mutant strains they were plated onto YPD media that had been adjusted to pH 6.0 or pH 8.0 with 0.125 M HEPES (Figure 5.6). All of the strains tested grew faster at pH 6.0 than pH 8.0, although the rate of growth in the two conditions was equal when media was supplemented with iron or copper. This suggests that the limitation of iron and copper are major limiting factors in the growth of *C. albicans* at alkaline pH. When the strains tested were compared the growth of the *CaaftΔΔ* mutant was indistinguishable from the wild-type. In contrast, the *CamacΔΔ* and *CactrΔΔ* strains displayed growth defects at pH 8.0, with the affect being more severe in the *CactrΔΔ* mutant. The growth defects were partially restored by iron, although the addition of copper more fully complemented the defects.

### 5.4 Impact of *CaMAC1* and *CaAFT* Deletion on the Expression of the Putative Ferric Reductases

In Chapter 4 it was shown that transcript levels of a number of the reductases increase in response to iron or copper limitation. The expression of these putative ferric reductases was examined in the *CamacΔΔ* and *CaaftΔΔ* mutants to determine whether the *CaAft1p* and *CaMac1p* were involved in regulating the cellular response to iron and copper limitation (Figure 5.7). When the expression of the putative ferric reductases was compared in wild-type, *Caaft1ΔΔ*, and *Camac1ΔΔ* mutant strains a number of differences were evident. The deletion of *CaMAC1* abolishes the large rise in *CaFRE12* transcript levels in response to copper limitation. When combined with the observation that the promoter contains putative CuREs this suggests that *CaMac1p* directly regulates the copper responsive expression of *CaFRE12*. In common with *S. cerevisiae* (DeFreitas *et al.*, 2004) the deletion of *CaMAC1* also
Figure 5.6 Effect of pH upon the growth of the Caaft ΔΔ and Camac1 ΔΔ mutants. Cells were grown in MD media supplemented with 50 μM BPS and BCS and harvested at a mid exponential phase. Cells were then washed twice and diluted to a cell density of $1 \times 10^7$ cell ml$^{-1}$. Serial dilutions were then spotted onto the appropriate media “using a hedgehog”. To determine whether growth defects were due to iron and copper deprivation growth was also tested on media supplemented with either 100 μM FeCl$_3$, 100 μM CuCl$_2$, or 100 μM of both metals. The strains and serial dilutions used are illustrated in (c).
Figure 5.7 Expression of putative ferric reductases in *C. albicans*. Cells were grown to mid exponential phase in MD media containing either BPS or BCS and then harvested, washed twice, and the RNA extracted. Iron restrictive media containing 100μM BPS, 100μM CuCl₂, and either 0μM or 100μM FeCl₃. Copper restrictive media containing 100 μM BCS and 100 μM FeCl₃ was supplemented with either 0μM or 100 μM CuCl₂. Samples of RNA were transferred to nylon membranes using a dot blotter, fixed, and hybridised with radio-labelled probes. The probes used were internal fragments of the genes *CaACT1*, *CaFRE5*, *CaFRE10*, and *CaFRE12* or fragments from the 5’ UTR of *CaFRE1* and *CaFRE2*. 

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results in the aberrant expression of iron regulated genes in response to copper limitation. This is shown by the increase in CaFRE10 transcript levels in response to copper limitation in the Camac1ΔΔ mutant. The deletion of CaAFT also affected the expression of CaFRE10 in response to low iron levels. While the response of CaFRE10 transcript levels to iron limitation was unchanged in the Caaft1ΔΔ mutant levels of transcript were elevated compared to those in the other strains. This suggests that the deletion of the putative CaAFT has some impacts upon iron uptake and metabolism.

5.5 Discussion
The aim of the work in this chapter was to investigate the role of CaMac1p and CaAftp in the transcriptional regulation of the ferric reductases. As shown in Chapter 3 the promoters of the putative reductases contain sequences that are identical or similar to those necessary for the binding of ScAft1/2p and ScMac1p to S. cerevisiae promoters. The expression of a number of the reductases is also regulated in response to the availability of iron and copper in the media. Therefore, as CaMac1p and the putative CaAftp are potential regulators of the ferric reductases the affect of their deletion upon the activity and expression of the putative reductases was examined.

To determine the involvement of CaMac1p and CaAftp in iron and copper uptake and metabolism the disrupted strains were examined for phenotypes associated with defects in the uptake of these metals in S. cerevisiae and other organisms. The Caaft1ΔΔ mutant generated during this study displayed phenotypes that were indistinguishable from the wild-type in all of the growth tests performed. This suggests that the Caaft1ΔΔ does not perform an identical function to ScAft1p, which is essential for the correct functioning of high affinity iron uptake. The deletion of CaAFT also has a different effect upon the regulation of iron responsive genes to that which has been observed in Scaft1ΔΔ mutants. While the majority of iron responsive gene regulation is abolished in Scaft1ΔΔ mutants (Yamaguchi-Iwai et al., 1996) the putative Caaft1ΔΔ mutant displays an elevated level of CaFRE10 transcript in iron replete and limited conditions. However, this does suggest that the deletion of CaAFT has an impact on cellular iron transport or metabolism. However, it is impossible to determine whether the putative CaAftp is directly involved in the expression of
CaFRE10, or the changes observed in the CaafIΔΔ are due to secondary effects. For example, it is thought that while ScAfI2p is not vital for the expression of many iron regulated genes it increases the efficiency of iron uptake and metabolism in *S. cerevisiae* (Courel *et al.*, 2005). If the putative *CaAFT* plays a similar role in *C. albicans* its deletion could decrease the efficiency of iron usage, leading to an up-regulation of other iron regulated genes.

While our laboratory has previously identified a number of phenotypes associated with the deletion of CaMac1p the mutant strain was further characterised during this study. In phenotypic growth assays the *Camac1ΔΔ* mutant grew poorly at high pH and in the presence of hydrogen peroxide. The mutant phenotypes can be complemented by the addition of copper to the growth media, and also iron in the high pH studies. These phenotypic changes are consistent with those observed for Scmac1ΔΔ mutants (Lamb *et al.*, 2001; Jungmann *et al.*, 1993). The *Camac1ΔΔ* mutant also displays differences in the levels of ferric iron reduction and uptake when compared to reference strains. The elevated levels of ferric iron reduction observed in the *Camac1ΔΔ* mutant corresponded to the changes in the expression of CaFRE10 also seen in this mutant. Interestingly, while iron uptake was initially impaired in the *Camac1ΔΔ* mutant it increased after 60-90 minutes until wild-type levels were restored. As the mutant grows poorly in a range of conditions this lag may be because it takes time to adapt to the assay media used. Alternatively the lag in iron uptake may represent the time required to mobilise intracellular copper stores and incorporate them into the multicopper oxidase required for iron transport. Another possibility is that the cell may cope with the loss of *Camac1ΔΔ* by utilising other metal transporters that do not require copper for their function such as the Nramp family of cation transporters. While members of the Nramp family have not been experimentally characterised in *C. albicans* there are a number of putative homologues present in the genome sequence database (d’Enfert *et al.*, 2005).

When all of the phenotypes are compared it is interesting to note that those associated with the deletion of *CaMAC1* are often less severe than in the *Cactr1ΔΔ* mutant. This could be explained if the majority of phenotypes associated with the *Camac1ΔΔ* mutant are caused by the lack of *CaCTR1* expression in response to copper limitation.
previously observed in this strain (Marvin et al., 2004). However, as the CaCTR1 ORF is still intact in the Camac1ΔΔ mutant it may still be expressed at low levels. The copper transported into the cell by these small amounts of CaCtr1p may therefore explain the less severe phenotype associated with the Camac1ΔΔ mutant.

In conclusion, the results presented in this chapter suggest that CaMac1p has a similar role in the regulation of copper uptake to its S. cerevisiae homologue. In contrast CaAftp does not have a similar role to ScAft1p as the major regulator of genes in response to iron limitation. It is therefore possible that CaAftp may have a similar function to ScAft2p in modulating gene expression in response to iron limitation. However, the lack of phenotypes associated with Scaft2ΔΔ mutants and the CaaftΔΔ mutant generated during this study mean that this theory will be very hard to prove.
Chapter 6
General Discussion

During the course of this study 17 putative ferric reductases were identified in the *Candida albicans* genome sequence through their homology to CaFre1p, CaFre2p, and ScFre1p. All of these putative proteins are transmembrane proteins containing conserved motifs that are consistent with a role in the reduction of iron. When the ferric reductases were compared to each other, and with those from *S. cerevisiae*, it is evident that they can be divided into four groups. The majority of the *C. albicans* ferric reductases are most similar to ScFre1-6p, suggesting that they have a similar function in the reduction of iron. The second group of reductases show the most homology to two uncharacterised ferric reductases in *S. cerevisiae*, Ygl160p and Ylr047c. Interestingly, CaFrp1 and CaFrp2p share relatively low levels of homology with the *S. cerevisiae* ferric reductases and are more similar to proteins in *S. pombe* and other fungi. The fourth group of ferric reductases has a single member, CaFre12p, which is the only ferric reductase with significant levels of homology to ScFre7p. These two proteins are also relatively short and share a number of similar features including the lack of an N-terminal signal sequence and low levels of homology to the other reductases, even in the conserved motifs.

To determine whether the number of ferric reductases in *C. albicans* is unusual the genome sequences of a number of other fungi were examined. This showed that while *C. albicans* has the most putative ferric reductase their numbers vary widely between species. Unfortunately, the small number of fungal genomes that have been sequenced makes it impossible to determine whether there is a correlation between particular evolutionary lineages and numbers of ferric reductases. As many of the genome sequences are in the early stages of assembly the number of ferric reductases within them will almost certainly increase. To draw any firm conclusions regarding whether there is any evolutionary significance in the number of ferric reductases will require the sequencing of further fungal genomes, and the extensive annotation of those currently available or nearing completion. In the two cases where different strains of the same species have been sequenced it is interesting to note that the number of ferric reductases differs. While this probably reflects the different stages of completion of
the various projects it will be interesting to re-examine the data in the future to determine whether there is any strain variation.

The expression of a number of the ferric reductases was examined in the course of this study with transcripts being detected for *CaFRE1*, *CaFRE2*, *CaFRE5*, *CaFRE10*, and *CaFRE12*. The results showed that *CaFRE10* and *CaFRE12* are the most highly expressed under the conditions tested. These two genes show contrasting patterns of expression, as *CaFRE10* is negatively regulated in response to levels of iron while *CaFRE12* is negatively regulated in response to levels of copper. While transcripts were detected for the other genes their response to iron and copper levels was much less clear. Extremely low levels of transcript for these genes also hampered their study.

There are a number of possible explanations for the apparent lack of transcript for some of the reductases. The most obvious reason is that the reductases may not be expressed in the conditions used. Alternatively, transcript levels may be extremely low or very unstable, preventing their detection by northern blotting. There are two alternative approaches that could be used to combat these problems. The first of these involves the use of RT-PCR to study the expression of the ferric reductases. This method is capable of detecting very low levels of transcript, although it is often necessary to confirm the data produced using Northern analysis. The large number of ferric reductases also makes the Northern blotting and RT-PCR based approaches to expression analysis extremely time consuming and labour intensive if multiple conditions are tested. An alternative approach for studying gene expression is the use of microarrays. A number of different *C. albicans* expression arrays are currently available and could be used for this purpose. However, unfortunately as many of them were designed using early assemblies of the *C. albicans* genome database a number of ferric reductases and other iron uptake genes are missing from the arrays. An alternative would therefore be to create mini-arrays containing the genes of interest (Marcus Marvin personal communication). In general the use of microarrays would save considerable amounts of time in analysing the expression of the ferric reductases in the various morphological forms of *C. albicans* and in response to a variety of conditions such as iron or copper limitation and pH. Several recent reports have also developed techniques for analysing expression patterns in cells isolated from infected
animals (Reviewed in Nguyen et al., 2004). A similar approach may provide useful insights regarding the importance of the ferric reductases within the host organism. An additional benefit of using this genome wide approach is that it would generate a wealth of data regarding the expression of other genes under the conditions tested.

To further investigate the function of selected ferric reductases \textit{CaFRE2}, \textit{CaFRE5}, and \textit{CaFRE10} were disrupted and the mutants examined for defects in iron and copper uptake and metabolism. A \textit{Cafre1\Delta \Delta} mutant created in a previous study in our laboratory was also examined (Hammacott, PhD thesis). In all of the phenotypic tests performed the \textit{Cafre1\Delta \Delta} and \textit{Cafre5\Delta \Delta} mutants were indistinguishable from the control strains used. In contrast the \textit{Cafre2\Delta \Delta} mutant displayed reduced levels of ferric reductase activity. However, this did not affect ferric iron uptake, or the ability of the mutant to grow in iron or copper limiting conditions. Taken together these phenotypes are reminiscent of those associated with the deletion of \textit{ScFRE2}, which also causes a small decrease in ferric reductase activity while not affecting iron uptake (Georgatsou & Alexandraki, 1994).

The deletion of \textit{CaFRE10} causes a number of phenotypic changes including the loss of the majority of the cell surface ferric reductase activity associated with \textit{C. albicans}. These defects in ferric reductase activity impact directly upon the ability of the \textit{Cafre10\Delta \Delta} mutant to acquire iron. This demonstrates that the reduction of iron is important prior to its transport across the plasma membrane in the conditions tested. However, while iron uptake is decreased in the \textit{Cafre10\Delta \Delta} mutant it does not display defects in its growth under iron or copper limited conditions. Growth in iron limited conditions despite severe defects in iron uptake has been observed in a number of other \textit{C. albicans} mutants (Ramanan & Wang, 2000; Knight et al., 2002). It has been suggested in the absence of high affinity iron uptake \textit{C. albicans} may possess sufficient stores of iron to survive for a prolonged period of time. Alternatively, the residual, but still considerable, ferric iron uptake observed in the \textit{Cafre10\Delta \Delta} mutant may be sufficient for growth under the conditions tested.

A recent study has also reported the effect of the deletion of \textit{CaFRE10} upon ferric iron reduction and uptake (Knight et al., 2005). In this study the \textit{Cafre10\Delta \Delta} mutant
generated displayed a 98% decrease in cell surface reductase activity and a 75% decrease in ferric iron uptake. These observations agree extremely well with the results generated during the course of this study. In common with this investigation it was also noted that the deletion of CaFRE10 did not affect growth in iron limiting conditions (Knight et al., 2005). In addition, Knight et al. observed that C. albicans was able to utilise transferrin as an iron source, and that CaFre10p and the iron permease CaFtr1p were required for this process.

It is evident from this study that CaFre10p is the major cell surface ferric reductase under the conditions tested. In contrast, its role in the reduction and transport of copper is unclear. While the basal level of cupric reductase activity is greatly decreased in the CaFre10AA mutant there is still an increase in response to copper limitation similar to that observed in control strain. However, as the increase in reductase activity in response to copper limitation is relatively small the basal level of activity provided by CaFre10p may be very important for copper uptake. One way to answer this question would be to determine the rate of copper uptake in the CaFre10AA mutant to that in control strains. However, radioactive isotopes of copper are not readily available, expensive, and their use is problematical as in contrast to iron they often have a half-life measured in hours instead of years.

Although phenotypic analysis of the mutants shows that CaFre10p and CaFre2p are cell surface ferric reductases, the function of CaFre1p and CaFre5p is still unknown. There are a number of possible explanations for the lack of mutant phenotypes associated with the CaFre1AA and CaFre5AA mutants. For example, they may be involved in the reduction of iron bound to specific substrates such as siderophores. It is also possible that the lack of phenotypes was due to functional redundancy among the ferric reductases. Alternatively, CaFre1p and CaFre5p could be involved in the intracellular trafficking of iron, in which case their deletion would not alter the reduction or uptake of iron at the cell surface. Evidence for the presence of intracellular reductases comes from S. cerevisiae where ScFre5p and ScFre6p localise to the vacuolar (Huh et al., 2003) and mitochondrial membranes respectively (Sickmann et al., 2003) respectively. However, no mutant phenotypes are associated with the deletion of these reductases. Once again this may reflect the fact that most of
the phenotypic tests performed will only detect mutants with defects in cell surface reduction. The easiest way to determine if any of the *C. albicans* ferric reductases are involved in the intracellular reduction of iron will therefore be to study their localisation. Experiments are currently underway in our laboratory to test the hypothesis that some of the ferric reductases are located intracellularly. This will utilise GFP tagged ferric reductase constructs to determine the localisation of the ferric reductases in live cells. It may also be possible to utilise these constructs to examine whether the ferric reductases are post-translationally regulated in a similar fashion to the *S. cerevisiae* ScFet3p/ScFtr1p complex (Felice *et al.*, 2005).

This study has shown that it is possible to determine the function of at least some of the ferric reductases by their disruption and phenotypic analysis of the resultant mutants. However, although the ‘URA-blasting’ based strategy used during this study is an extremely effective tool for the disruption of genes in *C. albicans* it must be noted that there are a number of problems associated with the use of this technique (reviewed in Staab & Sundstrom, 2003). One of the major problems involves the use of *CaURA3* as an auxotrophic marker as there are a number of mutant phenotypes associated with *Caura3ΔΔ* mutants including attenuated virulence, reduced adhesion, and increased filamentation (Bain *et al.*, 2001; Brand *et al.*, 2004; Cheng *et al.*, 2003; Lay *et al.*, 1998; Sundstrom *et al.*, 2002). Although the *CaURA3* gene is re-integrated in the ‘URA-blasting’ technique it has been shown that it often does not abolish all of the mutant phenotypes associated with *Caura3ΔΔ* mutant (Lay *et al.*, 1998). This has produced problems in a number of studies where the phenotypes associated with a number of disruption mutants were shown to be the result of reduced levels of CaUra3p activity due to positional affects (Bain *et al.*, 2001; Brand *et al.*, 2004; Cheng *et al.*, 2003; Sundstrom *et al.*, 2002). Indeed, it has been estimated that as many as 30% of all mutants created by ‘URA-blasting’ may be affected by this phenomenon. However, this problem can be solved by the reintegration of *CaURA3* at selected loci, *CaRPS10* (Brand *et al.*, 2004; Staab & Sundstrom, 2003) or *CaENO1*, where it is known to have sufficient activity to restore full virulence.

The second problem associated with ‘URA-blasting’ is that the majority of uridine requiring strain in which deletion mutants are created carry a partial deletion of a gene
adjacent to CaURA3 (Garcia et al., 2001). While little is known about this disrupted gene, CalRO1, it is thought to have a role in iron metabolism as it is capable of partially complementing the phenotypes associated with a ScaftIAΔΔ mutant (Chibana et al., 2005; Garcia et al., 2001). However, Calro1p does not appear to be a homologue of ScAf1p and its role in iron metabolism is poorly defined, although Cairo1ΔΔ mutants grow slightly slower in iron deplete conditions and display reduced virulence. The problems associated with this extra deletion have now been solved by the creation of a uridine auxotroph in which CalRO1 has been reconstituted (Noble & Johnson, 2005).

There are a number of alternative methods available for the disruption of genes in C. albicans that have recently become available that do not have the problems associated with the ‘URA-blasting’ method. These include the use of disruption cassettes by fusion PCR that contain the auxotrophic markers HIS1, ARG1, and LEU2 from Candida maltosa and Candida dublinensis (Noble & Johnson, 2005). Strains lacking all three of these auxotrophic markers only display a slight reduction in virulence compared to Caura3ΔΔ mutants, and the re-introduction of any of them using the disruption cassettes corrects this slight defect. These disruption cassettes also have the advantage that different markers can be used to disrupt the two alleles of the gene of interest. This removes the need for counter-selection and reduces the number of false positives obtained during the second round of gene disruption where the ‘URA-blaste’r cassette often targets the previously disrupted allele rather than the remaining wild-type copy. As the auxotrophic markers in the cassettes are not from C. albicans the chance of the cassette incorrectly integrating at the site of the remaining native marker sequence should also be greatly reduced.

A number of alternative disruption cassettes have also been developed containing positive selectable markers that avoid the problems associated with the use of auxotrophic markers. These include a recyclable cassette containing a nourseothricin resistance gene that has been codon optimised for use in C. albicans (Reuß et al., 2004; Shen et al., 2006). A cassette containing luciferase has also been developed where re-integrants are selected by their ability to produce light in the presence of specific substrates (Doyle et al., 2006). Unlike methods based upon the use of
auxotrophic markers these cassettes have the added benefit that they can be used for disrupting genes in wild-type strains of *C. albicans*.

An alternative method involves the use of publicly available disruption cassettes that have been generated by transposon mutagenesis of a *C. albicans* genomic library (Bruno & Mitchell, 2004). Plasmids containing all of the ferric reductases with transposons inserted within their sequence are freely available and simply require transformation into *C. albicans*. This approach has the added benefit that it allows the disruption of both alleles of the wild-type gene in a single step. However, unlike the complete disruption of the gene of interest the insertion of the transposon is not guaranteed to affect gene function.

An important question regarding any process in *C. albicans* is its relevance in the infection process. While this study has demonstrated that CaFre10p is the major ferric reductase in the *in vitro* conditions tested its relevance *in vivo* is unknown. Indeed, it is not even known whether the extracellular reduction of iron by ferric reductases is important for the establishment and progression of *C. albicans* infections. However, the observation that Cafrt1ΔΔ mutants are avirulent strongly suggests that reductive iron uptake is essential for the infection process (Ramanan & Wang, 2000). These questions can be addressed by testing the mutants created during this, and future studies, in animal models to determine whether they are capable of causing an infection. A number of cell culture models are also available to investigate the ability of *C. albicans* mutants to adhere to and invade tissues (Bernhart *et al.*, 2001; Kimura & Pearsall, 1978). Models are also available to determine the ability of *C. albicans* mutants to survive engulfment by macrophages (Prigneau *et al.*, 2004). Analysing the ferric reductase mutants using these approaches may therefore reveal their importance *in vivo*.

One of the aims of this project was to identify transcription factors involved in the regulation of the ferric reductases in *C. albicans*. One of the candidates for regulating the expression of the ferric reductases in response to copper limitation was CaMac1p, which was previously isolated and characterised in our laboratory (Marvin *et al.*, 2004). Evidence for its role in the regulation of the ferric reductases included the presence of putative CaMac1p binding sites in the promoters of *CaFRE3*, *CaFRE6*,

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Northern blot analysis has confirmed that levels of CaFRE12 transcript increase in response to copper limitation in a CaMac1p dependent manner. Studies in our laboratory have therefore shown that CaMac1p regulates the copper responsive expression of at least two genes, CaCTR1 (Marvin et al., 2004) and CaFRE12. Interestingly, although the CaFRE10 promoter contains a putative CaMac1p binding site its expression is unaffected by copper limitation in wild-type strains. One explanation for this is that CaMac1p may require at least two CuREs for efficient transcriptional activation of target genes. Evidence to support this theory includes the observation that two CuREs are required to support the activation of ScCTR1 and ScFRE1 in response to copper limitation (Joshi et al., 1999; Serpe et al., 1999). This is because ScMac1p dimerisation is thought to be essential for transcriptional activation, requiring the binding of ScMac1p to two sites within its target gene promoters. However, questions still remain regarding this process as the proposed ScMac1p target genes YFR055w and YJL217w only contain a single CuRE (DeFreitas et al., 2004). Unfortunately, after their initial identification as ScMac1p target genes using microarrays and Northern blot analysis they have been ignored in further studies.

Further work is currently underway in our laboratory to investigate CaMac1p mediated gene activation. This has initially involved the use of the β-galactosidase reporter gene tested during this study to examine transcriptional activation from the promoters of CaFRE10, CaCTR1, and CaFRE12. Deletion analysis of the CaCTR1 promoter, which contains three CuREs, suggests that they are all required to support CaMac1p mediated transcriptional activation. The importance of the CuREs is also being examined by mutating each of these motifs and examining its effect on transcriptional activation in response to copper limitation. To confirm the requirement of the CuREs for CaMac1p binding gel mobility shift assays are also planned. This will also hopefully reveal whether CaMac1p binds as a multimer to the promoters of its target genes. It will also be interesting to determine whether CaMac1p can bind to the single CuRE within the promoter of CaFRE10.

The promoters of some of the putative ferric reductases also contain sequences that are similar to binding sites for ScAft1p and ScAft2p. While a number of other studies
have identified genes, *CalROI* and *MNN5*, that are able to partially complement the phenotypes associated with the deletion of *ScAFT1* neither of these acts as a transcription factor (Bai *et al.*, 2005; Bai *et al.*, 2006; Garcia *et al.*, 2001). During this study the *C. albicans* genome sequence was therefore searched for the presence of homologues of these iron responsive transcriptional regulators. A single putative homologue was identified that showed low, but significant, levels of homology. The CaAftp also contained several features in common with ScAft1p including the presence of conserved cysteines, a glutamine rich region, and putative NES sequences. However, the deletion of *CaAFT* suggests that it is not a functional homologue of ScAft1p. In all of the phenotypic tests performed the *CaaftΔΔ* mutant is indistinguishable form wild-type strains. This is in stark contrast to *ScaftlΔΔ* mutants, which display a wide array of mutant phenotypes associated with defects in iron uptake and metabolism (Yamaguchi-Iwai *et al.*, 1995; Blaiseau *et al.*, 2001). However, although this study shows that CaAftp does not have an equivalent role to ScAft1p it may still be involved in the regulation of iron uptake. One possibility is that it may have a similar cellular role to the ScAft1p parologue ScAft2p. Although the precise role of ScAft2p is unclear it is the major regulator of a number of genes involved in intracellular iron transport and metabolism (Courel *et al.*, 2005; Rutherford *et al.*, 2003). It has also been suggested that ScAft2p may fine-tune the transcriptional response to iron limitation. In common with the *CaaftΔΔ* mutant the deletion of *ScAFT2* does not cause any phenotypic changes except for small changes in the expression of a small number of genes (Courel *et al.*, 2005; Rutherford *et al.*, 2003?). The best method of determining the role of *CaAFT* may therefore involve using microarrays to compare the expression profiles of the *CaaftΔΔ* mutant with wild-type strains. This approach has the advantage that it may provide information about the role of CaAftp if it is not involved in the regulation of iron uptake and metabolism.

The lack of phenotypes associated with the putative *CaaftΔΔ* mutant may also be explained by the results of a recent study demonstrating that iron dependent gene regulation in *C. albicans* is predominantly mediated by a GATA-like transcriptional repressor, Sfu1p (Lan *et al.*, 2004). This suggests that *C. albicans* employs a similar mechanism of iron regulated expression to those observed in *N. crassa* (Zhou *et al.*, 1999), *A. nidulans* (Haas *et al.*, 1999), *U. maydis* (Voisard *et al.*, 1993), and *S. pombe*.
Although the mode of Sfu1p mediated transcriptional regulation is still poorly understood it probably involves the recruitment of the general transcriptional repressor CaTup1p (Lan et al., 2004). This would explain the earlier observation that a number of iron regulated genes are de-repressed in tup1ΔΔ mutants (Knight et al., 2002). There is evidence that additional factors for the regulation of iron uptake may be present in some fungi, as iron responsive regulation is not totally abolished by the deletion of SRE in N. crassa (Zhou et al., 1999). In A. fumigatus the expression of a putative ferric reductase encoded by freA is also up-regulated independently of SREA (Oberegger et al., 2002). It is therefore possible that ScaAf1/2p like proteins may be present in these fungi in addition to the GATA-like transcription factors. An interesting future approach in C. albicans may therefore be to create a ScaAf1ΔΔsfu1ΔΔ mutant and examine its affect upon the expression of iron regulated gene expression.

The current evidence suggests that while the components of the iron uptake pathway in C. albicans are very similar to S. cerevisiae their regulation is more similar to other fungi such as S. pombe and U. maydis (Haas et al., 1999; Pelletier et al., 2005). Indeed, the identification of GATA-type transcription factors in an increasing number of fungi suggests that the method of transcriptional regulation in response to iron limitation utilised by S. cerevisiae may be unique, or confined to a small number of closely related species. It has been suggested that the main role of the iron responsive GATA-type transcription factors is in the control of siderophore biosynthesis and uptake (Pelletier et al., 2005). The discovery of Sfu1p in C. albicans is therefore interesting as all of the other fungi that utilise this regulatory system produce siderophores. This adds further fuel to the debate over whether C. albicans is capable of producing siderophores.

One of the questions raised by this study is why does C. albicans have so many ferric reductases compared with S. cerevisiae and S. pombe? One explanation is that the large number of ferric reductases may be required to allow C. albicans to cope with the wide variety of environmental conditions encountered within the human body. This has been suggested as a reason for the expansion of a number of gene families in C. albicans although this hypothesis has not been tested (Braun et al., 2005; Jones et al., 2004). The large number of ferric reductases may also be required to cope with
the complex mechanisms employed by the human host to prevent acquisition of iron by microbes.

The large ferric reductase gene family in *C. albicans* may also be a consequence of the regulatory mechanism employed. During this study it was observed that unlike *S. cerevisiae* none of the putative ferric reductases in *C. albicans* were regulated in response to both iron and copper limitation. It is possible that this may reflect the different mechanisms regulating the transcription of iron uptake in the two organisms. In *C. albicans* the presence of the transcriptional repressor Sfu1p at promoters may prevent the activation of transcription by activators such as CaMac1p. This would require *C. albicans* to possess a greater number of ferric reductases in order to respond to a similar number of environmental signals.

In conclusion this study has shown that the *C. albicans* genome contains 17 potential ferric reductases. Disruption of selected genes has confirmed that CaFre2p and CaFre10p are cell surface ferric reductases. The severe defects in the reduction and uptake of ferric iron associated with the CafrelOAA mutant show it is the major cell surface reductase under the conditions tested. Tests also show that CaFre10p is capable of reducing copper, although its expression is not altered by changes in copper levels. The cupric reductase activity associated with the CafrelOAA mutant also reveals that there is at least one copper responsive ferric reductase in *C. albicans*. Expression analysis of the ferric reductases revealed that CaFRE12 is regulated in response to changes in copper levels. The levels of CaFRE12 transcript increase considerably in response to copper limitation in a CaMac1p dependent manner. Analysis of the CaFre12p protein sequence and its expression profile suggest it is a homologue of ScFre7p, which is predicted to have a role in intracellular copper transport and metabolism (Hassett & Kosman, 1995). Another candidate is CaFRE3, whose promoter also contains a pair of CuREs. Although a role for the majority of the ferric reductases was not determined they may function in the intracellular reduction of iron or be expressed in response to different environmental signals. Future work looking at their expression, localisation, and the affect of their deletion will help determine the role of these proteins. Analysis of mutants in animal models will also tell us more about the role of the individual reductases, and the importance of iron
reduction in general, in the establishment and progression of *C. albicans* infections. This will increase our understanding of the precise role of iron acquisition in the virulence of *C. albicans* and other pathogenic fungi.
References


