Isolation and characterisation of ferric reductase genes from the pathogenic yeast *Candida albicans*

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by

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Jane Hammacott

Abstract

Candida albicans is a pathogenic fungus that causes both mucosal and systemic disease in immunocompromised patients. Iron levels in the host environment are highly restricted and consequently iron acquisition is often found to be a virulence factor in pathogenic microorganisms. One strategy employed by microorganisms is to utilise a cell surface ferric reductase to release iron from host sources. Previously, work from our laboratory has demonstrated that *C. albicans* possesses such a ferric reductase activity which is likely to play a role in iron acquisition. It is the aim of this study to isolate ferric reductase genes from *C. albicans* with a view to assessing the importance of iron acquisition in the virulence of this organism.

Saccharomyces cerevisiae has been used previously as a tool and a model for the isolation of genes from *C. albicans*. *S. cerevisiae* possesses a well characterised iron uptake system, which uses a reductive mechanism for acquiring iron. A cell surface ferric reductase is used to reduce Fe^{3+} to Fe^{2+} and ferrous iron is then taken up into the cell via a specific transporter. Several genes have been isolated in association with this system and have been shown to be regulated in response to iron. Iron uptake in *C. albicans* has not been extensively studied to date, but the ferric reductase activity identified in our laboratory has been shown to be regulated in a similar manner to that of *Saccharomyces cerevisiae*, suggesting that the *S. cerevisiae* system provides a good model for iron uptake in *C. albicans*.

This study has identified two *C. albicans* ferric reductase genes, which are capable of rescuing a *S. cerevisiae* mutant defective in a structural ferric reductase gene, *FRE1*. Both genes, *CFL1* (*C*andida *f*erric reductase *l*ike gene) and *CFL2*, encode proteins that show significant sequence identity with known ferric reductase proteins. Northern blot analysis has shown that *CFL1* is negatively regulated by both iron and copper but *CFL2* was not expressed under the conditions tested. Northern blot analysis has also been carried out on 6 other ferric reductase-like genes, which were identified through analysis of the *C. albicans* genome sequencing project (http://alces. med.umn.edu/Candida.html). Only one of those genes, *CFL95*, was shown to be expressed under the conditions tested and this gene was also negatively regulated by iron and copper. A *C. albicans* strain in which *CFL1* has been deleted has been constructed and phenotypic tests on this mutant have shown that it still possesses a cell surface ferric reductase activity indistinguishable from wild type as well as still being able to grow in low iron conditions. This suggests that it may be an intracellular reductase or that the effects of deleting this gene may be masked by the presence of other ferric reductase-like genes, which may have redundant function.

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Or do they?

Debate, endlessly.....

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Abbreviations

ABC-transporter	ATP-binding cassette transporter
AIDS	Acquired immuno deficiency syndrome
ARS	Autonomous replication sequence
BCS	Bathocuproine disulphonic acid
bp	Base pairs
BPS	Bathophenanthroline disulphonic acid
BSA	Bovine serum albumin
CEN	Centromeric sequence
dATP	Deoxyadenosine 5'-triphosphate
dCTP	Deoxycytidine 5'-triphosphate
dGTP	Deoxyguanosine 5'-triphosphate
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide 5'-triphosphate
DTT	Dithiothreitol
dTMP	Deoxythymidylate monophosphate
dUMP	Deoxyuridylate monophosphate
dTTP	Deoxythymidine 5'-triphosphate
ECM	Extracellular matrix
EDTA	(Ethylenedinitilo) tetraacetic acid
FAD	Flavin adenine dinucleotide
5-FOA	5-fluoroorotic acid
GADPH	Glyceraldehyde-3-phosphate dehydrogenase
HGT	High gelling temperature
HIV	Human immunodeficiency virus
kb	Kilobase pairs
kDa	Kilodaltons
LA	Luria agar
LB	Luria broth
M	Molar
MAP kinase	Mitogen-activated protein kinase
MOPS	3-(N-Morpholino) propanesulphonic acid
mRNA	Messenger RNA
nt	Nucleotides
ORF	Open reading frame
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
SD	Synthetic defined
SDS	Sodium dodoecyl sulphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SSC	Standard saline citrate
TE	Tris-EDTA buffer
TAE	Tris-acetate electrophoresis buffer
Tris	Tris(hydroxymethyl)aminomethane
v/v	Volume by volume
w/v	Weight by volume

Contents

Chapter 1
Introduction

1.1 Overview

Candida albicans is a pathogenic yeast which causes both systemic and mucosal infections in humans. Interest in *C. albicans* has risen over the past few years due to the increased incidence of disease caused by this organism (Jarvis, 1995). The overall aim of the work being carried out in our laboratory is to isolate and characterise genes involved with iron uptake in *C. albicans*. Since iron levels in the host environment are highly restricted, it is likely that iron acquisition may play a role in virulence. Many pathogenic bacteria have been shown to require specialised high affinity iron uptake systems to acquire iron in the host environment (reviewed in Crosa, 1999), and the same is likely to be true for *C. albicans*. In some pathogenic bacteria, the low iron environment of the host has also been found to act as a trigger for the expression of other virulence factors unrelated to iron acquisition (reviewed in Griffiths & Chart, 1999). If the same is true in *C. albicans* then the elucidation of iron acquisition mechanisms in this organism will provide insights into the regulation of other virulence determinants.

This introduction is divided into eight sections. Firstly, aspects of the pathogenicity of *C. albicans* are discussed, including its epidemiology and the virulence factors that have been implicated in its disease causing ability. The relationship of iron to the expression of these virulence factors is also discussed. The second section discusses importance of iron as a nutrient and is followed by a discussion of the iron acquisition mechanisms used by some pathogenic bacteria and fungi, with a view to giving a summary of iron acquisition mechanisms used by microbes. This is followed by a detailed discussion of the well-characterised iron uptake mechanism of the related yeast, *Saccharomyces cerevisiae*, which was used as a tool for isolating *C. albicans* genes and a model system in this work. The current knowledge of the iron acquisition mechanisms used by *C. albicans* is then examined. Finally the background to this project and the specific project aims are discussed.

1.2 *Candida albicans* as a pathogen

1.2.1 Epidemiology

Candida albicans is a common commensal microorganism found in the mouth, gastro-intestinal tract and vaginal tract, and is thought to be harboured by most humans at some stage during their life time. However, it has an ability to cause serious disease in immunocompromised individuals and is therefore regarded as an opportunistic pathogen. Diseases caused by this organism vary in severity from the relatively mild mucosal infections, affecting the mouth and vagina, to often fatal systemic infections. The incidence of infections caused by *C. albicans* and other *Candida* species is rising, and this seems to be due to increasingly aggressive medical techniques, which lead to immune system suppression in patients exposed to these treatments.

Commonly, *Candida* is known for its ability to cause vaginitis or 'thrush', but it can also infect the urinary tract, lungs, bloodstream and surgical wounds in certain classes of patients (Jarvis, 1995). *Candida* species are now the fourth most common group of pathogens isolated from the blood stream of intensive care patients in the USA and the sixth most common from hospital-wide patients (Jarvis, 1995). *C. albicans* accounts for 76 % of *Candida* species isolates (Jarvis, 1995). There is a high mortality rate of 30-70 % (Beck-Sague & Jarvis, 1993) associated with systemic *Candida* infections and thus these infections present a serious problem. *Candida* infections are difficult to diagnose and treat since *Candida* is often not detected in blood cultures in cases of disseminated candidiasis. This makes it difficult to distinguish between these infections and other causes of pyrexia that fail to respond to antibiotics (Matthews, 1994).

Mucosal *Candida* infections are often associated with underlying disorders such as AIDS, pregnancy and diabetes mellitus, although otherwise healthy individuals can also succumb to these infections. Systemic infections, on the other hand, are associated with predisposing factors such as acute leukaemia, cytotoxic chemotherapy, treatment with wide-spectrum antibiotics and organ transplantation and catheterisation (Wenzel, 1995). It is interesting to note that AIDS patients do not often succumb to systemic infections except in the very late stages of disease (Matthews, 1994). Similarly, patients with various cell-mediated immunity defects often suffer from chronic monocutaneous candidiasis, but not systemic forms of

infection (Matthews, 1994). This is perhaps a reflection of the specific nature of the immune system defects in these diseases, and suggests that different immune system defects may contribute to the type of disease caused by *Candida*.

The treatment of *Candida* infections poses a serious problem for two main reasons. Firstly, in the case of systemic infection, the difficulty of diagnosis often means that the disease is left untreated and secondly, since yeasts are eukaryotes, the drugs that are currently available have toxic side-effects in host tissues. Amphotericin B, which binds to sterols in the cell membrane causing cell leakage and cell death, is the main drug used for treating systemic infections. This has limited toxicity to the host since it primarily targets ergosterol, which is not present in mammalian cell membranes (Georgopapadakou & Walsh, 1994). Other drugs including flucanazole, which target ergosterol biosynthesis, are now also being used. Problems are encountered with resistance to these drugs and 5-10 % of isolates from AIDS patients are now resistant to flucanazole (Matthews, 1994).

1.2.2 Putative virulence factors

At the molecular level, a virulence factor may be described as a gene or protein that when removed from the pathogen results in the loss or depletion of virulence in a suitable animal model. The reconstitution of the factor into the mutant pathogen should restore virulence. *C. albicans* possesses a complex virulence profile and many different factors are thought to be involved in its pathogenesis (Cutler, 1991). Most of these factors are involved with survival in host tissues, and include adhesion to multiple different surfaces within the host, the production of proteolytic enzymes, the ability to produce hyphae and phenotypic switching. These virulence factors will be discussed below, together with evidence of their role in virulence.

Adherence

Adhesion to host tissues is required for *C. albicans* to colonise the host and a variety of different *C. albicans* adhesins are likely to play a role in mucosal and systemic disease. In disseminated candidiasis, it is likely that adhesion to the vascular endothelial layer, followed by penetration into the target organs is necessary, whilst in mucosal infections adherence to epithelial surfaces is required (Cannon *et al.*, 1995; Klotz, 1992). *C. albicans* has been shown to have a number of different binding activities and is able to adhere to both

endothelial cells and epithelial cells as well as many extracellular matrix (ECM) proteins including fibronectin, laminin, type IV collagen and fibrinogen (Klotz, 1990). Using antibodies raised against purified laminin and fibronectin receptors of *C. albicans*, it has been shown that these proteins are expressed in patients with both systemic and superficial mucosal *Candida* infections (Lopez-Ribot *et al.*, 1996). Therefore, it is likely that these proteins play a role in adhesion during the infection process.

Interestingly, one *C. albicans* cell surface protein that has been shown to bind fibronectin and laminin is glyceraldehyde-3-phosphate dehydrogenase (GADPH; (Gozalbo *et al.*, 1998). This protein is normally associated with the cytosol, but has been shown to be localised to the cell surface in other pathogens, including the Gram-positive bacteria *Staphylococcus aureus* where it is involved in transferrin binding (Modun & Williams, 1999), although there is no evidence that this is its role in *C. albicans*. A second class of *C. albicans* ECM binding proteins show similarity to human integrin proteins. Integrins are a family of cell surface proteins which bind specifically to short peptide sequences in ECM proteins (Hynes, 1992). Antibodies against human integrins cross-react with proteins on the cell surface of *C. albicans* (Gilmore *et al.*, 1988), suggesting that *C. albicans* uses similar proteins for adhesion to the host ECM. Furthermore, small peptides containing the ECM protein binding sequence compete with whole ECM proteins for binding to the *C. albicans* cell surface. This suggests that *C. albicans* cell surface receptors bind to the same ECM protein sequences as human integrins (Hostetter, 1998). A *C. albicans* gene, *INT1* (Integrin-like), encoding a protein showing similarity to a region of a human leukocyte integrin has been identified (Gale *et al.*, 1996) and is implicated in adhesion, since it confers the ability to adhere to epithelial cells when expressed in *S. cerevisiae* (Gale *et al.*, 1998). Interestingly, it has also been shown that a *C. albicans int1* deletion strain is defective in its ability to form hyphae and is no longer virulent in mice (Gale *et al.*, 1998).

A large family of at least 8 genes, which show similarity to the *S. cerevisiae* agglutinin genes, have been identified in *C. albicans*, (Hoyer, 1999). In *S. cerevisiae* the protein products of these genes are involved in cell-cell interactions during the mating process (Lipke *et al.*, 1989). The role of this *C. albicans* gene family in adhesion has not yet been assessed since the effect of deleting of one member of the family may be masked by the presence of the other members. However, two members of the family, *ALS1* (agglutinin-like sequence) and

ALA1 (agglutinin-like adhesin), have been shown to cause the non-adherent yeast, *S. cerevisiae* to become adherent to extracellular matrix proteins and endothelial and epithelial cells, suggesting that they do have some role in adhesion (Fu *et al.*, 1998; Gaur & Klotz, 1997). The protein product of *HWP1* (hyphal wall protein), another gene implicated in adhesion, is a substrate of the mammalian transglutaminase enzyme and is likely to play a role in the permanent covalent attachment of *C. albicans* cells to endothelial surfaces (Staab *et al.*, 1999), representing a novel adhesion mechanism for invading microorganisms. It has recently been shown that deletion of the *HWP1* gene from *C. albicans* results in a strain that shows reduced virulence in the mouse systemic model of candidiasis. This mutant is also unable to cause damage to endothelial cells under *in vitro* conditions (Tsuchimori *et al.*, 2000). Interestingly, *HWP1* is only expressed during the hyphal growth phase, and is required for normal hyphal development (Sharkey *et al.*, 1999). This suggests that there may be a link between hyphal formation and the ability to adhere to host cells.

Secreted aspartyl proteinases and phospholipases

It has long been known that *C. albicans* secretes proteolytic enzymes when grown in media containing bovine serum albumin (BSA) as the sole nitrogen source (Staib, 1965) and since these enzymes have a wide range of substrates such as keratin, collagen, albumin, haemoglobin and immunoglobulin A (Morschhauser *et al.*, 1997; Ruchel, 1986) they are implicated in virulence and the ability of the organism to invade host tissues. Moreover, there is a strong correlative link between proteinase production and virulence. Experiments have shown that *C. albicans* isolates from HIV^+ patients with oral candidiasis consistently secreted more proteinase than isolates from HIV^- individuals or asymptomatic HIV^+ individuals (De Bernardis *et al.*, 1996). De Bernardis and co-workers (1996) also showed that high proteinase producing strains were more pathogenic than low proteinase producing strains in a mouse model of systemic infection. Furthermore, in a rat model of candidal vaginitis, pepstatinA, which is an inhibitor of this family of proteinases (De Bernardis *et al.*, 1999), was able to cure the infection.

Nine secreted aspartyl proteinase (*SAP*) genes have been isolated from *C. albicans* and the expression of these genes has been shown to correlate with damage to epithelial cells during the experimental infection of reconstituted human epithelium, which is used as a model for oral candidiasis (Schaller *et al.*, 1998). Transcription, detected using RT-PCR, demonstrated

that different *SAP* genes were associated with different phases of infection, and, interestingly, the *SAP6* transcript was detected concomitantly with the appearance of hyphae and severe lesions of the epithelium 48 hours after infection. RT-PCR analysis of *SAP* gene expression carried out *in vivo*, where patients with oral candidiasis were compared to asymptomatic carriers, showed a similar association of different *SAP* gene expression with differing stages of disease (Naglik *et al.*, 1999). *SAP4-6* genes have also been shown to be expressed during phagocytosis by mouse macrophages (Borg von-Zepelin *et al.*, 1998), which is interesting since the pH optimum of Sap4-6p corresponds to the pH within the phagolysosomes, and thus they may be acting as cytolysins to facilitate the release of *C. albicans* from the macrophage (Borg von-Zepelin *et al.*, 1998).

Deletion mutants have been used to assess the role of *SAP* genes in virulence. Interestingly, all three of the *sap1*, *sap2* and *sap3* mutants showed attenuated virulence in mouse and guinea pig models of disseminated candidiasis, despite the fact that only *sap2* showed significant loss of proteinase activity under culture conditions (Hube *et al.*, 1997). A triple mutant of *sap4-6* also showed attenuated virulence in the same models of disseminated candidiasis (Sanglard *et al.*, 1997). In the rat vaginitis model (De Bernardis *et al.*, 1999) all three *sap1-3* mutants showed reduced virulence, being cleared from the host significantly faster than the parental strain, SC5314. However, the *sap4-6* triple mutant did not show attenuated virulence in the rat vaginitis model, despite showing reduced virulence in mouse and guinea pig models of disseminated infection. This suggests that these proteinases may play a specific role in systemic infection but not in vaginal infection.

C. albicans also produces phospholipase enzymes, which may play a role in virulence. These enzymes may exert their effect by disrupting host cell membranes, and have been shown to be virulence factors for several other pathogenic microorganisms. *Listeria monocytogenes*, for example, requires two phospholipases in order to escape from the host vacuoles and for cell-to-cell spread (Smith *et al.*, 1995). Phospholipase production has also been correlated with mucosal invasion in *C. albicans* (Barrett-Bee *et al.*, 1985). More recently, one *C. albicans* phospholipase B gene (*PLB1*) has been shown to be expressed during systemic infection of mice and disruption of this gene resulted in attenuated virulence in the mouse model of disseminated candidiasis (Leidich *et al.*, 1998). These workers also showed that the ability of the *plb1* mutant to penetrate host cell monolayers was severely impaired *in vitro*.

Hyphal formation

C. albicans can grow in different morphological forms: yeast-like blastoconidia, which look similar to other budding yeasts such as *Saccharomyces cerevisiae*, and filamentous forms, which range from pseudohyphae to hyphae. Pseudohyphae consist of strings of cells that show constrictions at the septa, whilst true hyphae are filaments with no constrictions at the septa between cells. Hyphae are thought to contribute to virulence through their ability to penetrate endothelial and epithelial cell layers (reviewed in Corner & Magee, 1997) and there is strong evidence that hyphal production is important for virulence. *C. albicans* clinical isolates that are unable to produce hyphae at 37 °C have been shown to be less able to produce vaginal infection in pseudoestrus rats than strains that can produce hyphae (Sobel *et al.*, 1984). Furthermore, a *C. albicans* strain that was unable to produce hyphae *in vitro* was shown to be less virulent in terms of lethality in the mouse model of systemic candidiasis (De Bernardis *et al.*, 1993). However, interestingly, this strain still caused chronic infection in these mice and was also capable of causing vaginitis in rats (De Bernardis *et al.*, 1993). This evidence suggests that whilst hyphal formation may play a role in virulence, the relationship is likely to be complex.

Recent studies have concentrated on isolating genes that encode protein components of the signal transduction pathways involved in the yeast-hyphal switch. *S. cerevisiae* has been used extensively in these studies since it too can switch to form pseudohyphae (but not true hyphae) under certain conditions (Gimeno *et al.*, 1992). Several *S. cerevisiae* genes encoding components of the signal transduction pathway implicated in the yeast to pseudohyphae switch have been identified (Liu *et al.*, 1993) and it was thought that similar proteins might be found in *C. albicans*. The first *C. albicans* gene to be isolated that was implicated in filament formation was *CPH1* (*Candida* pseudohyphal regulator). *CPH1* was found due to its ability to induce pseudohyphal growth in *S. cerevisiae* cells in high copy number (Liu *et al.*, 1994). Cph1p shows sequence similarity with Ste12p, a *S. cerevisiae* transcription factor that is required for both filament formation and mating, and is activated by a MAP kinase cascade. This suggests that Cph1p is involved in transcriptional activation. A *C. albicans* strain defective in both copies of *cph1* is unable to form hyphae on solid media, but is still able to form hyphae in liquid culture containing serum (Liu *et al.*, 1994). This implies that there may be several hyphal induction mechanisms in *C. albicans*. In fact a second gene, which shows similarity to *CPH1* has recently been cloned. Deletion of this gene, named

CZF1 (Candida zinc finger), leads to defective hyphal formation under certain conditions and deletion of both *czf1* and *cph1* leads to a more severe phenotype (Brown *et al.*, 1999). Again, consistent with the theory that *C. albicans* uses several different mechanisms to induce hyphal formation, another gene encoding a transcription factor, implicated in hyphal formation in *C. albicans* has been cloned (Stoldt *et al.*, 1997). This gene, *EFG1*, encodes a protein that shows sequence identity with the *S. cerevisiae* transcription factor, Phd1p, which is capable of over-riding a *ste12* deletion when over-expressed (Gimeno & Fink, 1994). This suggests that this protein may be responsible for regulating hyphal production in *C. albicans* when components of the MAP kinase cascade are deleted. Further evidence to support this comes from the *efg1* deletion strain, which shows the ability to form pseudohyphae but not hyphae when induced in serum (Lo *et al.*, 1997). Lo and co-workers (1997) also showed that a *cph1/efg1* double mutant could not form hyphae under any conditions tested, and, moreover, was avirulent in the mouse model of systemic infection. Another gene implicated in hyphal production is *TUP1* (Braun & Johnson, 1997). This gene is also found in *S. cerevisiae* where it encodes a global regulator responsible for turning off gene expression. Interestingly, a *C. albicans tup1* mutant is found to grow constitutively in a pseudohyphal form (Braun & Johnson, 1997), suggesting that it functions by maintaining the yeast growth form by suppressing hyphal genes.

Two components of the MAP kinase cascade, thought to be involved in signalling the yeast to hyphal switch, have been identified by functional complementation of *S. cerevisiae* mating mutants. Both genes, *CST20* and *HST7*, encode proteins that show similarity to components of the mitogen-activated protein (MAP) kinase cascade (Kohler & Fink, 1996). *C. albicans* strains defective in these genes again display an inability to produce hyphae under some conditions but still form hyphae in when grown in serum (Kohler & Fink, 1996). Virulence studies have shown that the *cst20* mutant shows reduced virulence in the mouse model of systemic candidiasis, although no difference is observed between the wild type and *hst7* mutants (Leberer *et al.*, 1996).

Another factor implicated in the ability of *C. albicans* to form hyphae is vacuole formation. It has been shown that as hyphae extend, the regions behind the growing tip become highly vacuolated (Gow & Gooday, 1984). This suggests that intact vacuoles may be required for growth in the hyphal form. Several genes, which are homologous to *S. cerevisiae* genes

implicated in vacuole biogenesis, have been identified in our lab. Currently, *C. albicans* strain carrying mutations in these genes are being constructed with a view to assessing the impact of these deletions on hyphal formation (G. Palmer, personal communication).

Phenotypic switching

Phenotypic switching is likely to play a role in virulence since it may aid the invading fungus evade the host immune system. *C. albicans* has been shown to undergo high frequency phenotypic switching which can be observed as alterations in colony morphology on agar plates. This type of switching results in pleiotropic phenotypic alterations, including changes in drug sensitivities and sugar assimilation, which suggests that the switch regulates multiple genes. The *C. albicans* strain WO-1, which was originally isolated from the blood and lungs of a patient suffering from systemic candidiasis (Slutsky *et al.*, 1987), is the best-characterised strain that can undergo phenotypic switching. This strain undergoes colony morphology switches between hemispherical creamy white colonies (the white phenotype) and flat grey colonies (the opaque phenotype). There are striking differences between the cell morphology of the two colony types with white cells being similar to the budding yeast cells of most *C. albicans* and *S. cerevisiae* strains and opaque cells being much larger and bean shaped, with an unusual pimpled surface (Anderson & Soll, 1987; Slutsky *et al.*, 1987). The phenotypic switch observed in this strain is different from hyphal production since both cell types are also capable of forming hyphae (Anderson *et al.*, 1989). WO-1 is not unique in its ability to undergo such a switch and strains of *C. albicans* showing the same colony morphologies have also been isolated from other candidiasis patients (Hellstein *et al.*, 1993).

Although the mechanism of the switch has not been elucidated it has been shown that deletion of the *SIR2* (silent information regulator) gene, which encodes a protein involved with chromosomal silencing, results in an increased frequency of colony morphology switching, suggesting that chromosomal silencing may have a role in this type of switch (Perez-Martin *et al.*, 1999). A link between virulence and phenotypic switching has not been clearly established, however, the mis-expression of white genes in opaque cells and vice versa has suggested a potential link. For example, the expression of the white specific gene, *WH11*, in opaque phase cells increases the frequency of opaque to white phase switching and increases the virulence of opaque phase cells in the systemic candidiasis mouse model (Kvaal *et al.*, 1997). Conversely, the expression of the opaque phase specific gene, *SAP1*, in white

cells led to a dramatic increase in virulence in the colonisation of skin in the mouse cutaneous model (Kvaal *et al.*, 1999). Although it is difficult to interpret this data at present it does suggest that some features of the opaque phase cells may aid in establishing superficial *Candida* infections.

Influence of iron availability on other virulence determinants

Iron is an important nutrient for *C. albicans* (Sweet & Douglas, 1991b) and efficient iron acquisition mechanisms may play a role in the virulence of this organism. Some studies have attempted to demonstrate a link between iron uptake and pathogenicity in *C. albicans* and early work showed that addition of sufficient iron to serum to saturate its iron-binding capacity relieved the growth inhibition of *C. albicans* by serum *in vitro* (Caroline *et al.*, 1964). Furthermore, a correlation was found between free iron in serum and candidiasis in leukaemic patients (Caroline *et al.*, 1969). More recent studies have shown that iron chelators, such as phenanthroline, reduce endothelial cell damage by *C. albicans*, apparently by reducing phagocytosis of *C. albicans* by the endothelial cells (Fratti *et al.*, 1998). This suggests that iron is required either for the endothelial cells to phagocytose *C. albicans* cells, or that *C. albicans* is capable of inhibiting phagocytosis in low iron conditions. It is interesting to note that some studies have provided evidence for a link between anaemia and candidiasis (Higgs, 1973), suggesting that it is low iron levels in the host that induce candidiasis. This perhaps reflects the complex nature of interactions between host and pathogen, and may be a sign of the reduced efficiency of the host immune system under these conditions.

The growth inhibition of *C. albicans* by serum appears to be due to transferrin since this organism cannot use transferrin as an iron source and transferrin alone has been shown to have the same inhibitory effect on growth rate as serum (Moors *et al.*, 1992). Ovotransferrin and lactoferrin have also been shown to have an inhibitory effect on *C. albicans* growth (Valenti *et al.*, 1986). *C. albicans* has been shown to be able to use haemoglobin, haemin and ferritin as iron sources (Manns *et al.*, 1994; Moors *et al.*, 1992). Iron levels have been shown to have an effect on growth, adhesion and hyphal formation of *C. albicans* (Sweet & Douglas, 1991b). The growth rate of *C. albicans* cultured in low iron conditions *in vitro* (0.026 μM) was reduced 26-59 % depending on the strain used, and maximal adherence to buccal epithelial cells was found to occur after growth in 0.2-0.4 μM iron. Hyphal induction

was maximal in *C. albicans* cultures grown in 0.2-0.4 μM iron (Sweet & Douglas, 1991b). These studies used iron concentrations ranging from 0.026 μM to 0.8 μM iron, where 0.8 μM iron was excess to requirements since the addition of extra iron did not alter growth rate. Whilst it is difficult to relate these iron concentrations directly to iron availability in the host environment, the results clearly show that iron has an effect on a number of factors that may play a role in pathogenicity.

It has also been shown that the cell surface protein profile is altered by growth in high and low iron conditions. These alterations appeared to be quantitative rather than qualitative when judged by 1 dimensional SDS-PAGE analysis (Sweet & Douglas, 1991b). Another study, which used antibodies from the sera of patients with systemic candidiasis to probe Western blots of cell wall proteins of *C. albicans* grown in high and low iron media, detected two antigens of 45 and 40 kDa that were present in the cell wall of *C. albicans* grown in low iron media at 37 °C. These antigens were not detected in cell wall extracts from *C. albicans* strains grown in iron replete media (Paul *et al.*, 1989), suggesting that changes in cell surface proteins do occur in low iron conditions and also suggesting that these two antigens are expressed during infection.

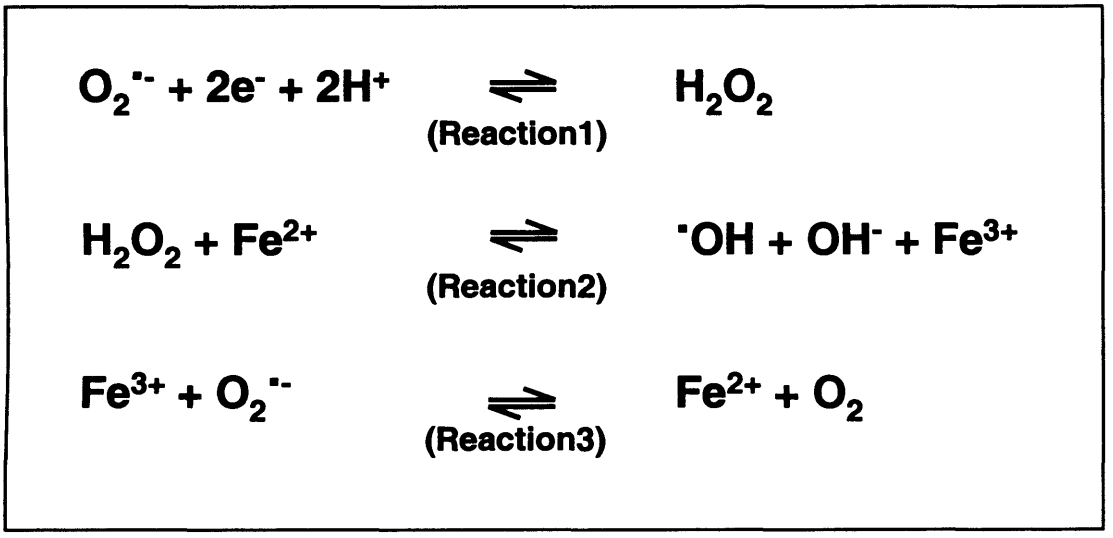
Interestingly, haemoglobin has been shown to affect *C. albicans* adhesion to extracellular matrix components such as fibronectin. It has been shown that growth of *C. albicans* in the presence of haemoglobin results in the expression of a saturable cell surface fibronectin binding activity (Yan *et al.*, 1996). This receptor is also capable of binding laminin and fibrinogen; a type IV collagen binding activity has also been shown to be induced under these conditions but via a different receptor (Yan *et al.*, 1998). The relevance of these observations to iron acquisition are not clear, but the induction does seem to be specific to haemoglobin since inorganic ferrous iron, protoporphyrin IX or haemin did not produce comparable effects, either individually or in combination with globin (Yan *et al.*, 1996). *C. albicans* may encounter free haemoglobin in host tissues due to tissue injury, which could in turn induce binding to extracellular matrix proteins, thereby facilitating tissue colonisation. Since *C. albicans* can produce a haemolytic factor, which allows it to use haemoglobin as an iron source, this too may play a role in inducing adhesion to extracellular matrix proteins.

1.3 Iron in nature: essential nutrient versus toxic metal

All living organisms, with the exception of lactic acid bacteria (Archibald, 1983; Pandey *et al.*, 1994), have an absolute requirement for iron. The importance of this transition metal is due to its ability to exist in two stable valencies, Fe^{2+} and Fe^{3+}, which allows it to facilitate redox reactions and makes it an essential cofactor for many enzymes. It is found, for example, in cytochromes as part of a haem cofactor, where its potential to switch between two oxidation states is used in electron transfer (Halliwell & Gutteridge, 1999). It is also found in enzymes such as catalase which catalyses the oxidation of hydrogen peroxide and other substrates to water (Halliwell & Gutteridge, 1999), as well as in ribonucleotide reductase and RNA polymerase (Reichard & Ehrenberg, 1983; Shoji & Ozawa, 1985). In its ferrous form it is essential for oxygen transport in higher eukaryotes, being an integral component of haemoglobin. However, its involvement in redox reactions makes it toxic at high concentrations due to its ability to catalyse Haber-Weiss-Fenton chemistry (Fig 1.1; (Halliwell & Gutteridge, 1999). The free radicals produced by this reaction can cause lipid peroxidation resulting in the breakdown of membranes and DNA strand breakage (Weinberg, 1989). Both of these reactions cause irreversible damage to cells and lead to cell death.

Despite being the second most abundant transition metal in the Earth's crust after aluminium, iron is highly insoluble. It is found mainly in the form of ferric oxyhydroxide polymers which have a solubility product of approximately 10^{-38} at pH 7, which limits the concentration of ferric iron in solution to 10^{-11} μM at neutral pH (Spiro & Saltman, 1969). As a result of the insolubility of ferric iron, coupled with the toxicity associated with excess intracellular iron, cells must possess mechanisms to solubilise ferric iron to allow transport into the cytosol, and additionally, they must be able to regulate that transport to keep intracellular iron concentrations within a tightly controlled range. These problems are faced by all living organisms and the restricted iron levels in the host environment present additional difficulties to the invading microorganism. In humans, levels of free iron which might be available for use by microorganisms are calculated to be approximately 10^{-18} M (Bullen *et al.*, 1978), a figure far below that required for growth. The majority of iron is found within cells complexed to haem, haemosiderin or ferritin (reviewed in Griffiths & Chart, 1999) and when found in extracellular fluids it is tightly bound to transferrin or lactoferrin (Morgan, 1981). Furthermore, during infection the amount of iron in serum is

Figure 1.1 Haber-Weiss-Fenton Chemistry.



Superoxide, a by-product of many electron transport chain reactions in the cell, can accept an electron and 2 protons to produce hydrogen peroxide (Reaction 1). Hydrogen peroxide can accept 1 electron from Fe(II), leading to the formation of the hydroxyl radical and hydroxide anion (Reaction 2). Superoxide can also react with Fe(III) to produce Fe(II), thus promoting the cycling of this reaction (Reaction 3). The hydroxyl radical is the toxic by-product of this set of reactions causing damage to DNA and lipid membranes.

reduced even further by a mechanism that apparently involves interleukin-1 (reviewed in (Ward & Bullen, 1999). This reaction, known as the hypoferraemic response, further reduces the amount of iron available to invading microorganisms.

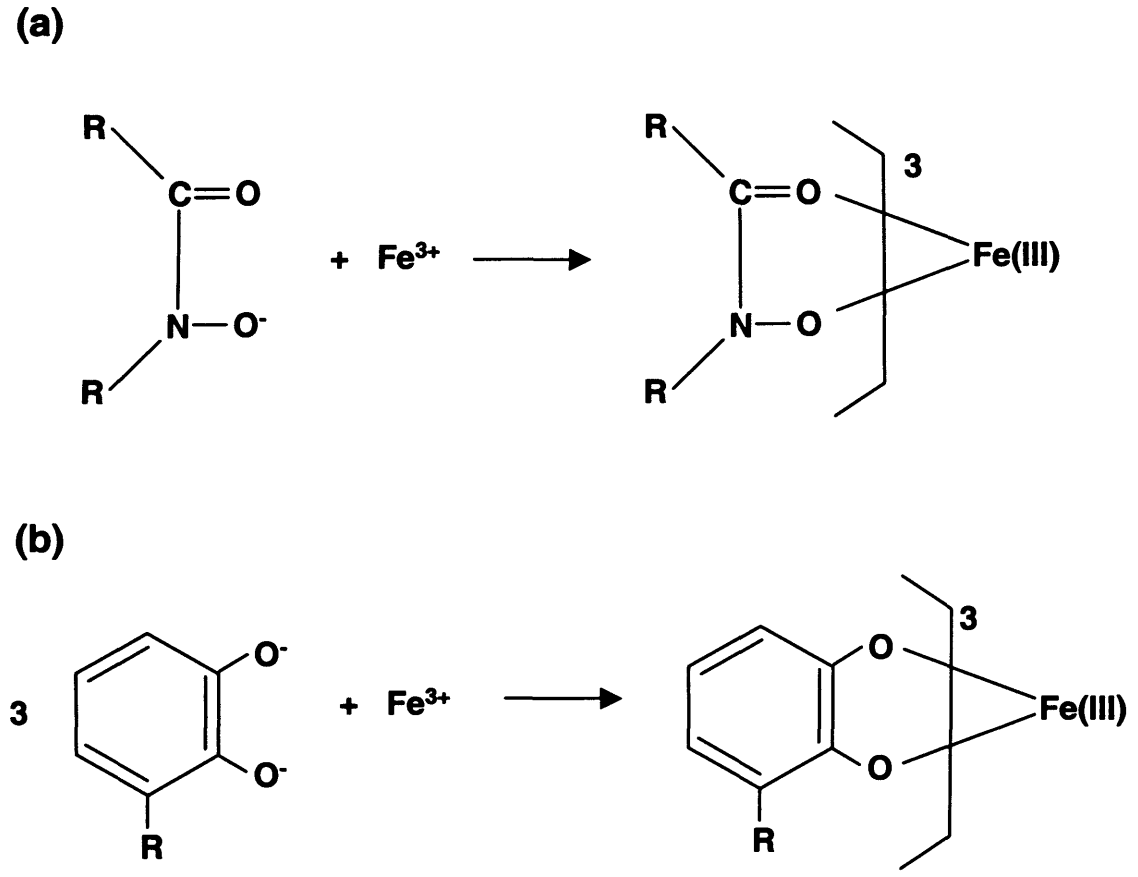
1.4 Microbial iron acquisition systems

As discussed in the section above, pathogenic microorganisms are presented with a particular problem in acquiring iron in the host environment. They therefore require specialised mechanisms for acquiring iron and such mechanisms may represent virulence factors affecting the pathogenicity of the invading microorganism. Indeed, the addition of iron to body fluids *in vitro* has been shown to abolish the antibacterial activity of these fluids (Williams & Griffiths, 1992) showing that the low level of iron found in the host represents a serious impediment to the survival of invading pathogens. Several mechanisms are employed by microbes in the assimilation of iron including: (1) the secretion of siderophores (low molecular weight iron-chelating compounds), (2) acquisition directly from host iron binding proteins such as haemoglobin, haem or transferrin and (3) the reduction of ferric iron at the cell membrane and subsequent transport of the more soluble ferrous iron. Each of these iron uptake mechanisms is discussed below by using specific examples from both pathogenic bacteria and fungi to give an overview of current knowledge in this field; this is followed by a discussion of other virulence factors whose expression are regulated by iron availability and the mechanisms controlling iron regulated gene expression.

1.4.1 Use of siderophore iron uptake system

A great many microbes including both bacteria and fungi rely on the production of siderophores for acquiring iron. Siderophores are low molecular weight compounds (500-1000 Da) with a high affinity for ferric iron and their biosynthesis is often found to be regulated by iron (Neilands, 1981). They generally fall into two families of compounds: phenolates or hydroxamates (Fig 1.2). Siderophores are secreted into the external environment where they scavenge and bind ferric iron. The ferric iron-siderophore complex then binds to specific receptors on the cell surface and is either taken up by the cell via

Figure 1.2 Generalised diagram of the two classes of siderophores



(a) Generalised structure of a hydroxamate siderophore. R indicates a organic side chain.

(b) Generalised structure of a phenolate siderophore where R indicates an organic side chain

siderophore specific transport proteins or is released at the cell surface for uptake (Neilands, 1982). Siderophore production has mainly been studied with respect to enterobacteria but other groups, including fungi, have been shown to produce them. Some microorganisms that are unable to synthesise their own siderophores, such as the yeast, *Saccharomyces cerevisiae*, and the bacterium, *Neisseria meningitidis*, are able to make opportunistic use of siderophores secreted by other organisms and possess specific transporters for taking them up into the cell (Lesuisse & Labbe, 1989; West & Sparling, 1987).

E. coli siderophore production has been studied extensively since it is easy to study due to the well-characterised genetics and biochemistry of this organism. *E. coli* produces two siderophores both of which have been detected in animals infected with this organism (Griffiths & Humphreys, 1980; Roberts *et al.*, 1989), suggesting that both siderophores play a role during infection. Both of these siderophores have also been shown to been produced by other *Enterbacteriaceae* including *Klebsiella pneumoniae*, *Salmonella typhimurium* and some *Shigella* species. This suggests that a common iron acquisition mechanism is shared between these bacterial species (reviewed in Griffiths & Chart, 1999). The first siderophore to be characterised with respect to *E. coli* was enterobactin (O'Brien & Gibson, 1970), a phenolate compound whose formation constant at neutral pH is 10^{52}, the highest recorded for a ferric iron chelator (Harris *et al.*, 1979). Enterobactin is used only once by *E. coli*, and once the ferric form is transported into the cell it is cleaved by a specific esterase (Greenwood & Luke, 1978). This is believed to raise the reduction potential enough to allow the release of iron from the chelator by reduction (Cooper *et al.*, 1978; Harris *et al.*, 1979). Pathogenic strains of *E. coli* have been shown to synthesize enterobactin (Rogers *et al.*, 1977) and enterobactin and its degradation products have been detected from peritoneal washings from guinea pigs lethally infected with *E. coli* (Griffiths & Humphreys, 1980).

A second siderophore produced by *E. coli* is aerobactin (Warner *et al.*, 1981), this was originally isolated from *Aerobacter aerogenes* (Gibson & Magrath, 1969), and belongs to the hydroxamate family of siderophores. In contrast to the biosynthesis genes for enterobactin, which are chromosomally encoded (Fleming *et al.*, 1985), the biosynthetic genes for this siderophore may be located either on a plasmid or in the chromosome (Valvano & Crosa, 1984; Williams, 1979). There is also some evidence that the aerobactin operon may be genetically mobile (de Lorenzo *et al.*, 1988). The production of aerobactin has been

correlated with virulence in *E. coli* and it has been shown that laboratory strains of *E. coli* carrying the aerobactin plasmid, ColV-K30, show greater virulence in mice than strains not carrying the plasmid. However, this effect is not observed if the mice are over-burdened with iron, suggesting that an enhanced ability to acquire iron is responsible for the greater virulence of the ColV-K30 carrying strains (Williams, 1979). Furthermore, *E. coli* isolated from humans and animals with *E. coli* infections are more likely to express the aerobactin-mediated iron uptake system than isolates from the faeces of healthy individuals (Jacobson *et al.*, 1988; Linggood *et al.*, 1987), and the production of this siderophore by *K. pneumoniae* has also been correlated with virulence (Nassif & Sansonetti, 1986).

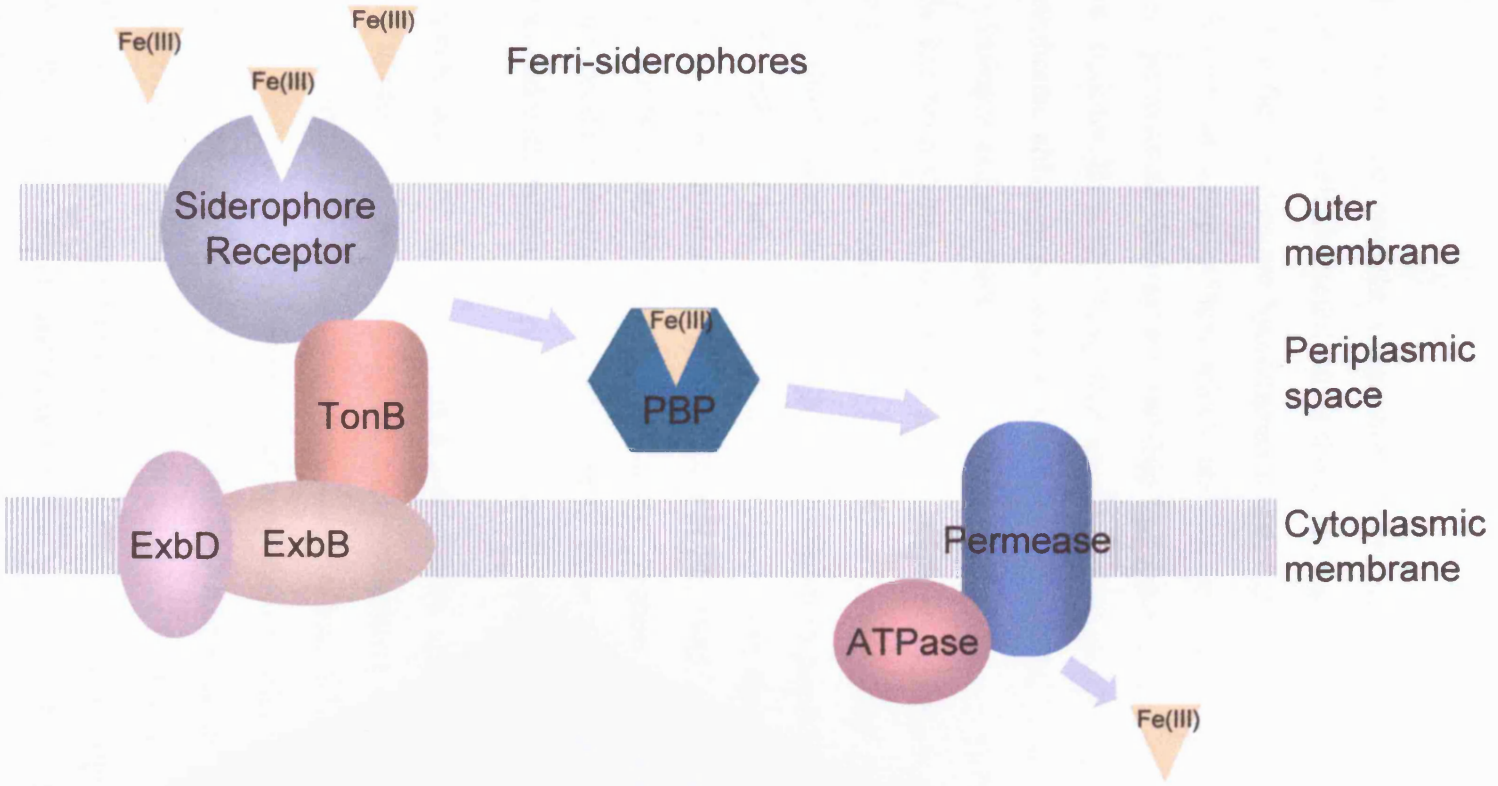
Several siderophore specific transporters have been identified in *E. coli* (Table 1.1) which are thought to be 'gated-pores', with extracellular domains that recognise specific ligands. These extracellular domains appear to form a cover over the surface of the pore, and it has been shown that deletion of these regions in the ferric enterobactin receptor (FepA) result in the loss of specificity of the pore (Rutz *et al.*, 1992). Transport through the periplasm is mediated via periplasmic binding proteins, which are specific for particular classes of siderophores; and across the cytoplasmic membrane by permease complexes, consisting of a permease and an ATP binding protein (see Fig 1.3 for an overview of siderophore uptake; Crosa, 1999). Again, the permeases are specific for particular classes of siderophores (Table 1.1; Fig 1.3).

Siderophore complexes are too large to cross the outer membrane of Gram-negative bacteria by simple diffusion, and an active transport system is therefore required to provide energy for this process. This is thought to be provided by a complex of proteins attached to the inner cytoplasmic membrane consisting of three components, TonB, ExbB and ExbD. TonB is thought to mediate the opening of the cell surface receptor whilst ExbB and ExbD may play a role in recycling TonB between an active and inactive conformation (Larsen *et al.*, 1994). It is thought that TonB is anchored in the cytoplasmic membrane by an uncleaved N-terminal signal sequence, and protrudes into the periplasmic space contacting cell surface receptors located in the outer membrane. Cross-linking experiments *in vivo* have shown that TonB interacts with the cell surface receptors for enterobactin and ferrichrome (Moeck *et al.*, 1997; Skare *et al.*, 1993) and this has been shown to occur through a highly conserved sequence in this family of receptors known as the TonB-box (Brewer *et al.*, 1990). The TonB-dependent

Table 1.1 Proteins used for siderophore uptake in *E. coli*

Siderophore	Outer membrane Receptor	Periplasmic binding protein	Permease	ATP binding cassette
Aerobactin	IutA	FhuD	FhuB	FhuC
Ferrichrome	FhuA	FhuD	FhuB	FhuC
Rhodoturulate/ Coprogen	FhuE	FhuD	FhuB	FhuC
Ferrioxamine B	FhuF	FhuD	FhuB	FhuC
Citrate	FecA	FecB	FecC/FecD	FecE
Enterobactin	FepA	FepB	FepG/FepD	FepC
Di-hydroxbenzylserine	Cir	FepB	FepD/FepG	FepC
Di-hydroxybenzylserine	Fiu	FepB	FepD/FepG	FepC

Figure 1.3 Generalised diagram of siderophore uptake in Gram negative bacteria



The ferric-siderophore is recognised by a specific cell surface receptor. The cell surface receptor is opened by an energy driven mechanism involving the TonB complex. The siderophore then passes into the periplasm where it is bound by a periplasmic binding protein, which transports the siderophore to a ABC transporter complex located in the cytoplasmic membrane. Diagram modified from Griffiths & Williams, 1999.

system of iron uptake is conserved in a wide range of Gram-negative bacteria and *N. meningitidis* utilises a TonB – ABC transporter system for internalising transferrin and iron from haem sources, as do *H. influenzae* and *Vibrio cholerae* (reviewed in Griffiths & Chart, 1999).

Many fungal species also produce siderophores, although the biosynthesis and transport mechanisms are not so well characterised as those of Gram-negative bacteria. Most fungal siderophores identified to date are hydroxamates, although a few are from a novel class of siderophores known as complexones, which are derived from citric acid (Winkelmann, 1992). Fungal hydroxamate siderophores fall into four main groups: ferrichromes, fusarines, coprogens and rhodotorulic acid. Fungi that are pathogenic to humans have been shown to produce siderophores, although no studies have been carried out to investigate the possible link between virulence and siderophore production. *Histoplasma capsulatum*, which causes histoplasmosis, has been shown to produce hydroxamate siderophores, and coprogen B and its breakdown products have been detected from this organism (Burt, 1982). Similarly, the human pathogen, *Blastomyces dermatitidis* has been shown to produce the same siderophore (Burt, 1983). *C. albicans* has been shown to produce both hydroxamate and phenolate siderophores in chemical colorimetric tests (Ismail *et al.*, 1985; Sweet & Douglas, 1991a), although these results must be treated with some caution since other by-products of biosynthesis may produce similar colour changes in these assays and ideally these results need to be associated with chemical structure data (see Section 1.6).

The non-pathogenic yeast, *S. cerevisiae* has a siderophore uptake system, even though it cannot produce siderophores itself. Although most exogenous siderophores appear to be reduced at the cell surface to release their iron (see Section 1.5; Lesuisse *et al.*, 1987), ferrioxamine B has been shown to be internalised, and the iron released by reduction within the cell (Lesuisse & Labbe, 1989; Lesuisse *et al.*, 1987). An open-reading frame, named *SIT1* (siderophore iron transport), encoding a member of the major facilitator superfamily has been identified which can rescue a mutant defective in ferrioxamine B transport (Lesuisse *et al.*, 1998). Recently, three more genes, encoding proteins related to Sit1p, have been isolated through the use of cDNA microarrays to identify iron-regulated genes (Yun *et al.*, 2000a). These have been named *ARN1* (*AFT1*-regulon), *ARN2* and *ARN4* (a fourth gene, *ARN3*, was also identified but this was found to be identical to *SIT1*). Studies of strains carrying

mutations in these genes have shown that Arn1p and Sit1p are responsible for the transport of ferrichrome and ferrichrome A, whilst Arn2p mediated the transport of triacetylfusarine (Yun *et al.*, 2000b). Interestingly, localisation studies showed that Arn1-4p and Sit1p were located in the endosomes of the cell suggesting that siderophores may be internalised by endocytosis and then taken up into the cytoplasm from endosomal compartments. This may be a reflection of the mechanism of energising the transport process. Members of the major facilitator family use chemiosmotic gradients to drive transport (Pao *et al.*, 1998) and the endosomes compartment may provide the necessary conditions for the transport process.

1.4.2 Use of host iron containing proteins

An alternative mechanism of iron acquisition is to scavenge iron directly from host iron binding proteins. Bacteria such as *N. meningitidis* and *N. gonorrhoeae* cannot produce siderophores but can remove iron directly from a number of host iron containing proteins including transferrin, lactoferrin and haemoglobin (reviewed in Griffiths & Chart, 1999). These organisms require direct contact with transferrin in order to acquire iron from this molecule (Archibald & Devoe, 1979), suggesting that they possess specific cell surface receptors for transferrin. *Haemophilus influenzae* has an absolute requirement for haem since it cannot synthesise it itself, and can use this as well as transferrin as a source for iron (reviewed in Griffiths & Chart, 1999). Gram-positive bacterial pathogens such as *Staphylococcus aureus* and *S. epidermidis* also utilise transferrin and haem but they use different cell surface receptors to those found in Gram-negative bacteria (reviewed in Griffiths & Chart, 1999). Little is known about whether pathogenic fungi can utilise host iron proteins, however, the pathogenic yeast, *C. albicans*, has been shown to be able to use haem, haemoglobin and ferritin as iron sources, but cannot use transferrin (see Section 1.6; Manns *et al.*, 1994; Moors *et al.*, 1992).

Transferrin and lactoferrin are likely to be important iron sources for the pathogens, *N. meningitidis* and *N. gonorrhoeae*, and both organisms can only utilise human forms of these proteins, suggesting that they have highly specialised mechanisms for acquiring iron from their normal host (Griffiths & Chart, 1999). There is evidence that transferrin and lactoferrin are important iron sources during infection since the injection of human transferrin into mice infected with *N. meningitidis* enhances the disease (Holbein, 1981; Schryvers & Gonzalez, 1989). Additionally, a *N. gonorrhoeae* mutant defective in the utilisation of

transferrin and haemoglobin as iron sources cannot grow in sub-cutaneous chambers implanted in mice (Genco *et al.*, 1991) and a mutant defective in transferrin uptake has been shown to be unable to cause urethritis in human male volunteers (Cornelissen *et al.*, 1998).

Two cell surface transferrin-binding proteins have been isolated from *N. meningitidis* and *N. gonorrhoeae* (Schryvers & Lee, 1989; Schryvers & Morris, 1988), named Tbp1 and Tbp2. These proteins are thought to form a complex at the cell surface and both are required for the efficient recognition and transport of transferrin. Tbp1 is typical of TonB controlled gated-pores and contains a TonB-box (Cornelissen *et al.*, 1992) indicating that Tbp1 is the pore that mediates transferrin uptake. This is interesting since it suggests that although these organisms acquire their iron in a different way to *E. coli* they use a similar mechanism to transport the iron into the cell. *N. gonorrhoeae* mutant strains lacking Tbp1 or Tbp2 showed that the loss of either protein leads to a reduction of transferrin binding, whilst loss of Tbp1 also means that transferrin can no longer be used as an iron source (Anderson *et al.*, 1994). It seems that Tbp2 is important in distinguishing between apo- and ferrated-transferrin since a *tbp2*$^-$ mutant is unable to distinguish between these two forms of transferrin, suggesting that the role of Tbp2 is to recognise iron-loaded transferrin (Cornelissen & Sparling, 1996).

Since the isolation of Tbp1 and the recognition of its similarity with known TonB dependent proteins, genes encoding TonB and its accessory proteins have been isolated from both *N. meningitidis* and *N. gonorrhoeae* (Biswas *et al.*, 1997; Stojiljkovic & Srinivasan, 1997). A periplasmic binding protein, Fbp (ferric binding protein), has also been identified which is capable of binding one molecule of ferric iron per molecule of protein (Berish *et al.*, 1992; Mietzner *et al.*, 1987). Mutants in the *tonB* gene are unable to use transferrin, lactoferrin or haemoglobin as iron sources, despite showing normal cell surface binding of these proteins, suggesting that transport of iron into the cell occurs via a similar mechanism to that found in *E. coli* for siderophore transport. *Neisseria* species can also use lactoferrin as an iron source and a gene encoding a component of the lactoferrin receptor has been cloned from *N. meningitidis* (Pettersson *et al.*, 1994).

Gram-positive bacteria such as *S. aureus* are also capable of using transferrin and lactoferrin as iron sources, although the cell surface receptors for transferrin uptake are not related to those found in Gram-negative bacteria. The transferrin receptor from *S. aureus* has recently been cloned and shows sequence homology to the cytosolic enzyme glyceraldehyde-3-

phosphate dehydrogenase (GADPH), and also displays the enzymatic activity of this enzyme (Modun & Williams, 1999). This is not the first example of this enzyme being found at the cell surface. *C. albicans* has also been found to express GADPH at the cell surface, where it is implicated in adhesion to extracellular matrix components such as laminin and fibronectin (section 1.2.2; Gozalbo *et al.*, 1998). The *S. aureus* transferrin binding protein has been shown to be expressed during infection using an intraperitoneal model of infection in rats (Modun *et al.*, 1998), suggesting that this is important for acquiring iron from the host during infection.

H. influenzae is capable of using transferrin as an iron source and cell surface transferrin binding proteins similar to those found in *Neisseriaceae* have been cloned (Gray-Owen *et al.*, 1995). However, *H. influenzae* is perhaps more interesting for its use of haem since it has an absolute requirement for this compound since it lacks most of the biosynthetic enzymes required to do to synthesise it itself (Coulton & Pang, 1983). It is therefore unsurprising that *H. influenzae* possesses multiple mechanisms for acquiring haem from a variety of different sources.

Haem is a cofactor for many different proteins in the human body. It is primarily thought of as a component of haemoglobin, the oxygen carrying protein located in erythrocytes in the blood stream, but a number of haemoglobin breakdown products are also found in the blood (reviewed in (Evans *et al.*, 1999). When haemoglobin is released from erythrocytes due to cell lysis it is rapidly bound to the blood borne protein haptoglobin, which is removed from the blood stream by the cells of the reticuloendothelial system. The trace amounts of haem that are released from haemoglobin are bound to haemopexin or serum albumin and are also removed from the blood stream. *H. influenzae* can scavenge haem from all of these blood-borne complexes. In order to acquire haem from the haem-haemopexin complex, a 100 kDa protein, HxuA (Cope *et al.*, 1994), is secreted that is able to bind this complex. The HxuA-haem-haemopexin complex is recognised by a specific cell surface receptor and internalised (Cope *et al.*, 1998). The *hxuA* gene is part of an operon containing two other genes, *hxuB* and *hxuC*, the second gene of which shows homology to a TonB-dependent outer membrane protein (Cope *et al.*, 1995), and possibly encodes the receptor for the HxuA-haemopexin-haem complex. The disruption of any of these genes results in the loss of ability to utilise haem-haemopexin as an iron source (Cope *et al.*, 1995).

The ability to use the haemoglobin-haptoglobin complex appears to depend on at least three genes encoding outer membrane proteins. These genes, designated *hgpA*, *hgpB* and *hgpC*, are each capable of mediating iron uptake from both the haemoglobin-haptoglobin complex and haemoglobin alone. Single and double mutants of these genes have no affect on the ability of *H. influenzae* to use haemoglobin-haptoglobin, but triple mutants have a reduced ability to use this complex (Morton *et al.*, 1999). HgpA is typical of a TonB-dependent protein suggesting that it mediates haem transport via a TonB-dependent mechanism. Indeed, it has been shown that *H. influenzae tonB* mutants are unable to utilise haem. Importantly, these mutants are also are found to be unable to produce invasive disease in animal models (Jarosik *et al.*, 1994). It is unsurprising that such a mutation should have an effect on virulence since it is likely to have pleiotropic effects, affecting iron acquisition from other sources as well as haem, and possibly uptake of other nutrients too. Another gene, *hhuA*, has been reported (MacIver *et al.*, 1996), the protein sequence of which shows 84 % identity to HgpA. In contrast to the three proteins discussed above, HhuA can only bind the haemoglobin-haptoglobin complex and not haemoglobin alone.

1.4.3 Use of reductases in iron uptake

Some organisms acquire iron by using extracellular ferric reductases to solubilise ferric iron and release it from chelators prior to uptake. The reductase activity may be either secreted or membrane bound and reduces ferric iron to its more soluble ferrous form, which is then transported across the cell membrane by specific transporters. Several pathogenic bacteria, including *Listeria monocytogenes*, *Legionella pneumophilia* and *Mycobacterium paratuberculosis* (Cowart & Foster, 1985; Homuth *et al.*, 1998; Johnson *et al.*, 1991) have been shown to possess ferric reductases as have the pathogenic fungi *Candida albicans, Cryptococcus neoformans* and *H. capsulatum* (Nyhus *et al.*, 1997; Timmerman & Woods, 1999).

Bacterial ferric reductases have not been well characterised, but *L. monocytogenes* has been shown to possess a ferric reductase. There is conflicting evidence as to whether the reductase is secreted or bound to the cell surface, or indeed, both (Barchini & Cowart, 1996; Deneer *et al.*, 1995). Significantly, the reductase is capable of removing iron from transferrin (Cowart & Foster, 1985) and also neurohormones such as catecholamines, which have iron binding capabilities (Coulanges *et al.*, 1997). This suggests that it may use the reductase to acquire

iron in the host environment. However, to date no experiments have been carried out to establish the role of the reductase(s) during infection. Interestingly, the ferric reductase produced by *M. paratuberculosis*, has been shown to be expressed in the gut tissue of naturally infected cattle, suggesting that this reductase may play a role in iron acquisition during the infection process, although a direct link with virulence has not been established (Homuth *et al.*, 1998).

Many fungal species produce ferric reductases which are capable of reducing iron bound to a wide range of chelates. The best characterised example of iron acquisition by this mechanism is that of *S. cerevisiae* which will be discussed in detail in Section 1.5. *Schizosaccharomyces pombe* also uses a reductive iron uptake mechanism. Several genes involved with this mechanism have been identified from this organism comparison of gene sequences with *S. cerevisiae* genes of known function (Askwith & Kaplan, 1997; Roman *et al.*, 1993). Three human pathogens, *C. albicans* (Morrissey *et al.*, 1996), *C. neoformans* (Nyhus *et al.*, 1997) and *H. capsulatum* (Timmerman & Woods, 1999) have also been shown to possess ferric reductase activities. The *C. albicans* ferric reductase system has been characterised biochemically, and this system is discussed in further detail in Section 1.6.

There are interesting parallels between the *S. cerevisiae* system and those of *C. neoformans* and *C. albicans*. The reductase activities of all of these organisms are regulated in response to iron and copper and are capable of reducing copper as well as iron (Dancis *et al.*, 1992; Morrissey *et al.*, 1996; Nyhus & Jacobson, 1999). This suggests that these organisms may possess similar iron uptake systems despite being evolutionarily distantly related. *C. neoformans* appears to possess two ferric reductase activities: one is secreted into the growth medium and is due in part to 3-hydroxyanthranilic acid, whilst the other is associated with the cell surface (Nyhus *et al.*, 1997). Both of these activities are negatively regulated in response to iron (Nyhus *et al.*, 1997). *C. neoformans* has also been shown to be able to transport ferrous iron and has two ferrous transporters, one with a high affinity for ferrous iron and one with a low affinity (Jacobson *et al.*, 1998). The high affinity system appears to be energy dependent since potassium cyanide abolishes activity, and it is also negatively regulated by iron (Jacobson *et al.*, 1998). Furthermore, it is apparently copper dependent since copper starvation also abolishes activity (Jacobson *et al.*, 1998). This is of interest since again, this is similar to the situation in *S. cerevisiae* where iron and copper transport are

inextricably linked (Section 1.5). Although no direct link has been shown between iron acquisition and virulence in *C. neoformans*, iron overload in the presence of deferoxamine has been shown to increase susceptibility to cryptococcosis in the experimental guinea pig model (Boelaert *et al.*, 1993). Moreover, the production of the cryptococcal polysaccharide capsule has been shown to be regulated by iron (Vartivarian *et al.*, 1993). The polysaccharide capsule represents a major virulence factor in this organism, helping it to evade the host immune system (Vartivarian *et al.*, 1993). Growth of *C. neoformans* in mammalian serum stimulates the production of the capsule, as does growth in iron-limited media, suggesting that the low iron conditions of the host may trigger production of the polysaccharide capsule. This is of interest since many bacterial virulence factors have been shown to be regulated by iron, but this is the first example of a fungal virulence factor being regulated by iron.

1.4.4 Iron responsive regulation of iron uptake genes and other virulence determinants

There are several different mechanisms used by different organisms to regulate gene expression in an iron dependent manner. The best characterised bacterial system is that of *E. coli*, and this same system appears to be conserved across a wide range of bacterial species (see below). The mechanisms of iron–regulated gene expression in fungi, however, appear to be more diverse and the systems used by the plant pathogen, *Ustilago maydis* (see this section) and the yeast, *S. cerevisiae* (see Section 1.5.4) have both been characterised. There are differences between the mechanisms employed by these organisms since *E. coli* uses a negative regulator, which binds to promoters of iron-regulated genes to prevent transcription in the presence of iron. However, *S. cerevisiae* uses a transcription factor which induces transcription from iron-regulated genes in the absence of iron. The *S. cerevisiae* mechanism of iron responsive gene regulation is described in more detail in Section 1.5.4.

The *E. coli* system uses a negative regulator protein named Fur (ferric uptake regulator), which binds to iron-regulated promoters in the absence of iron preventing transcription. The *fur* gene was cloned by mutant rescue of an *E. coli* mutant that showed constitutive derepression of *fhuA*, which encodes the outer membrane receptor for ferrichrome (Hantke & Zimmermann, 1981). The *fur* gene encodes a 148 amino acid, 17 kDa protein with a high histidine content (Bagg & Neilands, 1987). The Fur protein has been shown to bind the promoter of several iron-regulated genes including the aerobactin operon of the ColV-K30

plasmid, where a 78 bp region has been shown to confer iron-regulated gene expression and purified Fur has been shown to protect a 31 bp region within the larger region by DNase I foot-printing (de Lorenzo *et al.*, 1987). The Fur protein appears to bind to DNA as a dimer (Bagg & Neilands, 1987). *In vitro* translation studies using the aerobactin promoter fused to the *lacZ* gene in the presence of Fur showed that metal ions, such as Fe(II) or Mn(II) are also required to inhibit translation (Bagg & Neilands, 1987).

A Fur binding site has been identified in the promoters of genes regulated by Fur, which consists of a 19 bp consensus sequence showing dyad symmetry: 5'-GATAATGATAATCA TTATC-3'. When this oligonucleotide sequence was cloned into a *ompF* promoter fused to a *lacZ* gene it was shown to confer iron–regulated transcription on the *lacZ*, thus demonstrating that this region is sufficient for iron-regulation of gene expression (Calderwood & Mekalanos, 1988). Interestingly, the Fur binding site has been shown to overlap an RNA polymerase binding site and it has been proposed that the binding of Fur in the presence of iron prevents the binding of RNA polymerase, thereby preventing transcription. This has been shown to be the case using an *in vitro* translation system, where Fur and RNA polymerase were added sequentially (Escolar *et al.*, 1997). The addition of RNA polymerase prior to the addition of Fur and Mn(II) allowed one round of transcription to occur. However, addition of Fur followed by RNA polymerase prevented transcription (Escolar *et al.*, 1997), suggesting that the two proteins are indeed competing for overlapping binding sites.

Fur proteins are found in a wide range of Gram-negative bacteria including *Haemophilus influenzae*, *Neisseria meningitidis*, *Vibrio anguillarum* and *Pseudomonas aeroginosa* (reviewed in Crosa, 1999) and a Fur homologue has also been identified in the Gram-positive bacteria, *Staphylococcus epidermidis* (Heidrich *et al.*, 1996).

The mechanism of iron-regulated transcription in the fungus *Ustilago maydis*, a plant pathogen, has been partially characterised and a regulatory transcription factor has been isolated (Voisard *et al.*, 1993). The transcription factor, Urbs1 (*Ustilago* regulator of biosynthesis of siderophores), belongs to the GATA family of transcription factors, and is a 950 amino acid protein containing two putative zinc finger motifs. Strains that contain mutations in the *urbs1* gene show constitutive production of ferrichrome and mRNA of *sid1*, one of the biosynthetic genes involved with ferrichrome production (Mei *et al.*, 1993); section 1.4.1). Analysis of the promoter of *sid1* showed that a 63 bp region that contained

two GATA sequences located approximately 2.3-3.0 kb upstream of the ATG start site is responsible for iron–regulated expression of this gene (Leong *et al.*, 1995). Urbs1 has not been shown to bind iron and iron-responsive binding of Urbs1 to the promoter region of *sid1* has not yet been demonstrated. Given the large distance between the promoter and the regulatory region it is possible that Urbs1 controls chromatin structure.

Since the iron levels found in host organisms are low, it is perhaps unsurprising that bacteria appear to use low iron as a signal to regulate the expression of virulence factors unrelated to iron acquisition. Many bacterial toxins are regulated by iron, for example, diphtheria toxin, produced by *Corynebacterium diphtheriae,* Shiga-like toxins produced by *Shigella dysenteriae* and *E. coli* and α-haemolysins (which are also general cytolysins) produced by *E. coli* are iron-regulated. In some of these cases, the expression of these genes is regulated in a Fur-dependent manner. Since some virulence genes in pathogenic bacteria are regulated in this manner, a similar mechanism of gene regulation may be found in pathogenic fungi such as *C. albicans*. In terms of this project, it is hoped that the identification of *C. albicans* genes involved with iron uptake may lead to identification of regulatory elements, which may lead to the identification of other genes regulated by a similar mechanism.

At first glance, the diphtheria toxin produced by some strains of *C. diphtheriae* and the Shiga toxin and Shiga-like toxin 1 (SLT 1) produced by *S. dysenteriae* and *E. coli* respectively bear no relation to iron acquisition genes since all of three toxins inhibit protein synthesis in the host (Endo *et al.*, 1988; van Ness *et al.*, 1980). However, all three are found to be transcriptionally regulated by iron. Production of the diphtheria toxin has been shown to be regulated at the level of transcription (Leong & Murphy, 1985) and to be co-regulated with siderophore biosynthesis (Tsai *et al.*, 1990). The regulation takes place through the DtxR protein, which is the iron-responsive regulatory protein of this Gram-positive bacterium, and is functionally analogous to the Fur protein found in Gram-negative bacteria (Tao *et al.*, 1992). Both the Shiga toxin and SLT1 have been shown to be negatively regulated by iron (Jackson *et al.*, 1987; Strockbine *et al.*, 1988).

It is perhaps less surprising that the *E. coli* α-haemolysin should be regulated by iron, since the lysing of erythrocytes could present an mechanism of iron acquisition, however, it also capable of lysing a wide range of different human and animal cells including leukocytes. α-haemolysins are secreted into the growth medium and are pore-forming molecules. The

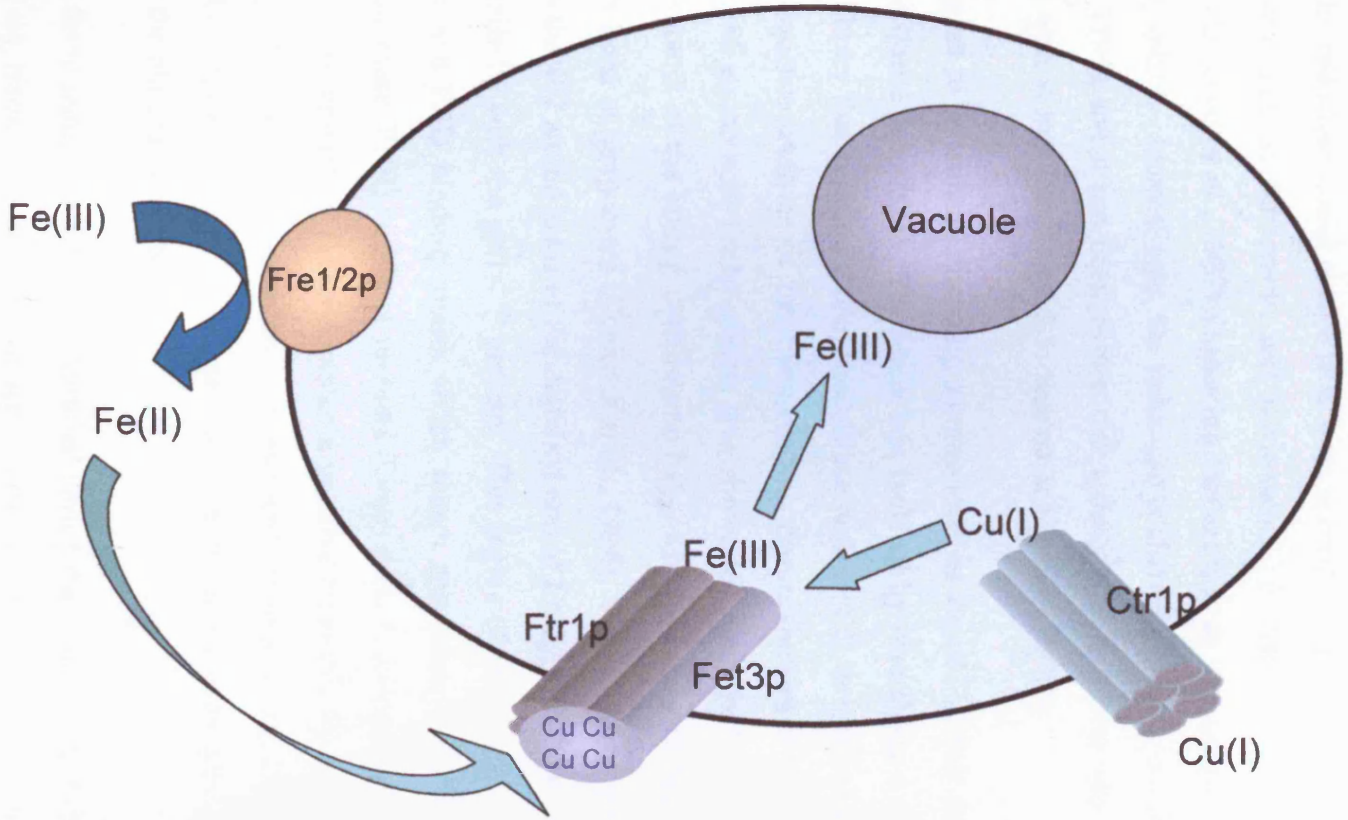
secretion of α-haemolysins has been shown to be regulated by iron levels in some strains of *E. coli* (Lebek & Gruenig, 1985).

1.5 *Saccharomyces cerevisiae* iron uptake system

The iron uptake system of *S. cerevisiae* is well characterised and many genes whose products play a role in this mechanism have been identified (Fig 1.4). This iron acquisition mechanism is of especial interest with regard to this project since *C. albicans* has been shown to possess a cell surface ferric reductase activity (Section 1.6; Morrissey *et al.*, 1996). The *C. albicans* ferric reductase activity is regulated in response to iron levels in a similar manner to the *S. cerevisiae* system suggesting that *C. albicans* may use a comparable mechanism for iron acquisition (Morrissey *et al.*, 1996). The extensive knowledge of the *S. cerevisiae* iron uptake system may therefore lead to valuable insights into the iron acquisition mechanisms used by *C. albicans*. The *S. cerevisiae* system uses a cell surface ferric reductase to reduce Fe^{3+} ions to Fe^{2+} ions, which are then taken up into the cell via a Fe^{2+} specific transporter. The transporter oxidises the iron back to Fe^{3+} during the transportation process. The components of both the ferric reductase and the transporter are regulated in response to iron levels.

Whilst the reductive mechanism of iron uptake is thought to be the major system used by *S. cerevisiae* to acquire iron, this organism has also been shown to possess a low affinity iron transporter, which operates in iron replete conditions and is partially regulated in response to iron (Dix *et al.*, 1997). *S. cerevisiae* can also take up some hydroxamate siderophores by a non-reductive energy-dependent mechanism (Section 1.4.1; Lesuisse & Labbe, 1989; Yun *et al.*, 2000b) and also secretes of anthranilate and 3-hydoxyanthranilate, both of which are capable of reducing iron and therefore may play a role in iron acquisition. However, this secreted ferric reductase activity is not regulated in response to iron (Georgatsou & Alexandraki, 1994; Lesuisse *et al.*, 1992) and its role in iron uptake has not yet been clearly elucidated.

Figure 1.4 Diagram of iron acquisition mechanism of *S. cerevisiae*



Iron is reduced to Fe(II) on the cell surface via the cell surface ferric reductase, coded for by the two genes, *FRE1* and *FRE2*. Fe(II) is then transported into the cell through the transporter complex, consisting of Fet3p and Ftr1p. Fet3p is a multicopper oxidase and requires copper for activity. Thus copper acquisition is a crucial step in iron uptake and iron and copper uptake are intimately linked (see Section 1.5.3; Fig 1.5). Once in the cell iron is transported to the vacuole from where it can be mobilised for use within the cell. Siderophore transport takes place through the Sit1p protein.

1.5.1 The Cell Surface Ferric Reductase

Early studies showed that *S. cerevisiae* possesses a cell surface redox system, which was capable of reducing extracellular ferricyanide (Crane *et al.*, 1982). Later studies demonstrated that iron is the physiological electron acceptor of this system and showed that the reductase activity is inducible by iron deprivation (Dancis *et al.*, 1990; Lesuisse *et al.*, 1987). The reductase could reduce iron from several different chelators including microbial siderophores such as ferricrocin and ferrioxamine B, both of which have high stability constants (Lesuisse *et al.*, 1987) suggesting that it plays an important role in iron acquisition by this organism. Interestingly, the reductase is also capable of reducing copper (Hassett & Kosman, 1995) and it has been shown that reduction is a crucial step in the acquisition of copper as well as iron (Section 1.5.3; Hassett & Kosman, 1995)).

The first gene to be isolated encoding a component of the cell surface ferric reductase system was *FRE1* (ferric reductase). This gene was isolated by identifying a clone from a wild type genomic library that rescued a ferric reductase deficient *S. cerevisiae* mutant (Dancis *et al.*, 1990). Sequence analysis of the open-reading frame showed it to be 2058 bp long. The predicted 686 amino acid Fre1p protein was shown to have significant similarity to $gp91^{phox}$, the large subunit of the human cytochrome b_{558}, which is an oxidoreductase involved in the respiratory burst of phagocytes (Chanock *et al.*, 1994). The sequence similarity is particularly marked in the 402 amino acids of the carboxyl end of Fre1p, which shares 17.9% identity and 62.2% similarity with the $gp91^{phox}$ protein. This region of the protein also contains potential NAD(P)H and FAD binding motifs which match consensus regions from the ferredoxin-$NADP^+$ reductase (FNR) family of proteins (Georgatsou & Alexandraki, 1994; Karplus *et al.*, 1991) The N-terminal of Fre1p contains a putative cleavable hydrophobic signal sequence suggesting that it is a membrane or secreted protein (Von Heijne, 1983); multiple hydrophobic regions suggest that it contains transmembrane domains and therefore that it resides in the plasma membrane.

A second ferric reductase gene was identified during the sequencing of chromosome XI. This open reading frame showed low but significant similarity to *FRE1* and was named *FRE2* (Casamayor *et al.*, 1995). Sequence analysis showed it to encode a 711 amino acid protein with 24.5% identity with Fre1p (Georgatsou & Alexandraki, 1994). Similarly to Fre1p, Fre2p possesses a hydrophobic N-terminal signal sequence and multiple hydrophobic

domains, which are likely to be transmembrane domains. It also contains the consensus regions for FAD and NADPH binding sites, both of which are thought to be important for electron transport (Georgatsou & Alexandraki, 1994). Deletion of both *FRE1* and *FRE2* leaves cells with a residual 2-10% reductase activity (Georgatsou & Alexandraki, 1994), which is consistent with the activity of the secreted phenolic compounds, anthranilate and 3-hydroxyanthranilate, which are capable of reducing iron and may play a minor role in iron uptake (Lesuisse *et al.*, 1992).

The Fre1p component of the ferric reductase has been shown to have a spectrum consistent with its being a cytochrome b (Lesuisse *et al.*, 1996; Shatwell *et al.*, 1996), suggesting that it must contain haem as a co-factor. This is in keeping with earlier observations that *hem1* strains of *S. cerevisiae*, which are unable to synthesise haem, also show defective ferric reductase activity (Lesuisse & Labbe, 1989). Mutational analysis of Fre1p has identified four histidine residues that are responsible for the haem binding activity of this protein. One pair of histidine residues separated by 13 hydrophobic amino acids appears in a hydrophobic domain, thought to be a transmembrane domain, in the N-terminal region of the protein. This hydrophobic domain is followed by an intervening hydrophobic domain, which in turn is followed by another hydrophobic region containing two histidine residues, again separated by 13 residues. Significantly, these histidine residues and their spacing is found to be highly conserved in this family of cytochromes, being similarly found in $gp91^{phox}$, the *S. pombe* orthologue, Frp1p, and Fre2p. By analogy with the stoichiometry of the human $gp91^{phox}$ (Quinn *et al.*, 1992; Segal *et al.*, 1992), the histidine residues are thought to co-ordinate two haem molecules, within the membrane and to mediate the transfer of electrons across the membrane. Although these histidine residues and their spacing is conserved in Fre2p, no haem spectrum has been observed for this protein even under conditions of over-expression (Shatwell *et al.*, 1996). The reasons for this are not yet clear.

Northern blot analysis showed that mRNA levels of *FRE1* were repressed in high iron conditions and induced in low iron (Dancis *et al.*, 1992), and similarly, a construct containing the *FRE1* promoter fused to a *lacZ* reporter gene was found to confer iron-responsive β-galactosidase activity on yeast cells carrying the construct (Dancis *et al.*, 1992). These results suggest that regulation occurs at the transcriptional level and not post-transcriptionally, as is the case for some iron-regulated genes in higher eukaryotes such as

transferrin (Klausner *et al.*, 1993). Additionally, it was shown that Fre1p expression was growth phase dependent with reductase activity peaking during exponential growth (Georgatsou & Alexandraki, 1994). The *FRE2* transcript is induced in low iron conditions in a similar way to *FRE1* although activity with respect to growth phase is found to be different. Reductase activity due to *FRE1* is found to peak during early log phase, whilst up to 80% of reductase activity found in later log phase is due to *FRE2* transcription (Georgatsou & Alexandraki, 1994).

There is evidence that cell surface ferric reductase activity may be positively regulated by the RAS/cAMP pathway (Lesuisse *et al.*, 1991) but it is not clear whether this is due to a direct interaction between protein kinase A and the ferric reductase or due to an indirect effect. It has previously been shown that the plasma membrane H^+-ATPase is regulated by cAMP (Ulaszewski *et al.*, 1989) and since the two systems have opposing activities: the H^+-ATPase moving hydrogen ions out of the cell, and the ferric reductase moving electrons out of the cell. It has therefore been suggested that the two systems may be coupled, and that the maximal ferric reductase activity requires fully derepressed H^+-ATPase activity, thereby explaining the apparent dependence of this system on cAMP (Lesuisse *et al.*, 1991).

Initial attempts to isolate the ferric reductase activity from the plasma membrane met with problems, as purifying to homogeneity led to loss of ferric reductase activity (Lesuisse *et al.*, 1990). It was therefore speculated that the ferric reductase complex might consist of several components, a view which is in keeping with the observation that Fre1p shows homology to gp91^{phox}, a component of the human phagocyte NADPH oxidoreductase. Human phagocyte NADPH oxidoreductase is a multicomponent complex consisting of a flavocytochrome b which has two membrane bound subunits gp91^{phox} and p21^{phox}, as well as other cytosolic components which play a role in the activation of the oxidase. Evidence to support the view that the yeast cell surface ferric reductase may also be a multicomponent complex came when a purified membrane fraction was shown to possess an NADPH ferric reductase activity, which was induced under iron deprivation (Lesuisse *et al.*, 1990). This activity did not co-purify with Fre1p and was also present in a *fre1Δ/fre2Δ* strain, leading to the suggestion that this represented a second component of the reductase system (Lesuisse *et al.*, 1996). It was subsequently found, however, that this activity was due to the 10-20 % of cytochrome P-450 which is associated with the inner leaflet of the plasma membrane (Lesuisse *et al.*, 1997). It

is now thought that Fre1p and Fre2p only account for the cell surface ferric reductase activity of whole cells.

Sequencing of the *S. cerevisiae* genome (http://genome-www.stanford.edu/Saccharomyces/) has identified 5 more genes with similarity to the two known ferric reductase genes, *FRE1* and *FRE2*. These have been named *FRE3-7* and their transcription has been shown to be negatively regulated by either iron (in the case of *FRE3-6*) or copper (*FRE7*) (Georgatsou & Alexandraki, 1999; Martins *et al.*, 1998). Since the deletion of both *FRE1* and *FRE2* results in almost complete abolition of cell surface ferric reductase activity, it seems likely that these genes are not cell surface reductases, but possibly play a role in intracellular iron transport. It seems likely that a ferric reductase activity may be found in the vacuole, since a transporter-ferroxidase complex comparable to the one found on the cell surface has been shown to be associated with this compartment (Section 1.4.2; Urbanowski & Piper, 1999). Additionally, early reports of ferric reductase activity associated with yeast cells showed that isolated mitochondria possess ferric reductase activity (Lesuisse *et al.*, 1990). This suggests that intracellular ferric reductases may be used to aid iron transport within the cell.

1.5.2 Iron transport

S. cerevisiae possesses two iron uptake systems which are biochemically and genetically separable. Both systems transport ferrous iron, as has been demonstrated by the fact that ferric reductase mutants are able to take up ferrous iron but not ferric iron (Dancis *et al.*, 1990); but one system has a high affinity with a K_M of 0.15-0.3μM for iron and the other a low affinity displaying a K_M of greater than 30μM (Eide *et al.*, 1992). The high affinity uptake system is found to be regulated in response to cellular iron content and is specific for ferrous iron (Stearman *et al.*, 1996), whilst the low affinity system is partially regulated by iron concentration and can transport other transition metals including cobalt, cadmium and nickel (Dix *et al.*, 1994).

High affinity iron uptake

The first gene to be isolated in association with the high affinity uptake system was *FET3* (ferrous transport; (Askwith *et al.*, 1994). This was isolated using a streptonigrin selection system, which has been used successfully in the past to isolate iron transport mutants in bacteria (Braun *et al.*, 1983). The system relies on the fact that streptonigrin diffuses into

cells where it can generate free radicals in the presence of iron, thus mutants defective in iron uptake are more resistant to the action of streptonigrin. *FET3* was found to rescue a mutant generated by the above procedure. Sequence analysis showed it to encode a 636 amino acid protein with significant similarity to the family of blue multicopper oxidases. Multicopper oxidases mediate the oxidation of their substrate, Fe(II) for example, during the 4 electron reduction of oxygen to water using copper as a cofactor (Solomon & Lowery, 1993).

$$4\ Fe(II) + O_2 + H^+ \rightarrow 4\ Fe(III) + 2H_2O$$

Multicopper oxidases contain 4 copper atoms in three spectroscopically distinct groups, known as type 1, type 2 and type 3 (Solomon & Lowery, 1993). Fet3p has been shown to contain all three groups and the stoichiometry of copper to protein has been shown to be approximately 4:1, as expected for this class of proteins (Hassett *et al.*, 1998).

Fet3p contains an N-terminal hydrophobic signal sequence (Von Heijne, 1983) and a hydrophobic sequence towards its C-terminal suggesting that it is a membrane bound protein with the majority of its mass protruding from the cell (de Silva *et al.*, 1995). Therefore, although *FET3* cannot code for the transporter itself, as it has only one putative membrane-spanning region, it may facilitate the transport of iron by oxidising ferrous iron to ferric iron. Northern blot analysis has shown that the *FET3* transcript is negatively regulated by iron concentrations in the media (Askwith *et al.*, 1994), supporting its proposed role in iron acquisition. Studies using polyclonal antibodies raised against small peptide sequences corresponding to C-terminal and N-terminal regions of Fet3p show that the N-terminal region of the protein is indeed extracellular as predicted by sequence analysis: cell lysates from spheroplasts treated with trypsin prior to detection with antibodies cross-reacted only with antibodies to regions of the protein predicted to be intracellular, whilst lysates from untreated spheroplasts cross-reacted with antibodies against both intracellular and extracellular regions of the protein (de Silva *et al.*, 1995). Furthermore, a recombinant Fet3p lacking the C-terminal hydrophobic domain is secreted into the extracellular media and displays ferroxidase activity (Hassett *et al.*, 1998). The finding that the ferroxidase domain is extracellular is consistent with all other known multicopper oxidases (Kojima *et al.*, 1990).

The presence of a ferroxidase activity on the cell surface presents an apparent paradox. Why should the cell first reduce Fe^{3+} to Fe^{2+} via the ferric reductase only to oxidise it back to Fe^{3+}

prior to uptake? It has been proposed that this mechanism imposes specificity on the transporter system (Askwith *et al.*, 1996). The ferrous transporter is the only part of the iron acquisition system of *S. cerevisiae* so far identified to show specificity for its substrate. The low affinity transporter can accept a wide range of transition metals, whilst the ferric reductase is known to reduce copper as well as iron, and may reduce other metals and compounds as well (Hassett & Kosman, 1995). The non-specificity of the reductase may be important since ferric iron may be presented to the cell bound to many different chelators. The function of the Fet3p protein may then be to introduce specificity to the system by recognising only un-chelated ferrous ions, which it oxidises and presents to the transporter protein. It has been suggested for organisms such as *S. cerevisiae*, which are unable to produce their own siderophores, that such a mechanism may be more efficient, as it means a wide range of chelators can be recognised and it avoids the need to take up "foreign" siderophores (Ecker *et al.*, 1986). Indeed the *S. cerevisiae* ferric reductase can reduce iron from ferric citrate, ferric EDTA (Lesuisse *et al.*, 1990) and from siderophores such as ferrioxamine B, ferricrocin (Lesuisse *et al.*, 1987), and rhodotorulic acid (Lesuisse & Labbe, 1989) and it appears that such non-specificity may be a general feature of reductive iron acquisition (Emery, 1987)

The gene encoding the transporter itself has also been cloned and named, *FTR1* (Stearman *et al.*, 1996). *FTR1* (ferrous transporter) encodes a 404 amino acid protein, which contains a N-terminal hydrophobic signal sequence and 6 putative membrane-spanning regions (Stearman *et al.*, 1996). The third putative transmembrane domain contains a 5 amino acid motif: REGLE, which is found to be conserved between homologous proteins from other organisms including *S. pombe* (Askwith & Kaplan, 1997) and *B. subtilis* (Stearman *et al.*, 1996) as well as with a similar open-reading frame from *S. cerevisiae*, named *FTH1* (*FTR1* homologue). This motif also bears resemblance to a motif found in mammalian ferritin light chains: REGAE, in which the glutamic acid residues have been shown to interact directly with iron and are thought to be involved in the formation of the iron core (Levi *et al.*, 1994; Trikha *et al.*, 1995). Site-directed mutagenesis of the glutamate residues in the REGLE motif of Ftr1p results in almost complete abolition of high affinity iron uptake, suggesting that they too may be important for the binding or recognition of iron (Stearman *et al.*, 1996). The 1.7 kb transcript of *FTR1* is induced by iron starvation as has been found for other components of this system (Stearman *et al.*, 1996).

Ftr1p has been shown to interact with Fet3p in a series of experiments in which both genes were cloned into multicopy vectors and were expressed either singly or doubly in yeast cells (Stearman *et al.*, 1996). Cells containing the multicopy vector encoding Fet3p did not show increased ferrous iron uptake and a similar result was seen for cells containing the multicopy vector encoding Ftr1p, however in those cells containing both the multicopy vectors an increase in iron uptake was observed. Interestingly, Ftr1p appears to have an additional role in the maturation of Fet3p. In the *ftr1-1* mutant, which is defective in Ftr1p expression a decrease in Fet3p oxidase activity is also observed. This seems to be due to the failure to insert copper into the maturing Fet3p since the addition of excess copper to the growth media restored ferroxidase activity (Stearman *et al.*, 1996). Fet3p maturation and copper loading still takes place correctly in yeast strains containing an allele of *FTR1* in which the terminal portion of this gene is truncated, resulting in the loss of ferrous iron uptake. This demonstrates that Ftr1p has a direct role in iron uptake and not merely an indirect role in the correct targeting of Fet3p (Stearman *et al.*, 1996). Conversely, site-directed mutagenesis of the *FET3* gene in the type 1 copper-binding site, results in loss of ferroxidase activity and high affinity iron uptake, but the correct localisation of Ftr1p and Fet3p is still observed (Askwith & Kaplan, 1998b). Thus, each protein is dependent on the other for correct localisation, and both are required intact for high affinity iron uptake.

Low affinity Iron Uptake

The low affinity iron uptake system is encoded by *FET4* (Dix *et al.*, 1994). This gene was isolated in a screen for multicopy suppressors of the slow growth on iron phenotype of a *fet3*Δ mutant. The predicted protein product of the gene is 552 amino acids in length and contains a high proportion of hydrophobic residues arranged in 6 regions, each large enough to constitute a membrane-spanning domain. Immunofluorescence microscopy using antibodies raised against Fet4p shows that it is localised to the periphery of the cell (Dix *et al.*, 1997). This, taken together with the fact that deletion of this gene destroys low affinity uptake and that an up-regulated mutant shows increased low affinity iron uptake (Dix *et al.*, 1994), suggests that *FET4* may encode the low affinity transporter and that it may represent the only component of the low affinity system. The low affinity system is negatively regulated by iron, with an approximately 3-fold increase in activity in iron-depleted conditions. However, the mechanism of regulation appears to be different to that of the high affinity system, since an *AFT1-1*^{up} mutant, which causes constitutive expression of genes

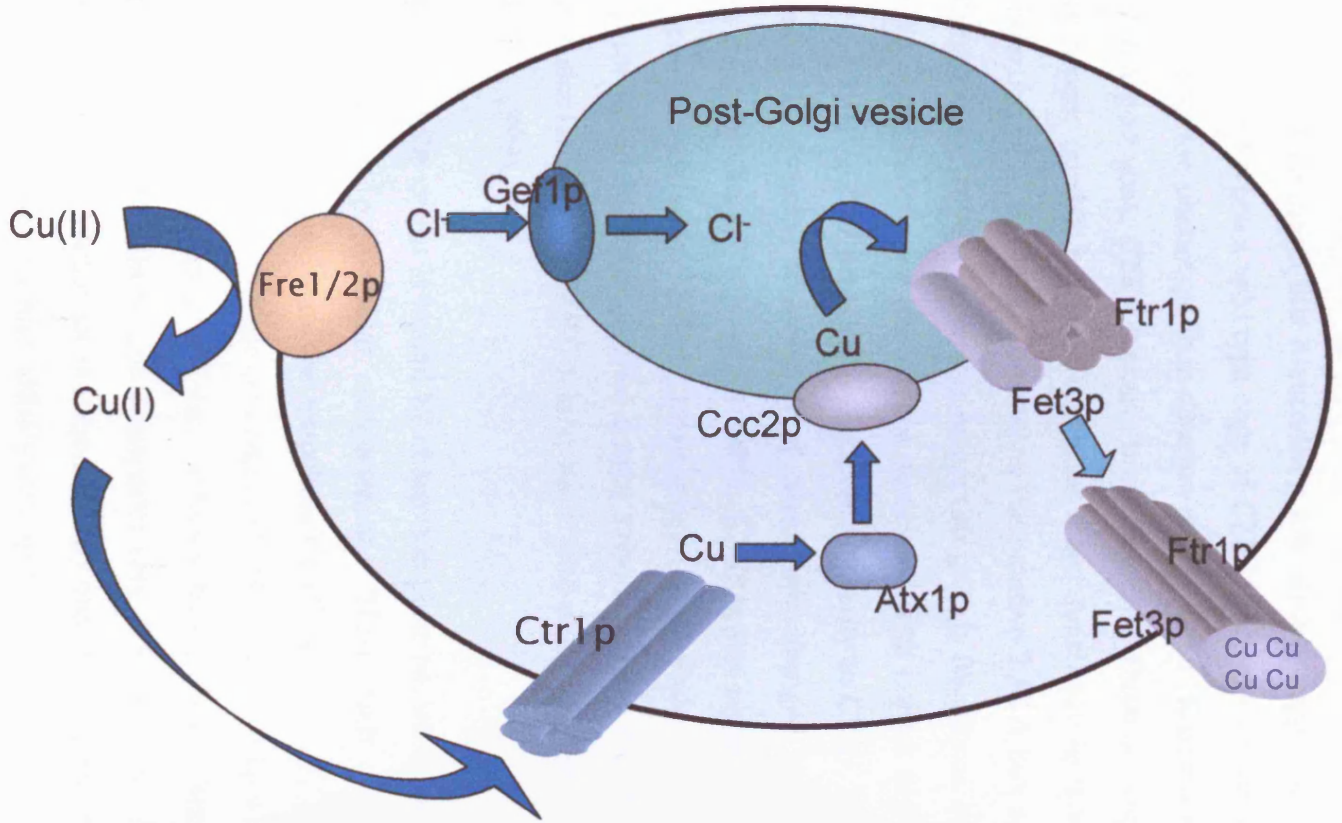
involved in the high affinity iron uptake system (see section 1.5.4), has no effect on the low affinity system (Dix *et al.*, 1997).

1.5.3 Copper transport and intracellular trafficking

An interesting early observation showed that disruption of the copper uptake pathway of the yeast cell resulted in pleiotropic effects on iron uptake. The *S. cerevisiae* copper uptake gene, *CTR1* (copper transporter), was isolated during a screen for mutants showing up regulation of *FRE1* transcription in high iron conditions (Dancis *et al.*, 1994a). Mutants defective *CTR1* were found to be unable to take up iron, although the addition of high concentrations of copper to the growth media could restore high affinity iron uptake, but not high affinity copper uptake (Dancis *et al.*, 1994a). This phenotype is consistent with observations that copper is an essential cofactor of Fet3p, a critical component of the iron transport complex, and provides evidence of an intimate relationship between iron and copper transport (see Fig 1.5). A further link between iron and copper uptake is found in the cell surface ferric reductase, which is capable of reducing copper as well as iron (Hassett & Kosman, 1995). The reduction of Cu(II) to Cu(I) is a necessary prerequisite for copper uptake by yeast cells and *FRE1*, which encodes a structural component of the cell surface ferric reductase activity, is found to be transcriptionally regulated by copper as well as iron (Hassett & Kosman, 1995).

Sequence analysis of the *CTR1* gene shows that the predicted protein has an unusual amino terminal domain consisting of 24% methionine, 38% serine, 6% threonine and 0% aromatic amino acids and which includes 11 repeats of the Met-X_2-Met repeat (X = any amino acid). Similar sequences have been found in bacterial copper handling proteins (Cha & Cooksey, 1991; Odermatt *et al.*, 1993) suggesting that this region of the protein may be important for the binding or recognition of copper. Although Ctr1p is suggested to be a copper transporter it contains only 2–3 transmembrane domains, whereas most transporter proteins contain between 6 and 12 membrane spanning regions. It has, therefore, been postulated that Ctr1p exists as an oligomer in the membrane and this has been confirmed by experiments using two *CTR1* constructs tagged with c-*myc* and the influenza haemagglutinin (HA) gene respectively. Lysates from cells expressing both forms of Ctr1p were immunoprecipitated using the HA tag and the products of the immunoprecipitation were analysed in a Western blot using an anti-c-myc antibody, and *vice versa*. In both cases cross-reactivity was observed suggesting that

Figure 1.5 The iron-copper connection: copper transport into the yeast cell



Copper is reduced to Cu(I) by the cell surface ferric reductase and transported into the cell via a copper specific transporter, Ctr1p. The imported copper is bound to an intracellular copper chaperone, Atc1p, which mediated the transport of copper to a post-Golgi vesicle. Copper is transported into the vesicle via a P-type ATPase, Ccc2p and then inserted into Fet3p, one of the components of the high affinity iron transporter. The Fet3p/Ftr1p complex is then transporter to the cell surface where it is responsible for high affinity iron transport into the cell.

Ctr1p does indeed exist as an oligomer (Dancis *et al.*, 1994b). Northern blotting showed that *CTR1* transcript levels are negatively regulated by copper concentrations in the growth media (Dancis, 1994b).

Two other copper transport genes have also been isolated. Following the cloning of a copper transporter gene, *COPT1*, from *Arabidopsis thaliana* a second *S. cerevisiae* gene, *CTR2*, has been identified by sequence homology with *COPT1* (Kampfenkel *et al.*, 1995). Disruption of this ORF led to the suggestion that it encoded a low affinity copper uptake protein since no phenotype is observed when a wild type copy of *CTR1* is present, but deletion of *ctr1*and *ctr2* results in a more severe phenotype than deletion of *ctr1* alone (Kampfenkel *et al.*, 1995). A third copper transport gene, *CTR3*, has also been identified which is thought to play a role in high affinity copper uptake; however, this gene is not functional in most laboratory strains due to the insertion of a Ty2 element between the putative TATA box and the beginning of the ORF (Knight *et al.*, 1996). In strains where this gene is functional, both *CTR1* and *CTR3* need to be disrupted before any phenotypes associated with copper deficiency are observed (Knight *et al.*, 1996). *CTR3* bears little sequence similarity to *CTR1*, however, it is cysteine rich, containing 11 cysteine residues, 6 of which are arranged in pairs, which may be conducive to metal binding (Winge *et al.*, 1985). *CTR3* is also regulated at the transcriptional level by copper (Knight *et al.*, 1996). Interestingly, a *S. pombe* copper transport gene has recently been identified and appears to be similar to both *CTR1* and *CTR3* of *S. cerevisiae*: its 5' end shows similarity with *CTR1*, whilst its 3' end shows higher similarity with *CTR3* (Labbe & Thiele, 1999).

Once copper enters the cell it is bound by chaperone proteins, which are responsible for its delivery to the correct intracellular compartment. Three such chaperones are known: Cox17p, which delivers copper to the mitochondria (Glerum *et al.*, 1996); Lys7p, which delivers copper to cytosolic dismutase (Culotta *et al.*, 1997) and Atx1p which delivers copper to the late Golgi where it is transported into the lumen by Ccc2p and then delivered to Fet3p, a component of the high affinity iron transporter (Fig 1.5; (Askwith & Kaplan, 1998a)). Yeast strains that contain deleterious mutations in any one of the genes: *CTR1*, *ATX1*, *CCC2* and *FET3* are unable to carry out high affinity iron uptake.

CCC2 ($\underline{C}a^{2+}$-sensitive $\underline{c}$ross-$\underline{c}$omplementer) was identified in a screen for genomic library clones capable of rescuing a calcium sensitive *S. cerevisiae* mutant in high copy number. It

encodes a P-type ATPase which is located in the late Golgi (Yuan *et al.*, 1997). Ccc2p mediates the transfer of copper into this intracellular compartment prior to its incorporation into Fet3p, the multicopper oxidase component of the high affinity iron transport complex (Fu *et al.*, 1995; Yuan *et al.*, 1995). In accordance with its apparent role in iron acquisition, *CCC2* transcription is regulated by Aft1p, the transcription factor mediating iron dependent gene expression (see Section 1.4.5), and not copper dependent transcription factors (Yamaguchi Iwai *et al.*, 1995; Yamaguchi Iwai *et al.*, 1996). An interesting feature of Ccc2p is that a mutant defective in this protein can be rescued by the addition of either iron or copper to the growth medium, confirming its role in both iron and copper metabolism (Yuan *et al.*, 1995). *ATX1* (anti-oxidant) was identified as a multi-copy suppressor of oxygen toxicity in yeast strains with defective superoxide dismutase activity (Lin & Culotta, 1995), but mutant defective in this gene were shown to have reduced high iron uptake. Sequence analysis shows that Atx1p possesses a sequence that is conserved in several bacterial mercury transporters: MTCXXC (X = any residue). This sequence has been shown spectroscopically to bind copper and mercury *in vitro* (Pufahl *et al.*, 1997). Atx1p has been shown to be located in the cytosol (Lin *et al.*, 1997), leading to the proposal that it functions as a copper chaperone delivering copper to Ccc2p. Ccc2p contains a motif similar to the one described above for Atx1p on its cytosolic facing domain, suggesting that copper may be transferred to this domain from Atx1p during the process of transferring the copper across the Golgi membrane. Two-hybrid experiments using the whole of Atx1p and parts of Ccc2p showed that an interaction occurred between Atx1p and the N-terminal region of Ccc2p that contains the putative copper-binding domain (Pufahl *et al.*, 1997). This interaction only takes place in the presence of copper, and addition of the copper binding chelator, BCS (bathocuproine disulphonic acid), prevents any interaction (Pufahl *et al.*, 1997).

A third protein that has been shown to have an effect on iron transport, Gef1p (glycerol/ethanol, Fe-requiring), was identified during a screen for mutants which showed reduced growth when supplied with glycerol or ethanol as a carbon source unless supplemented with iron (Greene *et al.*, 1993). This protein is again associated with the late Golgi compartment (Gaxiola *et al.*, 1998) and shows sequence similarity to a family of voltage-gated chloride channels (Greene *et al.*, 1993). The phenotype of a *gef1* mutant is similar to that of a *ccc2* mutant in that its slow growth phenotype can be rescued by the addition of either iron or copper to the growth medium (Gaxiola *et al.*, 1998). Since *GEF1*

encodes a putative chloride transporter it has been postulated that its role is to maintain the electroneutrality of Golgi, which would otherwise be disturbed by the transport of positively charged copper ions. In favour of this hypothesis is the observation that in the absence of chloride ions very little copper is loaded onto Fet3p (Davis-Kaplan *et al.*, 1998).

1.5.4 Iron and copper responsive regulation of *Saccharomyces cerevisiae* iron uptake genes

The genes involved in the high affinity iron uptake system have been shown to be regulated at the transcriptional level. It has been found that mRNA levels of these genes are so low as to be virtually undetectable in high iron conditions and to rise dramatically in the absence of iron (Dancis *et al.*, 1990; Dancis *et al.*, 1994b). A gene encoding a transcription factor has been cloned by isolating spontaneous mutants that showed constitutive expression from the *FRE1* promoter. The mutants were isolated from a *his3* yeast strain containing a plasmid borne *FRE1* promoter fused to a functional *HIS3* gene. When this strain was grown in high iron conditions transcription from the *FRE1* promoter was switched off, thus in the absence of histidine, most cells were unable to grow. However, when this strain was plated onto high iron medium lacking histidine, spontaneous mutants able to grow under these conditions were isolated after a few days. That these mutants were able to grow under these conditions, suggested that transcription from the *FRE1* promoter was occurring constitutively in these strains (Yamaguchi Iwai *et al.*, 1995). One of the semi-dominant mutations found in this manner corresponded to a gene named *AFT1* (altered ferrous transport), which encodes a protein with a highly basic amino terminal and a glutamine rich carboxyl terminal, both of which are characteristic of transcription factors (Frankel & Kim, 1991; Mitchell & Tjian, 1989). Although there is no recognised metal binding site within this protein, there is a distinctive pattern of histidine residues distributed throughout the sequence, with 10% of amino terminal residues and 10.5% of carboxyl terminal residues being represented by this amino acid. It is possible that these may play a role in any metal binding activity that might be associated with Aft1p. Two types of mutants have been isolated showing defects in this gene: *AFT1-1^{up}* mutants are found to have increased ferric reductase and ferrous iron uptake activity and are highly sensitive to growth on high iron, whilst *aft1* mutants, defective in Aft1p activity, are found to be unable to grow on low iron, have reduced ferric reductase activity regardless of the growth media and show no ferrous iron uptake (Yamaguchi Iwai *et al.*, 1995). The observation that *aft1* mutants are defective in both ferric reductase activity

and ferrous iron uptake suggests that Aft1p acts upstream of both of these activities, again signifying that it may indeed be a transcription factor controlling the expression of iron uptake genes. Supporting this is the observation that Northern blot analysis of *FRE1*, *FRE2*, and *FET3* expression in an *aft1* mutant show reduced levels of transcript with respect to the wild type (Yamaguchi Iwai *et al.*, 1995). The transcript produced by *AFT1* is 2.8 kb in length and is not regulated by iron (Yamaguchi Iwai *et al.*, 1995).

Further studies have identified a consensus sequence, PyPuCACCCPu, in the upstream regions of *FRE1*, *FRE2*, *FTR1*, *FTH1*, *CCC2* and *FET3*, and this has been shown to confer iron-regulated transcription in an Aft1p dependent manner (Yamaguchi Iwai *et al.*, 1996). Studies were initially carried out using the upstream region of *FET3* fused to a β-gal reporter gene in an *AFT1-1^{up}* mutant. A series of nested deletions in the Fet3p promoter showed that the Aft1p binding sequence was -263 to -234 bp upstream of the ATG. DNase foot-printing using oligonucleotides spanning this region confirmed this (Yamaguchi Iwai *et al.*, 1996). In addition *in vivo* genomic foot-printing showed that the Aft1p binding site was only occupied in low iron conditions, thus showing that the occupation of the site is iron regulated and suggesting that Aft1p is responsible for inducing the transcription of these genes Yamaguchi Iwai *et al.*, 1996).

Another transcription factor, known as Mac1p (metal binding activator), has also been implicated in the regulation of *FRE1* transcription (Jungmann *et al.*, 1993). Analysis of the predicted protein sequence of Mac1p shows that the amino terminal region is similar to the copper and DNA binding regions of two other known zinc finger-like transcription factors, *ACE1* (activator of *CUP1* expression) from *S. cerevisiae* (Szczypka & Thiele, 1989; Welch *et al.*, 1989) and *AMT1* (activator of metallothionein) from *Candida glabrata* (Zhou & Thiele, 1991). The two proteins encoded by these genes are copper fist transcription factors that play a role in copper homeostasis by activating the transcription of metallothionein genes in high copper conditions. Metallothioneins are responsible for chelating excess copper, thus preventing damage that might be caused to the cell by free radical formation catalysed by these ions. Immunofluorescence studies have shown that Mac1p localises to the nucleus, further supporting its role as a transcription factor (Jungmann *et al.*, 1993). *MAC1* mutants have phenotypes compatible with the view that this gene plays a role in copper metabolism: *MAC1^{up1}* mutants show increased reductase activity coupled to an increased sensitivity to

ferric reductase activity and are slow growing, respiratory deficient and hypersensitive to many transition metals.

Mac1p activates the transcription of *FRE1* (Jungmann *et al.*, 1993), *FRE7* (Martins *et al.*, 1998), *CTR1* (Graden & Winge, 1997) and *CTR3* (Labbe *et al.*, 1997). All of these promoters contain a sequence containing the palindromic TTTGCTCA.....TGAGCAAA sequence, which has been termed a CuRE (copper responsive element), since deletion of this sequence from the promoters of these genes results in loss of copper dependent transcription (Yamaguchi Iwai *et al.*, 1997). *In vivo* foot-printing has shown that this region of the promoter of *CTR3* is occupied during copper depleted conditions but not during copper replete conditions, and, consistent with this, that it is constitutively occupied in a $MAC1^{up1}$ strain (Labbe *et al.*, 1997). The number of CuRE elements in the promoters of these genes has been shown to be important, and increasing the number of elements produces a synergistic increase in expression levels (Jensen *et al.*, 1998), suggesting that the binding of multiple Mac1p proteins to the promoter increases transcription from these promoters. Furthermore, two species were detected in electrophoretic mobility shift assays (EMSA) using the *CTR1* promoter as a probe and it was inferred that the more slowly migrating species was due to a $(Mac1p)_2$.DNA complex, since deletion of one of the CuREs resulted in the loss of the slow migrating species (Joshi *et al.*, 1999).

Since Mac1p shows sequence similarity to Ace1p and Amt1p, both of which are involved in the transcriptional regulation of metallothionein genes, the question of whether Mac1p binds copper, and the location of its DNA binding domain has been of interest. The mechanism of action of Amt1p and Ace1p is well established: under high copper conditions, 4 Cu(I) ions bind to Ace1p or Amt1p and activate their DNA binding domains via a conformational change in the protein (Furst *et al.*, 1988; Graden *et al.*, 1996). It was suspected that a cysteine rich region of Mac1p may be the copper binding domain of this protein and this was tested by constructing a fusion construct consisting of the Gal4p DNA binding domain and Mac1p lacking its DNA binding domain. This construct was capable of mediating the copper dependent expression of a *GAL1* promoter/*lacZ* reporter gene construct (Graden & Winge, 1997). Furthermore, when the cysteine rich region of Mac1p was purified from *E. coli* as a GST fusion protein, metal analysis showed it to contain 7.7 ± 0.4 bound copper ions per molecule of Mac1-GST fusion protein (Jensen & Winge, 1998).

The DNA binding domain is located at the N-terminal of Mac1p and was identified by its similarity to the DNA binding domains of Ace1p and Amt1p. That this region represents the DNA binding domain was conclusively proved by the fusion of the first 159 amino acids of Mac1p to the herpes simplex VP16 activation domain. This fusion protein is capable of activating transcription from a *CTR1* promoter fused to a *lacZ* reporter gene in a copper independent manner (Jensen *et al.*, 1998). Metal analysis of the purified N-terminal of Mac1p shows it to contain 2 zinc ions, consistent with it being a zinc finger protein, as is predicted from its similarity to Ace1p and Amt1p (Jensen *et al.*, 1998).

1.5.5 Intracellular iron transport and storage in *Saccharomyces cerevisiae*

Iron metabolism within the cell is not well characterised, however, it is known that the majority of iron used by the cell is required in the mitochondria, where it is inserted into porphyrin to produce haem, a vital prosthetic group for the many mitochondrial cytochromes. Recent evidence also suggests that iron-sulphur (Fe-S) clusters, which are important cofactors for many mitochondrial and cytosolic enzymes, are synthesised in the mitochondria (Kispal *et al.*, 1999). Iron taken up by the cell can be stored in the vacuoles prior to use, possibly in the form of polyphosphates (Raguzzi *et al.*, 1988).

Mitochondrial iron acquisition

Many genes have been shown to have an effect on mitochondrial iron metabolism. Interestingly, one of them was identified through its sequence similarity with a human gene implicated in causing Friedrich's ataxia, a neurodegenerative disease. The yeast nuclear gene, *YFH1* (yeast frataxin homologue), encodes a 174 amino acid protein containing a mitochondrial targeting sequence (Babcock *et al.*, 1997). Mutants lacking a functional *yfh1* show hypersensitivity to iron in the growth media, and iron levels in the mitochondria of these cells has been shown to be 10-fold greater than that of wild type cells. This has led to suggestions that Yfh1p may be involved in regulating iron efflux from the mitochondria. An alternative suggestion has been that Yfh1p may play a role in the biosynthesis of Fe-S clusters. It has been observed that although *yfh1* mutants grown in standard media show decreased activity of all respiratory complexes including both cytochromes and Fe-S containing proteins (a likely side effect of the increased mitochondrial iron concentration resulting in increased production of free radicals), *yfh1* mutants which are grown in low iron conditions do not show defects in cytochrome oxidase, a haem containing enzyme, but still

containing proteins (a likely side effect of the increased mitochondrial iron concentration resulting in increased production of free radicals), *yfh1* mutants which are grown in low iron conditions do not show defects in cytochrome oxidase, a haem containing enzyme, but still show decreased activity mitochondrial aconitase, an Fe-S cluster containing enzyme (Foury, 1999). This suggests that the reduced activity of cytochromes seen in this mutant is due to the excess free radical activity but that Fe-S cluster proteins are more directly affected. Aconitase contains a 4Fe-4S cluster, and it is possible that Yfh1p is involved in the biogenesis of this enzyme. Interestingly, Yfh1p shows some similarity to a protein from *Clostridium acetobutylicum*, which belongs to a family of proteins implicated in tellurite resistance. Tellurium is a metal which is closely related to sulphur and it is therefore possible thatYfh1p may play some role in sulphur metabolism.

Many other proteins with possible roles in Fe-S cluster formation are also found within the mitochondria, and many of them show similar mutant phenotypes to that of *yfh1*, with high levels of iron being accumulated in the mitochondria. Nfs1p is a mitochondrial protein showing similarity to bacterial cysteine desulphurase, NifS (Zheng *et al.*, 1993), which initiates the biogenesis of Fe-S clusters by releasing elemental sulphur from cysteine (Kispal *et al.*, 1999). Nfs1p seems to be required for the synthesis of both mitochondrial and cytosolic Fe-S proteins, since the activities of these proteins are found to be reduced in *nfs1* mutant strains. It is also interesting to note that deletion of *ATM1*, an gene encoding an ABC transporter protein associated with the inner membrane of the mitochondria, results in loss of activity of cytosolic proteins bearing Fe-S clusters, but not mitochondrial Fe-S proteins; these mutants also show abnormally high levels of iron in the mitochondria (Kispal *et al.*, 1999). This suggests that Atm1p is responsible for the efflux of Fe-S clusters from the mitochondria.

Whilst mutations in the proteins discussed above, which have a putative role in the biogenesis of Fe-S cluster containing proteins, result in accumulation of iron in the mitochondria and consequent oxidative damage, a similar effect is not seen for haem biosynthesis proteins. Mutants defective in haem biosynthesis do not accumulate iron in the mitochondria and indeed it appears that the transport of iron into the mitochondria for insertion into protoporphyrin is tightly regulated (Lange *et al.*, 1999).

The apparent differences in the regulation of iron uptake into mitochondria for insertion into these two different prosthetic groups may reflect their comparative importance. Haem is

wider range of proteins that require iron-sulphur clusters may make these prosthetic groups more important to the cell, thus the effect of eliminating their biosynthesis may be more marked.

Iron storage

A cytosolic protein showing similarity to bacterioferritin has been identified in yeast (Raguzzi *et al.*, 1988), thus presenting the possibility that iron is stored in the cytosol in a ferritin-like molecule. However, this protein was shown to contain 50-100 iron atoms per molecule, which was thought to be too low for it to be an important storage molecule. Moreover, its iron content was shown to be independent of cellular iron content: in conditions of high iron concentrations in the culture medium the iron content of the cell increased, but that of the ferritin molecule did not (Raguzzi *et al.*, 1988). Studies of the intracellular iron distribution show the vacuoles accumulate 8.8 times more iron when grown in iron rich media than when grown in low iron media, whereas other intracellular compartments show no marked increase in iron content (Raguzzi *et al.*, 1988). It has therefore been suggested that the vacuoles are the site of iron storage within the cell. Furthermore, it has also been shown that iron stored in the vacuoles can be mobilised for use during mitochondriogenesis, thus demonstrating that the vacuoles are not merely a store for excess iron but play an active role in cellular iron metabolism (Raguzzi *et al.*, 1988). Further studies using a *vps16* mutant, which has defective vacuolar structure, showed that iron uptake was impaired in such mutants, whilst a mutant deficient in vacuolar acidification, *vps6*, was shown to be able to acquire iron normally (Bode *et al.*, 1995). This suggested that iron was transported into the vacuole via a mechanism other than the cation-anion exchange mechanism shown for other metal ions destined for storage in the vacuole.

Interestingly, other work has shown that mutants lacking vacuoles show constitutive ferric reductase activity. A *vps11* mutant, which lacks visible vacuoles, has been shown to have a 6-fold increase in ferric reductase activity and *FRE1* gene expression, suggesting that vacuole deficient strains are iron starved (Amillet *et al.*, 1996). However, the same workers showed that the iron content of these cells did not alter significantly when compared to wild type cells (Amillet *et al.*, 1996), suggesting that the vacuole may serve as an important sensor of intracellular iron concentration. Work in our laboratory has shown that a mutant originally identified by its constitutive reductase activity was defective in *VPS18* (J. Morrissey, personal

communication). *VPS18* has been previously shown to be essential for vacuolar biogenesis and is from the same class of vacuolar mutants as *vps11*, both of which lack vacuole-like structures (Preston *et al.*, 1991; Robinson *et al.*, 1991). Mutants defective in *vps18* and *vps11* have also been shown to be sensitive to copper and to accumulate greater levels of this metal than wild type cells, suggesting that the vacuole is a storage or detoxification site for copper, and again possibly a sensor of intracellular copper concentrations (Szczypka *et al.*, 1997).

The iron transporter homologue, Fth1p, has been shown to be located in the vacuolar membrane along with the Fet3p homologue, Fet5p (Urbanowski & Piper, 1999). This suggests that these two proteins may play a role in moving iron either in or out of the vacuole. Examination of the topology of these proteins showed that they are orientated to pump iron into the cytosol, implying that they are involved with the mobilisation of iron stored in the vacuole. An *fth1Δ/fet5Δ* mutant was shown to be unable to support the switch from growth in glucose (fermentative metabolism) to growth in ethanol and glycerol (respiratory metabolism), a change that requires mitochondriogenesis and the synthesis of cytochromes (Urbanowski & Piper, 1999). Again, this supports the theory that the Fth1p/Fet5p complex is responsible for the mobilisation of iron from the vacuole.

1.6 Iron acquisition in *Candida albicans*

C. albicans has been shown to be capable of utilising iron from many sources, including siderophores and host iron binding proteins such as haemoglobin and ferritin. It can bind to complement-coated sheep erythrocytes via a complement-receptor-like molecule on the yeast cell surface and is able to acquire iron from this source (Moors *et al.*, 1992). Although complement is a host mechanism for detecting invading microorganisms, which functions by the binding of complement components to the cell surface of the pathogen marking it as an invader, complement proteins can also bind to host tissues in the vicinity of the complement activation: the so-called 'by-stander' mechanism. It has been shown that the presence of *C. albicans* can induce the binding of complement to human erythrocytes (Moors *et al.*, 1992), suggesting that *C. albicans* may have hijacked this host defence mechanism for its own ends. *C. albicans* is also capable of producing a haemolytic factor, which it apparently secretes from the cell since culture supernatants can lyse erythrocytes (Manns *et al.*, 1994;

1992), suggesting that *C. albicans* may have hijacked this host defence mechanism for its own ends. *C. albicans* is also capable of producing a haemolytic factor, which it apparently secretes from the cell since culture supernatants can lyse erythrocytes (Manns *et al.*, 1994; Watanabe *et al.*, 1999). Purification of the factor suggested that it was a mannoprotein, and H^1-NMR analysis of the mannan moiety of the mannoprotein implied that it was cell wall derived (Watanabe *et al.*, 1999). Haemolysis was inhibited in the presence of 4,4'-diisothiocyanatosilbene-2,2'-disulphonic acid (DIDS), which binds specifically to the erythrocyte cell surface band 3 protein, suggesting that this protein may mediate the binding of the haemolytic factor to the erythrocyte cell surface (Watanabe *et al.*, 1999).

Siderophores present an alternative mechanism of iron acquisition available to *C. albicans*. There is some debate as to whether *C. albicans* can produce its own siderophores since all studies to date have relied on colorimetric assays to show siderophore production (Ismail *et al.*, 1985; Sweet & Douglas, 1991a) and no structural studies have been carried out on purified compounds. However, one study has shown that the amount of 'siderophore' in culture supernatants capable of removing iron from a high affinity iron chelator is increased when the cells are cultured in low iron conditions (Sweet & Douglas, 1991a), suggesting that *C. albicans* does indeed produce siderophores. *C. albicans* has been shown to be capable of utilising ferrichrome as an iron source when provided exogenously, suggesting that even if it cannot make its own siderophores it is capable of using those produced by other organisms (Minnick *et al.*, 1991).

Although the secretion of siderophores and lysis of erythrocytes are both potential iron acquisition mechanisms, the transport of iron into the cell has not been studied in depth. Work from our laboratory has shown that *C. albicans* produces a cell surface ferric reductase activity which is negatively regulated by both iron and copper in a similar manner to the *S. cerevisiae* ferric reductase described in section 1.4 (Morrissey *et al.*, 1996). The ferric reductase is also capable of reducing copper, again suggesting similarities with the *S. cerevisiae* iron uptake system (Morrissey *et al.*, 1996). Furthermore, a gene showing similarity to the *S. cerevisiae* gene, *FET3*, which encodes a multicopper oxidase and is required for high affinity iron uptake in *S. cerevisiae*, has been cloned from *C. albicans* (Eck *et al.*, 1999). Deletion of the *CaFET* gene resulted in a strain that was unable to grow in low iron media, but no differences were observed in its pathogenicity in the mouse model of

stages of this work, two ferrous transporter-like genes were isolated from *C. albicans* (Ramanan & Wang, 2000). These genes, named *CaFTR1* and *CaFTR2*, show similarity to the *S. cerevisiae* ferrous transporter gene, *FTR1*, and *CaFTR1* is found to be negatively regulated by iron. Interestingly, deletion of *CaFTR1* results in a strain which is no longer virulent in the mouse model of systemic infection (Ramanan & Wang, 2000). It therefore seems likely that *C. albicans* uses a reductive iron uptake mechanism to acquire iron during infection and that high affinity iron acquisition is crucial to the virulence of this organism.

1.7 Background to the project

The aim of this project was to isolate and characterise ferric reductase genes from the pathogenic yeast *C. albicans*. Previous studies carried out in our laboratory have shown that *C. albicans* possesses a ferric reductase activity at its cell surface (Morrissey *et al.*, 1996). This reductase activity is negatively regulated by both iron and copper in a similar way to the ferric reductase of *S. cerevisiae* and has been shown to be able to reduce copper as well as iron. It therefore seems likely that *C. albicans* possesses a reductive iron and copper uptake mechanism similar to that of *S. cerevisiae*.

The identification of ferric reductase-like genes from *C. albicans* is considered important for several reasons. Firstly, this method of iron uptake may represent an important iron acquisition mechanism for *C. albicans*. It may therefore play a part in iron acquisition in the host environment, potentially influencing pathogenicity. Secondly, iron-restriction has been shown to be a key factor in the expression of virulence factors unrelated to iron acquisition in several pathogenic bacteria. The expression of these virulence genes is mediated through the same regulatory proteins that regulate iron uptake genes (Griffiths & Chart, 1999). Some virulence determinants in *C. albicans* may also be regulated by iron and the identification of regulatory sequences in the promoters of iron-regulated genes may lead to the identification of genes with a potential role in virulence.

1.8 Summary of project objectives

The aims of this project are:

- To isolate *C. albicans* ferric reductase genes capable of rescuing a *S. cerevisiae* ferric reductase mutant.
- To analyse expression of the ferric reductase genes by Northern blotting.
- To construct *C. albicans* mutant strains carrying deletions of the ferric reductase genes identified during the course of the study.

Chapter 2
Materials and methods

2.1 Strains and plasmids

Table 2.1 *S. cerevisiae* and *C. albicans* strains used in this study

Organism	Strain	Genotype	Source/reference
Saccharomyces cerevisiae	S150-2B	*MATa, leu2-3, leu2-112; his3-Δ; trp1-289; ura3-52*	J. Hicks, Cold Spring Harbour Laboratory, New York
Saccharomyces cerevisiae	JHS1	*MATa, leu2-3, leu2-112; his3-Δ; trp1-289; ura3-52,Δfre1::HIS3*	This study
Saccharomyces cerevisiae	BY4733	*MATa, his3Δ0, leu2Δ0, met15Δ0, trp1Δ63, ura3Δ0*	(Brachmann *et al.*, 1998)
Saccharomyces cerevisiae	JHS2	*MATa, his3Δ0, leu2Δ0, met15Δ0, trp1Δ63, ura3Δ0, Δfre1::HIS3, Δfre2::URA3*	This study
Candida albicans	S/01		R. Matthews, Department of Microbiology, University of Manchester, UK
Candida albicans	SC5314		Clinical isolate from a patient with disseminated candidosis (Gillum *et al.*, 1984)
Candida albicans	CAF2	*Δura3::imm434/URA3*	(Fonzi & Irwin, 1993)
Candida albicans	CAI4	*Δura3::imm434/Δura3::imm434*	(Fonzi & Irwin, 1993)
Candida albicans	JHC1	*Δura3::imm434/Δura3::imm434, Δcfl1::hisGURA3hisG/CFL1*	This study
Candida albicans	JHC3	*Δura3::imm434/Δura3::imm434, CFL1/Δcfl1::hisGURA3hisG*	This study
Candida albicans	JHC1.1	*Δura3::imm434/Δura3::imm434, Δcfl1::hisG/CFL1*	This study
Candida albicans	JHC3.1	*Δura3::imm434/Δura3::imm434, CFL1/Δcfl1::hisG*	This study
Candida albicans	JHC1.2	*Δura3::imm434/Δura3::imm434, Δcfl1::hisG/Δcfl1::hisGURA3hisG*	This study

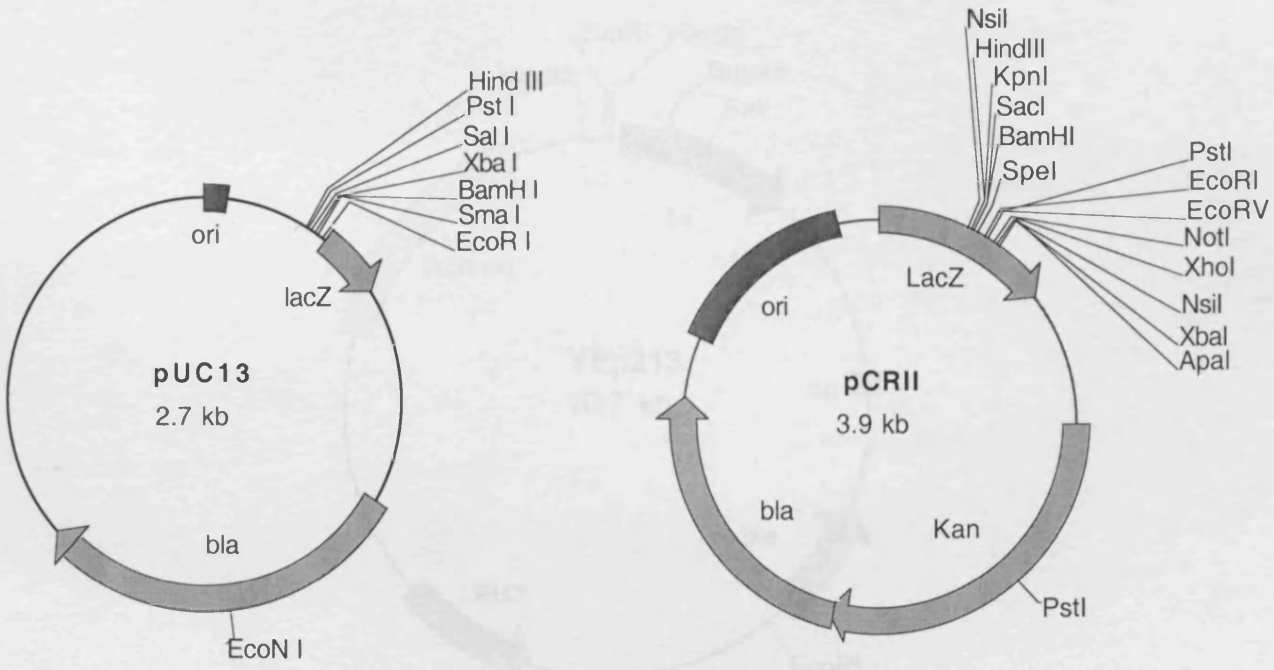
Table 2.1 *E. coli* strains used in this study

Organism	Strain	Genotype	Source/Reference
E. coli	DH5α	*ϕ80lacZΔM15, recA1, endA1, gyrA96, thi-1, hsdR17(r_K^-, m_K^+), supE44, relA1, deoR, Δ(lacZYA-argF)U169*	(Hanahan, 1983)
E. coli	DH1	*recA1, endA1, gyrA96, thi-1, hsdR17(r_K, m_K^+), supE44, relA1*	(Hanahan, 1983)
E. coli	MH755	*recA1, endA1, gyrA96, thi-1, hsdR17(r_K, m_K^+), supE44, relA1, srl::Tn10recA*	(Hanahan, 1983)

Table 2.3 Plasmids and vectors used in this study

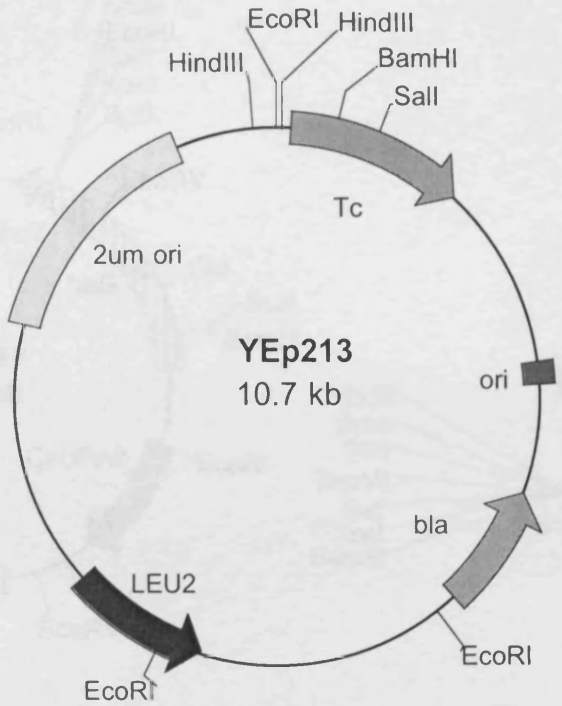
Plasmid	Genotype	Source or reference
pUC13/18	*lacZα$^+$, amp^R*	(Yanisch-Perron *et al.*, 1985)
pCR II	*lacZα$^+$, amp^R*	Invitrogen,
YEp312	*amp^R, tet^R, 2 μM ORI , LEU2*	(Broach *et al.*, 1988)
pMB7	*hisGURA3hisG* cassette carried on pUC18	(Fonzi & Irwin, 1993)
p5921	*hisGURA3hisG* cassette carried on pUC18	(Gow *et al.*, 1994)
pYRG17	*amp^R, 2 μM ORI,HIS3*	P. Meacock
pYRG24	*amp^R, 2 μM ORI,URA3*	P. Meacock
YpB1	*amp^R, 2 μM ORI, C. albicans URA3, C. albicans* ARS	(Goshorn *et al.*, 1992)
pJD3	*FRE1* gene carried on pUC13	This study
pJD3.2	*Δfre1::HIS3* cassette carried on pUC13	This study
pJD7	*FRE2* gene carried on pUC13	This study
pJD7.2	*Δfre2::URA3* cassette carried on pUC13	This study
pJDF1.3	*CFL1* gene carried on YEp213	This study
pJDF2.3	*CFL2* gene carried on YEp213	This study
pJD8.1	5' flank of *CFL1* on pMB7	This study
pJD8.2	*Δcfl1::hisGURA3hisG* cassette on pMB7	This study
pJD9.1	5' flank of *CFL1* on p5921	This study
pJD9.2	*Δcfl1::hisGURA3hisG* cassette on p5921	This study

Figure 2.1 *E. coli* vectors used in this study



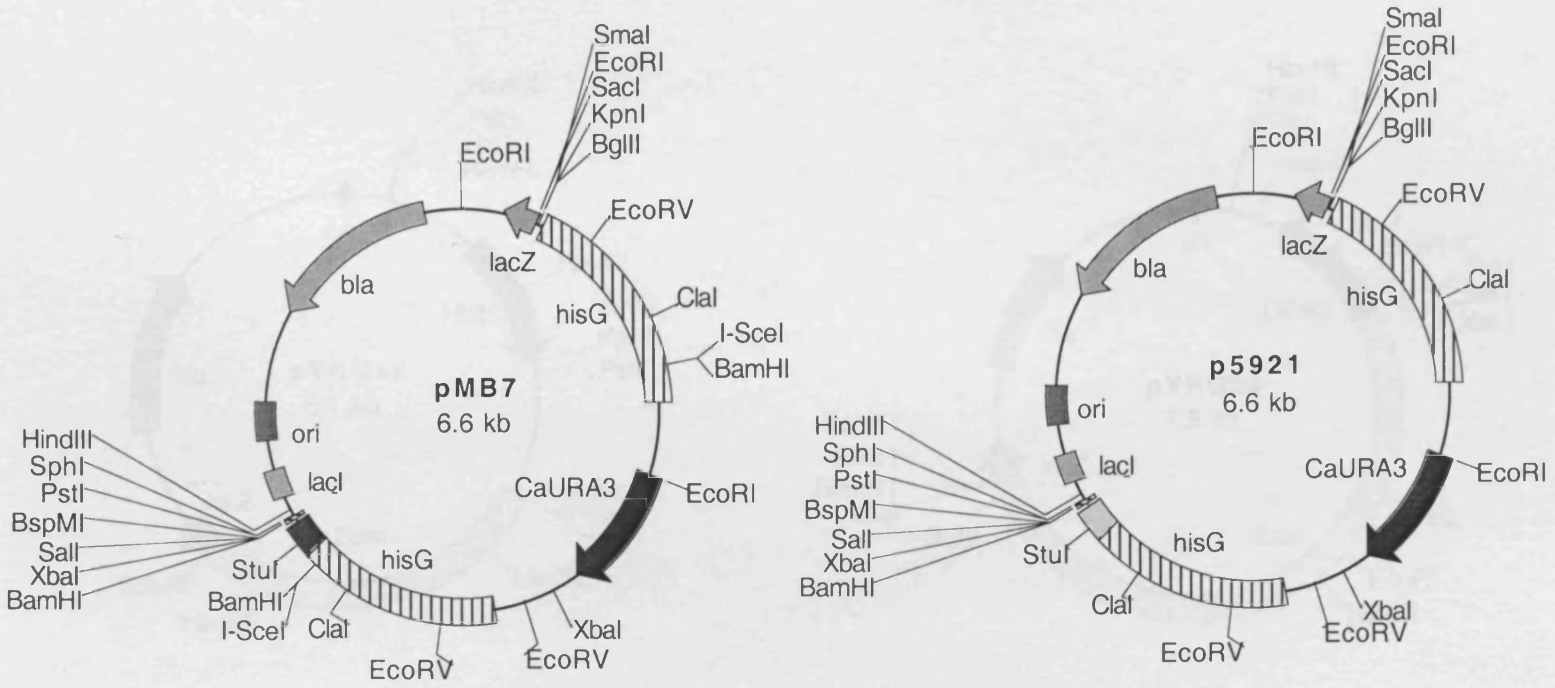
Restriction enzyme sites in the multi-cloning sites are shown as are other relevant sites. *bla*: ampicillin resistance gene for selection in *E. coli*; *Kan*: kanamycin resistance gene for selection in *E. coli*; *ori*: origin of replcation; *lacZ*: beta galactosidase gene. The sizes of the plasmids are indicated in kilobase pairs. Source: pUC13, Yanisch-Perron *et al.* (1985); pCRII, Invitrogen.

Figure 2.2 Yeast shuttle vector used in this study



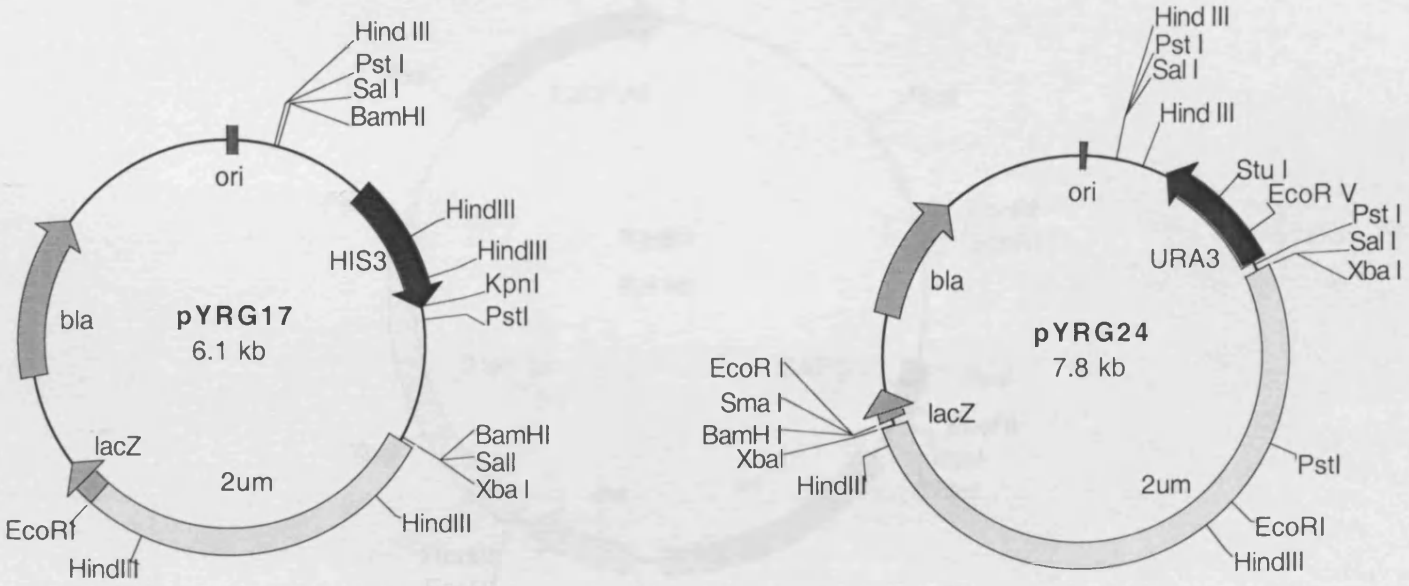
YEp213 is the host vector of the *C. albicans* genomic library used in this study. Relevant restriction sites are shown. *Amp*: ampicillin resistance gene for selection in *E. coli*; *Tc*: tetracyclin resistance gene for selection in *E. coli*. The activity of this gene was disrupted by the insertion of *C. albicans* genomic clones into the *Bam*HI site. *ori*: bacterial origin of replication; 2μm *ori*: yeast replicative regions from the 2μm plasmid; *LEU2*: yeast auxotrophic marker gene. Broach *et al.*, (1988).

Figure 2.3 *URA*-blasting plasmids



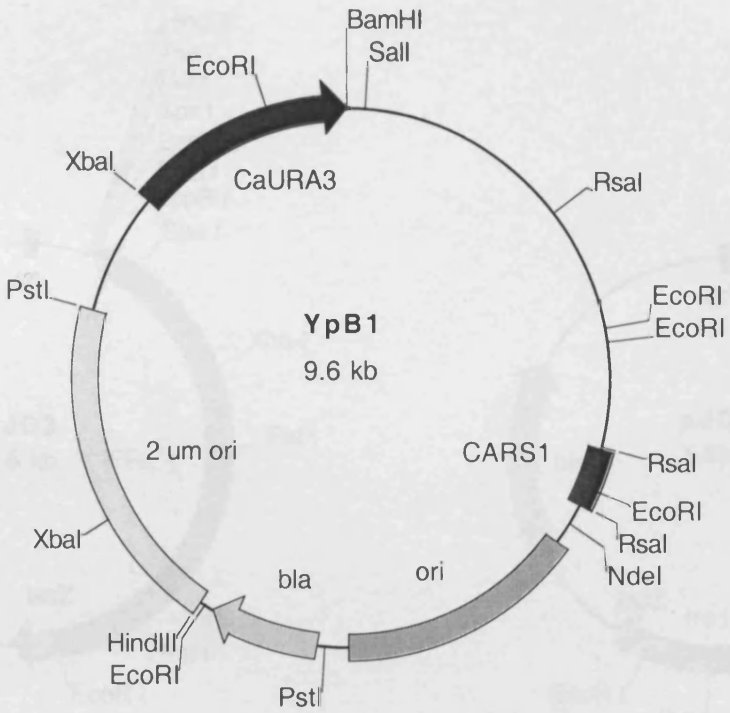
Relevant restriction sites are indicated. *bla*: ampicillin resistance; *lacZ*: beta galactosidase; *ori*: origin of replication; *CaURA3*: *C. albicans URA3* gene; *hisG*: *hisG* gene from *S. typhimurium* arranged in repeats to allow *CaURA3* to be removed by recombination between the two *hisG* repeats after 5-FOA selection (see Chapter 6). pMB7: Fonzi & Irwin (1993); p5921: Gow *et al.* (1999).

Figure 2.4 Yeast shuttle vectors used in this study



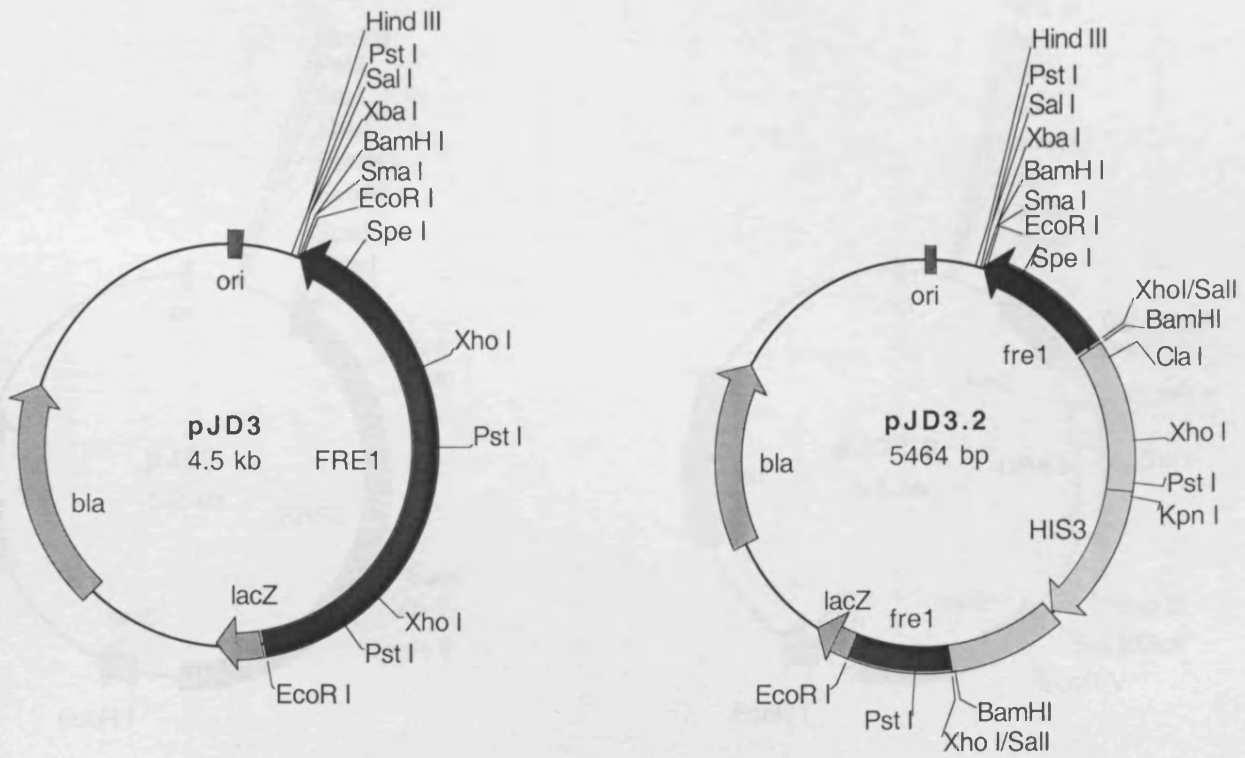
Both of these vectors are based on pUC13 (see figure 2.1). Relevant restriction sites are shown. *bla*: ampicillin resistance gene; *lacZ*: beta galactosidase gene; *ori*: bacterial origin of replication; 2μm: yeast replicative regions from 2 μm plasmid; *URA3*: yeast marker gene; *HIS3*: yeast marker gene. Source: P. A. Meacock.

Figure 2.5 *C. albicans*/ *S. cerevisiae*. *E. coli* shuttle vector used in this study



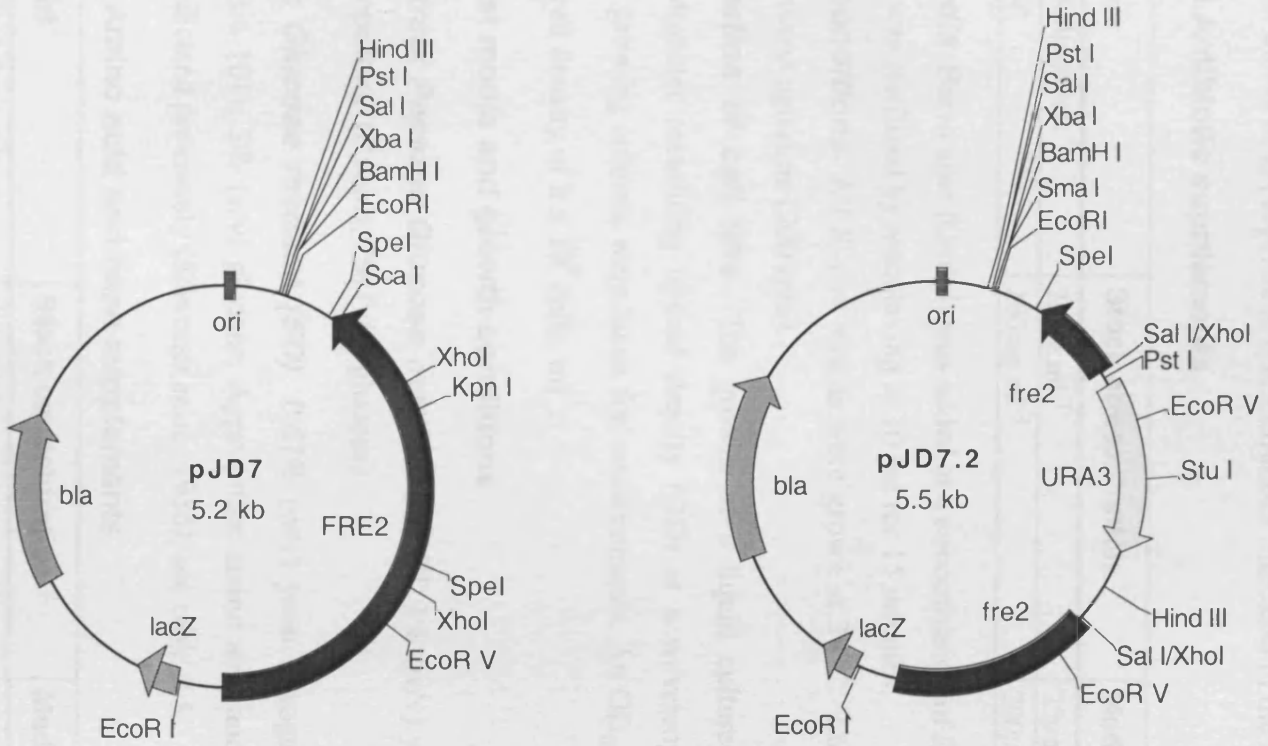
This vector was used as a source of the *C. albicans URA3* gene which was used as a loading control for Northern blotting. *CaURA3*: *C. albicans URA3* gene; *CARS1*: *C. albicans* autonomously replicating sequence; ori: bacterial origin of replication; *bla*: ampicillin resistance gene for selection in *E. coli*; 2 μm *ori*: *S. cerevisiae* replicative regions from the 2μm plasmid. Goshorn & Scherer (1992).

Figure 2.6 *FRE1* disruption plasmids



bla: ampicillin resistance gene for selection in *E. coli; ori*: origin of replication for *E. coli*; *lacZ*: beta galactosidase gene for blue/white colony selection in *E. coli*; *FRE1*: cloned *FRE1* sequences from *S. cerevsiae*; *fre1*: disrupted *FRE1* sequence; *HIS3*: yeast marker gene conferring histidine prototrophy Relevant restriction sites are shown. Source: this study.

Figure 2.7 *FRE2* disruption plasmids



bla: ampicillin resistance gene for selection in *E. coli; ori*: origin of replication for *E. coli*; *lacZ*: beta galactosidase gene for blue/white colony selection in *E. coli*; *FRE2*: cloned *FRE2* sequences from *S. cerevisiae*; *fre2*: disrupted *FRE2* sequence; *URA3*: yeast marker gene conferring uracil prototrophy Relevant restriction sites are shown. Source: this study.

2.2 Growth media and conditions

2.2.1 Bacterial media and growth conditions

Luria-Bertani medium (LB) 1% Bacto-tryptone (Oxoid); 0.5% Bacto-yeast extract (Oxoid); 0.5% sodium chloride; pH adjusted to 7.2 with sodium hydroxide. Antibiotics were added to the media as appropriate at concentrations indicated in the table 2.4.

Table 2.4 Antibiotic supplements

Antibiotic	Stock concentration	Media concentration
ampicillin sodium salt	25mg.ml^{-1}	25μg.ml^{-1}
streptomycin	200mg.ml^{-1}	200μg.ml^{-1}

Solid media Bacto agar (Oxoid) was added at a concentration of 2% (w/v).
All media was sterilised by autoclaving at 10 psi for 15 minutes.

Growth conditions All *E. coli* strains were grown at 37°C. Liquid cultures were grown with continuous agitation (200 rpm).

Determination of cell titre The growth of a liquid culture was monitored using a spectrophotometer measuring optical density (OD) at a wavelength of 600nm. One ml aliquots of growing cultures were taken for measurements. An OD_{600} of 1.0 is approximately equal to a cell density of 8×10^8 cells. ml^{-1}.

2.2.2 Yeast media and growth conditions

Yeast extract Peptone Glucose medium (YPD) 1% (w/v) yeast extract (Oxoid); 2% (w/v) Bactopeptone (Oxoid); 2% (v/v) glucose)

Synthetic Glucose medium (SD) 0.67% (w/v) yeast nitrogen base with ammonium sulphate (Bio 101); 2% (v/v) glucose; Appropriate amino acid and base supplements were added as indicated previously (Sherman *et al.*, 1986), see table 2.5.

Table 2.5 Amino acid and base supplements

Supplement	Stock concentration	Media concentration
adenine hemisulphate salt	2mg.ml^{-1}	20μg.ml^{-1}
histidine	8mg.ml^{-1}	20μg.ml^{-1}
leucine	12mg.ml^{-1}	30μg.ml^{-1}
methionine	2mg.ml^{-1}	20μg.ml^{-1}
tryptophan	8mg.ml^{-1}	20μg.ml^{-1}
uracil	2mg.ml^{-1}	20μg.ml^{-1}
uridine	5mg.ml^{-1}	50μg.ml^{-1}

Minimal Defined medium (MD) Based on Wickerham's nitrogen base recipe (Wickerham, 1951) with modifications from Eide and co-workers (1992). 10% (v/v) salt and trace solution; 0.1% (v/v) vitamin solution; 7mM calcium chloride; 20mM tri-sodium citrate pH4.2; 2% (v/v) glucose; amino acid supplements (as above).

This media was rendered iron restricted by the addition of either 1mM EDTA, pH8.0 (MD-EDTA) or 120μM di-pyridyl (MD-dipyridyl) or BPS (MD-BPS) (concentrations added indicated in the text). To create high iron conditions $FeCl_3$ was added back to the media at concentrations indicated in the text.

Low copper conditions were created by using salt and trace solution lacking copper sulphate and by adding BCS to concentrations as indicated in the text as appropriate.

Table 2.6 Components of salt and trace solution

Compound	Stock concentration	Final concentration
ammonium sulphate	75.7 mM	7.57 mM
potassium dihydrogen orthophosphate	50.2 mM	5.02 mM
di-potassium hydrogen orthophosphate	9.2mM	0.92 mM
magnesium sulphate	20. 3mM	2.03 mM
sodium chloride	17.1mM	1.71 mM
boric acid	1.62μM	162 nM
cupric sulphate	0.4μM	40 nM
potassium iodide	0.6μM	60 nM
zinc sulphate	2.44μM	244 nM

Table 2.7 Components of vitamin solution

Compound	Stock concentration	Final concentration
d-biotin	8.19μM	8.19 nM
thiamine hydrochloride	1.19mM	1.19 μM
pyridoxine hydrochloride	1.95mM	1.95 μM
myo-inositol	11mM	11 μM
d-pantothenic acid calcium salt	0.84mM	0.84 μM

Solid media Bacto agar(Oxoid) was added at a concentration of 2% (w/v).

All media was sterilised by autoclaving at 10 psi for 10 minutes prior to use.

Growth conditions All *S. cerevisiae* and *C. albicans* strains were grown at 30°C. Liquid cultures were grown with continuous agitation (200 rpm).

Determination of cell titre The growth of a liquid culture was monitored using a counting chamber with modified Thoma ruling. A 10μl sample, diluted when necessary, was placed over the grid and covered with a counting chamber cover glass.

2.3 Transformation procedures

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

Cultures grown to a titre of 0.45 to 0.55 (OD_{600}) were harvested by centrifugation at 4,000 rpm for 5 minutes at 4 °C, washed in 0.5 × culture volume of ice cold 100 mM calcium chloride and again harvested by centrifugation. The cells were then resuspended in 0.5 × culture volume of ice cold 100 mM magnesium chloride and incubated on ice for 20 minutes. After harvesting again, the cells were resuspended in 0.05 × culture volume of ice cold 100 mM calcium chloride and incubated on ice or stored in 50% glycerol at –80 °C until use.

For transformation 200 μl of competent cells were mixed with 90 μl of 1 × TE (pH 8.0) and 1 μg of transforming DNA. A negative control was also set up using 1 × TE (pH 8.0) in place of the transforming DNA. The suspensions were mixed, incubated on ice for 30 minutes and then heat shocked at 42 °C for 2 minutes before incubating on ice for another 20 minutes. 2 ml of LB was added to the cell suspension and the mixture incubated at 37 °C with agitation for 60-90 minutes. The cells were then harvested by centrifugation at 14,000 rpm and resuspended in an appropriate volume of LB. 100 μl aliquots were plated onto LA plates containing the appropriate antibiotic for selection of transformed colonies. Incubation was carried out at 37 °C overnight until colonies were formed.

Transformation efficiencies of between 1×10^5 to 1×10^7 colony forming units per microgram of transforming plasmid DNA were obtained using this protocol.

2.3.2 Lithium acetate transformation of *Saccharomyces cerevisiae* (Gietz *et al.*, 1992)

The cell titre of an overnight YPD culture was determined and diluted to a concentration of 2×10^6 cells. ml^{-1} in 50 ml fresh pre-warmed YPD. Incubation was then continued until a cell titre of approximately $1\text{-}2 \times 10^7$ cells. ml^{-1} was attained. The cells were harvested by

centrifugation at 3,000 rpm and the supernatant discarded. The cells were then washed by resuspension in first 1 ml of sterilised distilled water and then 1 ml of 1 × TE-lithium acetate solution (1 × TE; 0.1 M lithium acetate, pH 7.5). The cells were then resuspended in 1 × TE-lithium acetate solution at a cell concentration of 2×10^9 cells. ml^{-1}.

For transformation, 50 μl of the cell suspension was gently mixed with 1 μg of transforming DNA, 50 μg of denatured salmon sperm DNA and 300 μl of 40% PEG 3,350 in 1 × TE-lithium acetate solution. A zero DNA control was also set up using sterilised distilled water in place of DNA. The cells were incubated at 30 °C for 30 minutes and were then heat shocked at 42 °C for 15 minutes and harvested by centrifugation at 13,000 rpm. The supernatant was discarded and the cells were washed in 1ml of 1 × TE and finally resuspended in an appropriate volume of 1 × TE (500 μl for a standard transformation or 10 ml for a library transformation). The transformation suspension was dispensed onto selective SD plates in 100 μl aliquots and the plates were then incubated at 30 °C for 2-5 days until colonies were observed.

2.3.3 Lithium acetate transformation of *Candida albicans* (Gow *et al.*, 1999)

Cultures growing exponentially in YPD media were harvested at 3,000 rpm, washed in 0.5 volumes of 1 × TE-lithium acetate solution and then resuspended in 0.1 volumes of 1 × TE-lithium acetate solution. The suspension was incubated at 30 °C for 30 minutes and 100 μl of the cell suspension was then transferred to a fresh microfuge tube containing 100 μg of denatured salmon sperm DNA and 1 μg of transforming DNA. A zero-DNA control was also set up containing sterile distilled water in place of the transforming DNA. The cell suspension was incubated at 30 °C for 30 minutes and after this time 700 μl of 40 % PEG 3,350 in 1 × TE-lithium acetate solution was added and the suspension incubated for a further 1 hour at 30 °C. The suspension was then heat shocked at 42 °C for 15 minutes and the cells harvested by centrifugation at 13,000 rpm. The cells were washed once in 500 μl of 1 × TE buffer, resuspended in 100 μl of 1 × TE buffer and dispensed onto selective SD plates. The plates were incubated at 30 °C for 3-5 days until colonies appeared.

2.4 DNA and RNA preparations

2.4.1 Large scale DNA preparations from *Escherichia coli*

All large scale plasmid extractions were carried out using a Plasmid Midi (100) Kit from QIAGEN (available from QIAGEN Ltd.). The protocol provided by the manufacturers was followed. This method combines the alkaline lysis method (Birnboim & Doly, 1979) with ion exchange chromatography to isolate purified plasmid DNA from *E. coli*.

2.4.2 Small scale plasmid DNA preparations from *Escherichia coli* (Ish-Horowicz & Burke, 1981)

1.5 ml of an overnight culture, grown with the appropriate antibiotic, was harvested, resuspended in 100 µl of solution I (50 mM glucose; 25 mM Tris-HCl, pH 8.0; 10 mM EDTA, pH 8.0) and left at room temperature for 5 minutes. 200 µl of solution II (0.2 M sodium hydroxide; 0.1 % SDS) was then added with gentle mixing and the suspension placed on ice for 5 minutes. 150 µl of ice cold solution III (5 M potassium acetate) was added and the mixture placed on ice for 5 minutes, followed by centrifuging at 13,000 rpm for 2 minutes. The supernatant was transferred to a fresh tube and the nucleic acids precipitated by the addition of 2 volumes of ethanol. The nucleic acids were pelleted by centrifugation at 13,000 rpm and then washed in 70 % ethanol before resuspending in 50 µl of sterile distilled water. This preparation results in the isolation of RNA as well as DNA so RNase A was added to subsequent restriction enzyme digests.

2.4.3 Large scale preparation of total genomic DNA from *Saccharomyces cerevisiae* and *Candida albicans* (Cryer *et al.*, 1975)

50 ml of an overnight cultures were harvested at 3,000 rpm, resuspended in solution I (1.2 M sorbitol; 25 mM EDTA, pH 8.0, 175 µM DTT) and incubated at 30 °C for 30 minutes. The cells were then harvested at 3,000 rpm and resuspended in 5 ml solution II (1.2 M sorbitol; 0.1 M sodium citrate; 10 mM EDTA, pH 8.0) to which 1 mg of zymolyase was added. The cell suspension was incubated at 30 °C for approximately 1 hour or until spheroplasts were observed and then harvested again and washed gently in 1.2 M sorbitol to remove any traces of DTT. The cell pellet was resuspended in 2 ml of solution III (3 % sarkosyl; 0.5 M Tris-HCl; 0.2 M EDTA. pH 8.0) and 0.2 mg of proteinase K added. The suspension was incubated at 37 °C for 45 minutes and then the volume made up to 5 ml with 1 × TE. 5 ml of phenol-chloroform was added and the mixture shaken and then centrifuged for 7 minutes at 13,000 rpm. The aqueous phase was then removed and the extraction process repeated until white

proteinaceous matter was no longer observed at the interface. A final extraction with chloroform was then carried out to remove any phenol contamination and the DNA was precipitated with 2 volumes of ethanol. The DNA was pelleted by centrifugation at 13,000 rpm and washed in 70 % ethanol before resuspending in 0.5 ml of sterile distilled water. 200 µg of RNase A was then added and the solution incubated for 1 hour at 37 °C. The DNA was precipitated by the addition of 2 volumes of ethanol and pelleted and washed as before. Finally the DNA was resuspended in 100 µl of sterile distilled water.

2.4.4 Preparation of genomic DNA from *Candida albicans* for colony PCR

This method was used for the rapid preparation of genomic DNA from *C. albicans* colonies for colony PCR screening. Cells were obtained from a colony by touching a yellow Gilson tip to the surface of the colony. The cells were resuspended in 10 µl of incubation solution (1.2 M sorbitol; 10 mM Tris-HCl, pH 7.5) and 50 µg of zymolyase was added. The suspension was incubated at 37 °C for 30 minutes. 2 µl of suspension was used in a 50 µl PCR reaction.

2.4.5 Small scale plasmid DNA preparations from *Saccharomyces cerevisiae* (Holm *et al.*, 1986)

The following method was used for the preparation of yeast genomic DNA enriched for plasmids for transformation into *E. coli.*

Overnight cultures of 5 ml were centrifuged at 3000 rpm and resuspended in 1 ml of sterile distilled water. The suspension was spun in a microcentrifuge and the cells resuspended in β-ME buffer (50 mM sodium phosphate buffer, pH 7.5; 0.9 M sorbitol; 1 µl β-mercaptoethanol per ml of buffer) to which 25 µl of 10 mg. ml^{-1} zymolyase was added. The suspension was incubated at 37 °C for 30 minutes, checked for spheroplast formation, and then incubated at 70 °C for 20 minutes. 200 µl of potassium acetate was added and the suspension mixed by inversion. After incubation on ice for 45 minutes the suspension was centrifuged and the supernatant was transferred to a fresh tube. 0.55 ml of propan-2-ol was added and the mixture incubated at room temperature for 5 minutes. The DNA was then pelleted by spinning in a microcentrifuge for 15 minutes and the supernatant removed. The pellet was washed in 70 % ethanol and resuspended in 20 µl sterile distilled water. 10 µl of the suspension was used in subsequent transformations into *E. coli.*

2.4.6 RNA preparations from *Candida albicans* (Schmitt *et al.*, 1990)

200 ml of exponentially growing cultures were harvested by filtration and washed in 5 ml DEPC (diethyl pyrocarbonate) treated water. The cell pellets were then resuspended in 400 µl of AE buffer (50 mM sodium acetate, pH 5.3; 10 mM EDTA) and 80 µl of 10 % SDS added. The suspension was vortexed for 30 seconds prior to the addition of 480 µl of phenol equilibrated with AE buffer. The suspension was again vortexed for 30 seconds and then incubated at 65 °C for 4 minutes followed by chilling in dry ice/ ethanol until phenol crystals appeared (about 3 minutes). This freeze/ thaw process was repeated 3 times with a final 4 minute incubation at 65 °C. The aqueous layer was separated by centrifugation at 13,000 rpm for 5 minutes and was removed into a separate tube. An equal volume of phenol/chloroform was then added and the aqueous layer again separated by centrifugation. Phenol/chloroform extractions were repeated until no proteinaceous matter was no longer observed at the interface. The RNA was then precipitated by the addition of 2 volumes of absolute ethanol and was pelleted by centrifugation at 13,000 rpm for 15 minutes. The RNA was resuspended in 50 µl of DEPC-treated water and the concentration determined by UV spectrophotometry at 260 nm (the extinction coefficient of RNA at this wavelength is 0.025).

> ***DEPC-treated water*** DEPC (diethyl pyrocarbonate) was added to sterile distilled water at 0.1 % v/v. The water was left overnight in a fume hood and then autoclaved the following morning. This water was used to prepare all solution required for RNA work.

2.5 Bacterial transposon mutagenesis (Sedgwick & Morgan, 1994)

In order to pinpoint the location of a rescuing gene within the insert of a rescuing clone the bacterial mutagenesis method of Sedgwick and Morgan (1994) was employed. The plasmid of interest was transformed into *E. coli* strain DH1 (Sm^S) harbouring the conjugative plasmid R388::*Tn1000(HIS3)*. Cointegrate formation between the two plasmids was then selected for by conjugal mating with a streptomycin resistant strain, MH1578 (Amp^S, Sm^R).

Donor and recipient cells were grown overnight in LB containing the appropriate antibiotics. Cells were then diluted to an OD_{600} of 0.05 in 10 ml LB and allowed to grow with aeration until an OD_{600} of 0.5 was reached. 0.5 ml of both donor and recipient cells were then mixed

in a microcentrifuge tube and the cell suspension applied to a nitrocellulose filter (25 mm diameter, 0.45 μm pore) attached to a vacuum. The nitrocellulose filter was then placed on a pre-warmed LA plate and incubated for 1 hour at 37 °C. The filter was then transferred to a microcentrifuge tube, 0.5 ml of water added and the tube vortexed to detach the cells. The cells were then pelleted by centrifugation, resuspended in 100 μl of water and plated onto LA plates containing the ampicillin and streptomycin to select for recipient cells which had received the cointegrate from the donor cells.

2.6 DNA Manipulations

2.6.1 Restriction analysis

Restriction endonucleases were obtained from Gibco-BRL Ltd. Digestion of DNA was carried out using the React buffers supplied by the manufacturer at the recommended temperature following the manufacturers instructions.

1-2 μg of plasmid DNA was digested for 1-2 hours with 1 unit of enzyme per reaction. Yeast genomic DNA digestions were carried out in 20 μl volumes for 3 hours with 2 units of enzyme. This was followed by further digestion with an additional 1 unit for 2 hours. The reaction volume was increased to 30 μl using restriction buffer and distilled water.

2.6.2 DNA agarose gel electrophoresis

DNA was visualised and fragments were purified from gels made up and run in 1 × Tris acetate electrophoresis buffer (TAE) containing 0.5 μg/ml ethidium bromide. Stocks of TAE were made up at 50 × concentrate (2 M Tris-base, 1 M Sodium acetate (trihydrate), 0.5 M EDTA, pH 8.2 to using glacial acetic acid). Standard gels were made from 0.7-1.5 % Seakem HGT agarose, depending on the fragments requiring separation.

10× loading buffer 0.4 % Bromophenol blue

50 % glycerol

2.6.3 Recovery of DNA from agarose gels

Samples of restriction digested DNA were electrophoresed on a gel such that 5 μg-10 μg of DNA was present in the band of interest. The band of interest was excised using a scalpel

and placed in a microfuge tube. A QIAGEN gel extraction kit (available from QIAGEN Ltd.) was then used to recover the DNA from the gel according to the manufacturers instructions.

2.6.4 DNA ligation

Where it was necessary to phosphatase the vector prior to setting up the ligation reaction, shrimp alkaline phosphatase (USB) was used. Phosphatase reactions were carried out in volumes of 20 µl using 1 µg of vector DNA and 0.1 units of shrimp alkaline phosphatase. The reaction buffer provided by the manufacturer was used. The reaction was incubated at 37 °C for 30 minutes after which a further 0.1 units of enzyme were added, the reaction volume made up to 30 µl and the reaction incubated at 37 °C for further 30 minutes. The reaction was terminated by incubation at 65 °C for 15 minutes.

T4 DNA ligase (Gibco-BRL, 1U. μl^{-1}) was used. Reactions were carried out in a total volume of 20 µl, containing 1 µl T4 ligase, 4 µl T4 ligase buffer (250 mM Tris-HCl, pH 7.5; 50 mM $MgCl_2$, 50 mM DTT, 5 mM ATP, 125 µg. ml BSA) plus the DNA being ligated. Samples were incubated overnight at 4 °C and then transformed into competent *E. coli* cells. Generally 2 reactions were set up in parallel, one containing vector:insert in the molar ratio of 1:1 and the other 1:3 (Dugaiczyk *et al.*, 1975).

2.7 Southern and Northern blotting

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

The method used to transfer DNA to a nitrocellulose filter was essentially that of Southern (1975) with a depurination step before alkaline denaturation to facilitate the transfer of large DNA fragments (Wahl *et al.*, 1979).

After visualisation the gel was washed briefly in distilled water. It was then washed twice for 10 minutes in depurinating solution (0.25 M HCl) and twice for 10 minutes in denaturing solution (0.5 M NaOH, 1 M NaCl) with rinsing in distilled water between washes. Finally the gel was washed twice in neutralising solution (0.5 M Tris-HCl (pH 7.4), 3 M NaCl) for 15 minutes and was then was mounted on a glass sheet that was covered with Whatman 3MM to act as a wick over a reservoir of 20 × SSC. The edges of the gel were covered with plastic film in order to prevent "short circuiting". A nitrocellulose filter cut to the size of the gel was wetted in 6 × SSC and then placed on the gel and covered with 5 sheets of Quickdraw

blotting paper (available from Sigma Chemical Company Ltd). A glass plate was placed on the top with a weight placed on it to distribute the weight evenly. This was left overnight to elute the DNA onto the filter.

After the transfer was complete the filter was removed and placed on a piece of Whatman 3MM paper and allowed to dry completely. The DNA was then cross-linked to the filter by exposure to ultra violet light in a UV cross linker (Amersham Life Science) at 254 nm and 70×10^3 microjoules.cm^{-2}.

2.7.2 Denaturing gels for RNA separation

RNA for Northern blotting was separated on formaldehyde-denaturing gels. Gels were made containing 1.5 % agarose in 1 × MOPS (3-[N-morpholino]propanesulphonic acid), 5 % formaldehyde and were run in 1 × MOPS. RNA samples of 30 μg were prepared for loading by the addition of 2 μl of 5 × MOPS, 10 μl of deionised formamide, 3.5 μl of 40 % formaldehyde. The samples were then incubated at 65 °C for 10 minutes in order to denature the RNA and then snap cooled on ice. 2.5 μl of 10 × loading buffer was then added and the samples loaded onto the gel. The samples were run in triplicate where two samples were used for probing and one was post-stained with ethidium bromide to visualise the RNA.

2.7.3 Northern transfer

The gel was mounted on a glass sheet covered with Whatman 3MM to act as a wick over a reservoir of 20 × SSC. The edges of the gel were covered with plastic film in order to prevent "short circuiting". A nitrocellulose filter cut to the size of the gel was wetted in 6 × SSC and then placed on the gel and covered with 5 sheets of Quickdraw blotting paper (available from Sigma Chemical Company Ltd). A glass plate was placed on the top with a weight was placed on it to distribute the weight evenly. This was left overnight to elute the RNA onto the filter.

After the transfer was complete the filter was removed and placed on a piece of Whatman 3MM paper and allowed to dry completely. The RNA was then cross-linked to the filter by exposure to ultra violet light in a UV cross linker (Amersham Life Science) at 254 nm and 70×10^3 microjoules.cm^{-2}.

2.8 Radioactive labelling and detection of probes

2.8.1 Preparation of labelled probe

Radiolabelled DNA probes were prepared for filter hybridization using random hexamer priming (Feinberg & Vogelstein, 1983). α-^{32}P-dCTP was incorporated into the DNA in the presence of the other unlabelled nucleotides.

The DNA to be labelled was denatured by boiling for 3 minutes and then snap cooled on ice. To 25 ng of the denatured DNA 5 µl of oligo-labelling buffer (OLB; see below), 1 µl of BSA (10 mg.ml^{-1}), 1 µl Klenow and 2.5 µl α-^{32}P-dCTP. The mixture was incubated at room temperature for 4 hours and then fractionated in order to separate the un-incorporated nucleotides away from the labelled probe. The fractionation was carried out using a column made from a Pasteur pipette plugged with polymer wool containing Sephadex G-50 (medium) beads (Pharmacia) suspended in 1 × TE. The volume of the labelling reaction was made up to 100 µl by the addition of 1 × TE and the solution added to the top of the column. Aliquots of 100 µl of 1 × TE were then added to the top of the column and the individual 100 µl fractions collected in microfuge tubes. Each aliquot was tested for radioactive emission using a Geiger counter and the first group of aliquots showing high emissions were pooled for use as the probe. Since Sephadex beads separate the oligonucleotides from un-incorporated nucleotides by the size exclusion principle the labelled probe passes through the column faster than the un-incorporated nucleotides and is found in the first peak of radioactive material collected from the bottom of the column. Finally, the DNA was denatured by boiling for 3 minutes and was then added directly to the hybridisation chamber.

OLB was made up from solutions A, B and C in the ratio 2:5:3.

Solution A 100µl solution O (1.25 M Tris-HCl, pH 8.0; 0.125 M $MgCl_2$), 18 µl 2-mercapto-ethanol, 5 µl each of dATP, dTTP, and dGTP.

Solution B 2 M HEPES, titrated to pH 6.6 with 4 M sodium hydroxide

Solution C Hexadeoxynucleotides resuspended in 1 × TE at 90 OD units. ml^{-1}.

2.8.2 Filter hybridisation for Northern and Southern blots

Filters were incubated in 50 ml of prehybridisation solution (Southern blots) or 10 ml of Church-Gilbert buffer (Northern blots; Church & Gilbert, 1984) for 4 hours at 65 °C in a hybridisation chamber in a rotating hybridisation oven. The prehybridisation solution was then replaced with 25 ml of hybridisation solution (Southern blot only) and the denatured

labelled probe added. Hybridisation was continued at 65 °C overnight and stringency washes were carried out the following day. In general, three stringency washes (3 × SSC, 0.1 % SDS; 1 × SSC, 0.1 % SDS and 0.5 × SSC, 0.1 % SDS) were carried out at 65 °C for 30 minutes each. The filters were then wrapped in Saran wrap, placed in X-ray cassettes with X-ray film. The film was exposed at –80 °C for 1 to 28 days prior to developing.

Prehybridisation solution 6 × SSC, 0.5 % SDS, 5 × Denhardt's solution, 0.1 mg. ml^{-1} denatured salmon sperm DNA

Hybridisation buffer 6 × SSC, 0.5 % SDS, 5 × Denhardt's solution, 10 mM EDTA, 0.2 mg. ml^{-1} denatured salmon sperm DNA

50 × Denhardt's solution 1 % (w/v) Ficoll (mw 40,000), 1 % (w/v) bovine serum albumin, 1 % (w/v) polyvinylpyrididone (mw 40,00)

Church-Gilbert buffer 0.5 M sodium phosphate, pH 7.4; 7 % SDS; 1 mM EDTA

2.9 DNA sequencing and polymerase chain reaction

2.9.1 DNA sequencing

Custom made primers were supplied by PNACL, Leicester University, as described in table 2.7. DNA was sequenced on an ABI model 373A sequencer by PNACL, Leicester University, following preparation using the PRISM Ready Reaction DyeDeoxy Terminator cycle sequencing kit.

Sequencing reactions were carried out in a total volume of 20 µl (overlaid with liquid paraffin) containing 8.0 µl terminator premix, 200 ng template DNA and 3.2 pmol primer DNA. The cycling reaction was carried out in a Hybaid Omni-E cycler, and consisted of 45 cycles of a denaturation step (96 °C, 30 sec), an annealing step (50 °C, 15 sec) and an extension incubation (60 °C, 4 min). After the PCR the reaction mix was isolated from under the paraffin layer by pipetting and extension products precipitated by the addition of 50 µl of absolute ethanol and 2 µl sodium acetate (pH 5.6). The precipitated DNA was washed twice with 70 % ethanol and the dried pellet submitted to PNACL for analysis on the DNA Sequencer.

Terminator premix 1.58 μM A-DyeDeoxy, 94.74 μM T-DyeDeoxy, 0.42 μM G-DyeDeoxy, 47.37 μM C-DyeDeoxy, 78.95 μM dITP, 15.79 μM dATP, dCTP, dTTP, 168.42 mM Tris-HCl (pH 9.0), 4.21 mM NH_4SO_4, 42.1 mM $MgCl_2$, 0.42 units. μl^{-1} AmpliTaq DNA polymerase.

2.9.2 Polymerase chain reaction

PCR was used for the amplification of DNA fragments and was performed using either a Hybaid Omni-E thermal cycler or a Sanyo MIR-D30 DNA amplifier. A typical reaction was carried out in a final volume of 50 μl containing 200 ng of each oligo-nucleotide primer, 50 ng template DNA, 1 unit Taq polymerase and 5 μl reaction buffer. The reaction was overlaid with liquid paraffin to prevent evaporation during the course of the PCR temperature cycling. Custom made primers were made by PNACL, Leicester University and are described in table 2.7.

10 x PCR buffer 450 mM Tris-HCl (pH 8.8), 110 mM NH_4SO_4, 45 mM $MgCl_2$, 67 mM 2-mercaptoethanol, 44 μM EDTA (pH 8.0), 10 mM dATP, 10mM dCTP, 10 mM dGTP, 10 mM dTTP, 1.13 mg. ml^{-1} BSA.

A typical reaction profile consisted of 35 cycles of a denaturation step (95 °C, 1 min), an annealing step (50-60 °C, 1 min) and an extension step (72 °C). The length of the extension incubation time was calculated by using a rule of 1 minute per kb of DNA being amplified. The products of PCR were visualised following agarose gel electrophoresis.

Table 2.6 List of synthetic oligonucleotides used for sequencing used in this study

Name	Sequence (5' → 3')	Target
	Standard sequencing primers	
δ		Tn1000 LTR (86 to 68 from δ terminal)
γ		Tn1000 LTR (57 to 37 from γ terminal)
TetA		tetracycline resistance gene of YEp213 (356 to 373 of Yep213)
	pJDF1.3 sequencing primers	
TetA.1	caa tca cgg ctc tga ttc ttc	*CFL1* (-750 to -30)
1.3F3	gat aat aat acg tga acg ccg	*CFL1* (-508 to -488)
1.3F4	gtt tgc ggt agt att aat cg	*CFL1* (+ 29 to +48)
505.F	taa gac ttc tgc tag tgc	*CFL1* (+377 to +394)
1.3F5	ggc agt aac agc agt act ag	*CFL1* (+456 to +475)
1.3F6	caa tat gct act aag aat gc	*CFL1* (+939 to +961)
cflf.f	aca aga ggt taa aca agc	*CFL1* (+2132 to +2149)
1.3R5	cga cag att cga taa gtt ttg g	*CFL1* (-555 to –566)
1.3R4	caa atg aat gtg ata tat gcg	*CFL1* (-218 to –238)
1.3R3	ctg aga ttg ttc caa acc c	*CFL1* (+207 to +189)
581r	cat gag cat ttg tgg acg	*CFL1* (+456 to +439)
1.3R7	cgt aga aat atc tgg acg cc	*CFL1* (+2123 to +2104)
1.3R6	cat caa cgg cct cca aga caa g	*CFL1* (+2602 to +2581)
	pJDF2.3 sequencing primers	
2.3F1.3	gca ctt gca tat aat cgg tgc c	*CFL2* (-1117 to –1096)
2.3F1.2	ctt tga ggt tga cga taa ttg g	*CFL2* (-639 to –618)
2.3F1.1	ctt aca gat acg tat aca atc c	*CFL2* (-231 to –210)
2.3db	caa cac caa gaa gag ttt c	*CFL2* (+168 to +186)
2.3F1	gca aaa ccc tgt cta cga aag c	*CFL2* (+586 to +607)
2.3F2	gat tct tcg atg gtc ttg tcc c	*CFL2* (+955 to +976)
2.3F3	gtc gct acc att tgt gct gg	*CFL2* (+1350 to +1369)
2.3F4	cag tct ggg tat ttg atc gag c	*CFL2* (+1522 to + 1543)
2.3F5	ctc tga atg tgt tga ttt agc c	*CFL2* (+1937 to +1958)
2.3F7	gaa aca gac cgt ggt gca ctt ggg ttc g	*CFL2* (+2274 to +2298)
2.3F9	ggg ctt aac ttg ctg gga ttg tta gtt acc	*CFL2* (+2404 to +2433)
2.3R4	gct acc gtc tag tgt ttt gtg gt	*CFL2* (-759 to –780)
2.3R3	gaa agc taa tta aga ggc cg	*CFL2* (-278 to –298)
2.3R2	gat gag gca aaa caa act agc	*CFL2* (-55 to –75)
2.3R1	ctg atg ctt tcg tag aca ggg	*CFL2* (+612 to +592)
2.3R1.1	gca ttc ttg tat ggt gtg aac g	*CFL2* (+721 to +700)
2.3R1.2	gtc acc ata ccc ttg gtc g	*CFL2* (+1502 to +1484)
2.3R6	cgt aac gaa ccc aag tgc acc g	*CFL2* (+2303 to +2282)
2.3R1.3	cct caa ttc atc aac cat ggc	*CFL2* (+2336 to +2316)
2.3R5	cca aag taa gtg ata act gta cc	*CFL2* (+2690 to +2668)

Table 2.7 List of synthetic oligonucleotides for PCR used in this study

Name	Sequence (5' → 3')	Target site	Special features
SF1.2	ggg gta cct gct tga cgg gtt tgc agc	*FRE1* ORF (+1 to +27))	*Kpn*I site incorporated into 5' end
SF2	ttc aac gtc aat ctt caa gga aga gtc	*FRE1* ORF (+2034 to +2008)	
SF2.L	cgg aat tcc cat gcg aca cac tac gac	*FRE2* ORF (+2326 to + 2308)	*Eco*RI site incorporated into 5' end
SF2.R	cgg gat ccg gct caa tga tgc tag tgg g	*FRE2* ORF (-304 to –290)	*Bam*HI site incorporated into 5' end
CFL376	gaa ctt ttt gaa gat cta tta ctg aag aac c	*CFL1* ORF (-932 to –903)	
CFL739	ggc att gga cag atc tgt tga ctt gg	*CFL1* ORF (+431 to + 406)	*Bgl*II site incorporated into middle of primer
CFL3278	ctc agg att agc atg ctt tga aca cg	*CFL1* ORF (+1970 to +1995)	*Sph*I site incorporated into middle of primer
CGT4219	gtc aac tcc cct gca tgc ccc ttt aaa gtt gg	*CFL1* ORF (+3311 to +3280)	*Sph*I site incorporated into middle of primer
CFL2220	gta agg cac tga aga gac ccg	*CFL1* ORF (+641 to +661)	
CFL347	caa gaa agc aaa gaa tcc tta ata cc	*CFL1* ORF (-961 to –936)	
hisG	cat gct ttc atg cac cac tgg	*hisG* ORF (-89 to –108)	
CFL91.F	tgt att gga gtc tgg ttt gg	*CFL91* ORF (+1854 to + 1835)	
CFL91.R	gaa tgg tgg gta ttt gta gc	*CFL91* ORF (+1237 to + 1256)	
CFL93.F	gct atc aag gta ata tcg gc	*CFL93* ORF (+1310 to + 1291)	
CFL93.R	tgg taa ttg ttc tcc atg cc	*CFL93* ORF (+731 to + 750)	
CFL94.F	tcc ttg tat tat gga gca gg	*CFL92* ORF (+469 to + 488)	
CFL94.R	ata aat gca tga cca ccg gg	*CFL92* ORF (+1379 to + 1360)	
CFL95.F	ata acg gta aag gta ttg gc	*CFL95* ORF (+987 to + 1006)	
CFL95.R	gtt tga gca ggt tgt tga gcc	*CFL95* ORF (+1530 to +1510)	
CFL96.F	ctt cat aat acc att gta cc	*CFL96* ORF (+1774 to +1756)	
CFL96.R	ttg gat tcc aat tgg caa cc	*CFL96* ORF (+1307 to + 1326)	
CFL97.F	cta aat ata atg tga cta tac c	*CFL97* ORF (+875 to +854)	
CFL97.R	gta tga tta atg cat tca ttg g	*CFL97* ORF (+582 to +603)	

2.10 Detection of ferric reductase activity

2.10.1 Qualitative ferric reductase assay

A modified version of the qualitative solid phase ferric reductase assay described by Dancis and co-workers (1990) was used to identify extracellular ferric reductase activity. Cells were streaked out onto solid SD media, grown for 3 to 5 days at 30 °C. The colonies were then replica plated onto Hybond-N nylon filter discs and placed on MD-dipyridyl media, to which ferric chloride had been added to a final concentration of 300 μM. The plates were then incubated at 30 °C for 5 hours. After incubation the nylon filters were removed and incubated with the colony side facing upwards for 5 minutes on Whatman 3MM paper soaked in assay buffer (50 mM sodium citrate pH 6.5; 5% (v/v) glucose). The nylon filters were then transferred to a second sheet of 3MM paper soaked in assay buffer containing 1 mM $FeCl_3$ and 1 mM of the iron chelator BPS. Incubation was then carried out for 5 minutes. Colonies with ferric reductase activity stained the filter red due to the formation of the $[Fe^{2+}(BPS)_3]$ complex.

2.10.2 Quantitative liquid ferric reductase assay

Small starter cultures were grown in YPD for approximately 8 hours, and were then used to inoculate a fresh culture in liquid MD media at a cell density of 3.6×10^4 cells.ml^{-1} for *C. albicans* or 1×10^5 cells.ml^{-1} for *S. cerevisiae*. These cultures were grown for approximately 16 hours and then diluted to 2×10^6 cells.ml^{-1} in either MD-dipyridyl in the case of *C. albicans* or MD-EDTA in the case of *S. cerevisiae*. Iron chloride was added back to this media to create high iron conditions at concentrations indicated in the text.

Quantitative reductase assays were carried out at appropriate time points as follows. Samples of the culture removed and the cell density determined by counting. A sample of 1×10^7 cells was then removed and harvested by filtration. The filter was transferred to a microfuge tube, 0.5 ml of sterile distilled water added and the tube vortexed to remove the cells from the filter. The cell suspension was then centrifuged at 13,000 rpm and the supernatant removed. The cells were resuspended in 0.8 ml of assay buffer (50 mM sodium citrate pH 6.5; 5% (v/v) glucose) to which 20 μl of 5 mM $FeCl_3$ and 200 μl of 50 mM BPS was added. A blank tube was also set up at this point containing no cells, but all the other components. The cell suspension and blank were then incubated at 30 °C for 10 minutes and the cells harvested by

centrifugation. The supernatant and the blank were then transferred into 1 ml cuvettes and the optical density measured at 520 nm. The principle of this assay is the same as that of the solid phase assay but allows the quantitative measurement of the rate of formation of Fe^{2+} ions.

A standard curve was constructed in order to allow the relationship between OD_{520} and the concentration of Fe^{2+} ions to be determined. A series of tubes containing the same mixture as the blank described above but supplemented with varying concentrations of $FeCl_2$ were used. The OD_{520} of each sample was measured and plotted against the $FeCl_2$ concentration, thus showing that a linear relationship exists between the two parameters.

Chapter 3
Construction and analysis of a *Saccharomyces cerevisiae fre1* mutant

3.1 Introduction

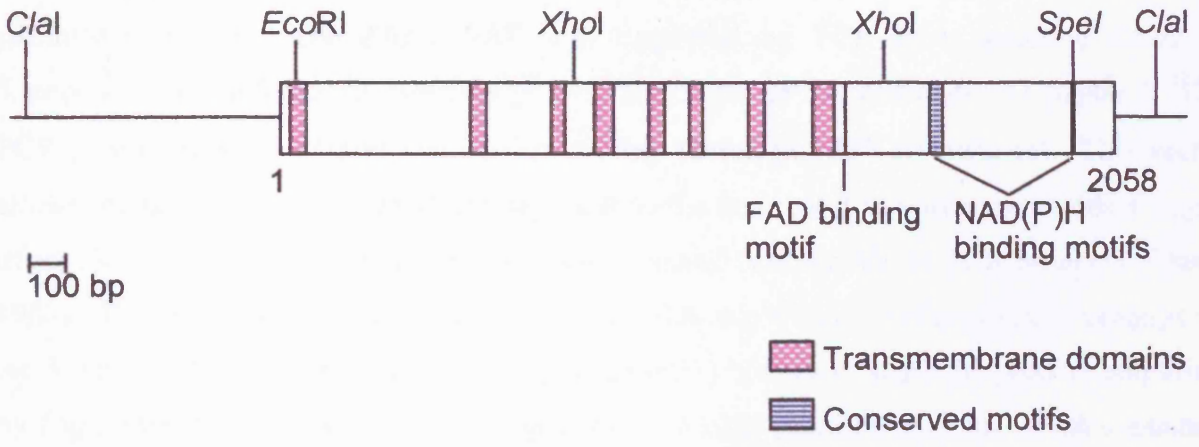
The aim of the work outlined in this chapter was to construct and analyse a *S. cerevisiae* strain with defective ferric reductase activity by deleting the *fre1* gene, which encodes a structural component of the cell surface ferric reductase. The purpose of constructing this strain was to create a mutant background that could be used to screen for *C. albicans* genomic clones capable of complementing the loss of ferric reductase phenotype associated with a *fre1* mutant.

S. cerevisiae has a well-characterised iron uptake system which makes use of a cell surface ferric reductase to reduce the relatively insoluble Fe^{3+} ions to Fe^{2+} ions. Two main components of this reductase are encoded by the genes *FRE1* and *FRE2* (Dancis *et al.*, 1992; Georgatsou & Alexandraki, 1994). These components are both capable of conferring reductase activity individually, as shown by deletion experiments where each gene is knocked out in turn. The resultant mutants still display reductase activity but the activity is temporally different and it has been shown that Fre1p activity peaks in early to mid log phase whilst Fre2p activity peaks in late log (Georgatsou & Alexandraki, 1994). The deletion of *FRE1* results in the loss of up to 98 % ferric reductase activity, depending on growth phase (Dancis *et al.*, 1990). *FRE1* was therefore considered an appropriate deletion target for constructing a mutant with defective reductase activity. Although reports of such mutants are present in the literature, unfortunately none were available for our use.

3.2 Construction of *fre1* disruption cassette

Fre1p is a member of the family of ferredoxin-NAD(P)H reductases that are found in a wide variety of organisms (Segal *et al.*, 1992). A number of motifs implicated in NAD(P)H binding and FAD binding are found to be conserved within this group of proteins (Karplus *et al.*, 1991) and in Fre1p these motifs are found clustered towards its C-terminal end (Fig 3.1). The deletion strategy was therefore designed with this in mind to ensure that these motifs were either removed by the deletion or were downstream of the inserted marker gene.

Figure 3.1 Schematic diagram of *FRE1*



The *FRE1* ORF is shown as a box. The conserved motifs implicated in FAD and NAD(P)H binding and putative transmembrane regions are shown as coloured blocks on the ORF. The *Xho*I sites used for excising the central portion of the gene are shown as are the *Cla*I sites which were used by Dancis and co-workers (1992) to produce a complete deletion of *FRE1*.

Previous studies have used *Xho*I sites internal to the *FRE1* gene to construct deletion cassettes (Dancis *et al.*, 1992). This results in the deletion of 838 bp from the centre of the gene. This region encodes the motif implicated in FAD binding as well as 5 putative transmembrane domains (Fig 3.1). A mutant strain constructed in this way has been shown to have an identical phenotype to one produced by a complete deletion of the gene using *Cla*I sites which lie outside the ORF (Fig 3.1; Dancis *et al.*, 1992). Thus, this small deletion may be regarded as producing a complete null phenotype.

A one-step gene disruption method was used to construct the *S. cerevisiae fre1* mutant strain (Rothstein, 1983). The *FRE1* ORF was amplified by PCR from genomic DNA of *S. cerevisiae* strain S150-2B using the primers SF1.2 and SF2 as described in Chapter 2. The PCR product was sub-cloned into the TA-cloning vector, pCRII™ (Invitrogen). This vector allows the direct cloning of PCR products due to the fact that *Taq* polymerase adds a single deoxyadenosine residue onto the 3' hydroxyl group of both strands of PCR products (Clark, 1988). The vector pCRII is provided as a linear molecule with deoxythymidine overhangs on the 3' strand of both ends thus providing complimentary ends to any PCR product amplified by *Taq* polymerase. However, this vector contains a large multi-cloning site which contained restriction sites required for the subsequent disruption strategy. Consequently, a 1.8kb *FRE1* *Eco*RI fragment was sub-cloned from the pCRII-*FRE1* plasmid into pUC13, resulting in the plasmid pJD3 (Fig 2.6).

The plasmid pJD3 was prepared for construction of the deletion cassette by digestion with *Xho*I. This resulted in the removal of the central 838 bp of *FRE1* leaving the rest of the plasmid intact. The intact plasmid was recovered by gel extraction from an agarose gel after the fragments had been separated by electrophoresis. This was then treated with shrimp alkaline phosphatase and used in ligation reactions to introduce the selectable marker.

The marker gene used to insert into pJD3 in place of the 838 bp *Xho*I fragment was *HIS3*. This was obtained from the plasmid pYRG17 (see Chapter 2), a yeast shuttle vector derived from pUC13, which contained the *HIS3* gene inserted into the *Sal*I site. Since *Sal*I and *Xho*I have compatible 5' overhangs this was a suitable choice for the disruption. The *HIS*3 gene was excised from pYRG17 by digestion with *Sal*I and recovered by purification from an agarose gel after the digest had been electrophoresed to separate the fragments.

Ligation reactions which were set up using the phosphatase treated plasmid and the *HIS3* gene were transformed into *E. coli* strain DH5α. Colonies were picked for further analysis

and 4 colonies were identified containing the *HIS3* gene disrupting *fre1*. One of these plasmids, which was named pJD3.2 (see Fig 2.6), was used to provide the disruption cassette for the construction of the *S. cerevisiae* mutant.

3.3 Allele replacement of *FRE1* by one step gene disruption

The *fre1* disruption cassette was linearised by digestion of the plasmid pJD3.2 with *Eco*RI and *Spe*I. These restriction sites were both present in the cloned *FRE1* fragment (Fig 3.1) and therefore the disruption cassette contained no vector sequences that might interfere with homologous recombination. The enzymes were inactivated by heating and the DNA used to transform the *S. cerevisiae* strain S150-2B. A total of 1770 colonies were obtained with a transformation efficiency was 885 colonies . μg of DNA^{-1}.

Thirty-six colonies were chosen for further analysis and each was resuspended in 5 μl of water in wells of a microtitre dish. The wild type strain, S150-2B, was also included in one well as a positive control. The colonies were stamped out in triplicate onto SD plates. Once colonies had grown two of the plates were used to replica plate colonies onto nylon filters for use in the solid phase reductase assay. The third plate was retained as a reference plate. The nylon filters were placed onto MD-dipyridyl plates containing 300 μM iron (with the colony side facing upwards) and incubated at 30 °C for 5 hours. After this time the filters were removed and soaked in assay buffer (see Chapter 2) for 5 minutes followed by 5 minutes in assay buffer containing iron chloride and BPS. Where ferric reductase activity is present Fe^{3+} ions from the iron chloride are reduced to Fe^{2+} ions, which are chelated by BPS forming a pink compound that stains the filter. Thus, colonies that have lost ferric reductase activity can be identified since they do not stain the filter because they cannot generate Fe^{2+} ions.

Of the 36 colonies checked for ferric reductase activity 7 showed a loss of reductase activity as compared to the wild type control. One of these was then picked for further analysis by Southern blotting. The genome sequence surrounding *FRE1* was known and three restriction enzymes were selected on the basis that they produced reasonable sized fragments containing the *FRE1* gene and that the wild type bands were easily distinguishable from the expected mutant bands. This strain was named JHS1.

Genomic DNA from both S150-2B and JHS1 were digested with *Cla*I, *Eco*RI and *Kpn*I then analysed by Southern blotting using a 1.8 kb *Eco*RI fragment of pJD3 as a probe (see Chapter

2). The observed band sizes for each of the enzymes and strains were as predicted, confirming the correct insertion of the disruption cassette (Fig 3.2, Table 3.1). This strain was therefore used in further studies requiring a *S. cerevisiae* ferric reductase mutant and was named JHS1.

3.4 Phenotypic analysis of JHS1

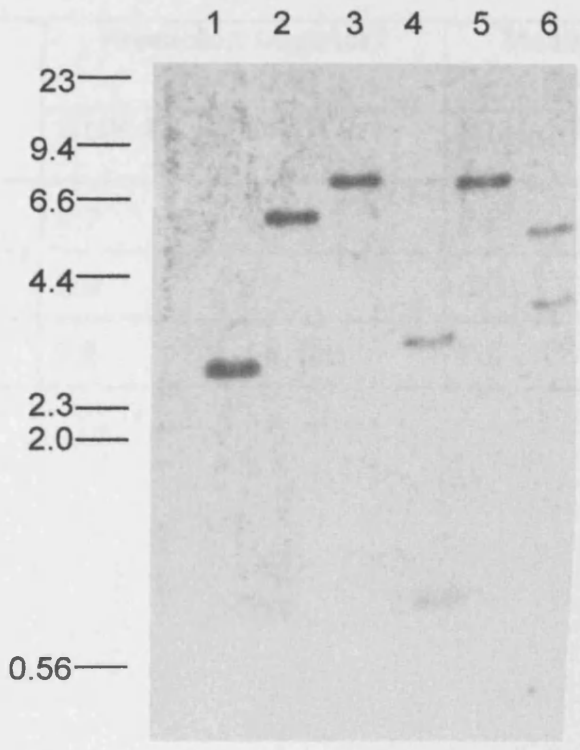
Further phenotypic tests were carried out on JHS1. Firstly, the solid phase reductase assay was used to confirm that JHS1 showed loss of ferric reductase activity. It was observed that the mutant was indeed defective in reductase activity (Fig 3.3a). Secondly, JHS1 was tested for its ability to grow in low iron. Cultures of both S150-2B and JHS1 were grown to saturation in YNB and their cell densities determined by counting. The cultures were then harvested and the cells resuspended at 2×10^7 cells $.ml^{-1}$. Three 1/10 dilutions were then made and 5 µl of each dilution was spotted onto MD-EDTA media supplemented with varying amounts of iron. As can be seen from Figure 3.3b the wild type grows well when supplemented with iron concentrations ranging from 200 µM to 800 µM; whereas JHS1 grows less well when supplemented with 400 µM iron and fails to grow at all when supplemented with 200 µM iron. The addition of 800 µM iron restores the growth of the mutant to almost wild type levels thus indicating that the lack of growth observed on the low iron media is due to the low levels of this metal present. This phenotype is consistent with that of other *fre1* mutants reported in the literature (Dancis *et al.*, 1992).

3.5 Summary

The purpose of the work described in this chapter was to create a *S. cerevisiae* mutant with defective ferric reductase activity such that it could be used in further studies to identify *C. albicans* rescuing clones by functional complementation. A gene encoding a structural component of the cell surface ferric reductase, *FRE1*, was targeted for disruption since previous studies had shown that disruption of this gene resulted in the loss of up to 98 % of cell surface ferric reductase activity during early to mid log phase.

The resultant *fre1* mutant, JHS1, has been shown to possess the phenotypes expected of a ferric reductase mutant. Ferric reductase activity was found to be lost and the strain showed reduced growth in low iron conditions. Both of these results have been observed in similar

Figure 3.2 Southern blot analysis of JHS1



S150-2B genomic DNA and JHS1 genomic DNA were digested with *Cla*I, *Eco*RI and *Kpn*I and electrophoresed in a 0.8 % agarose gel. The gel was blotted onto Hybond-N filter paper and probed with the 1.8 kb *Eco*I fragment from the cloned *FRE1* fragment in pJD3. After over-night hybridisation the filter was washed using the standard stringency conditions described in Chapter 2 and exposed to X-ray film, which was developed after 7 days exposure at -80 °C. Lane 1: S150-2B, *Cla*I; lane 2: S150-2B, *Eco*RI; lane 3: S150-2B, *Kpn*I ; lane 4: JHS1, *Cla*I ; lane 5: JHS1, *Eco*RI; lane 6: JHS1, K*pn*I. The predicted band sizes for each of these enzymes is shown in Table 3.1.

Table 3.1 Actual and predicted band sizes detected by Southern blotting of S150-2B and JHS1 with an *FRE1* probe

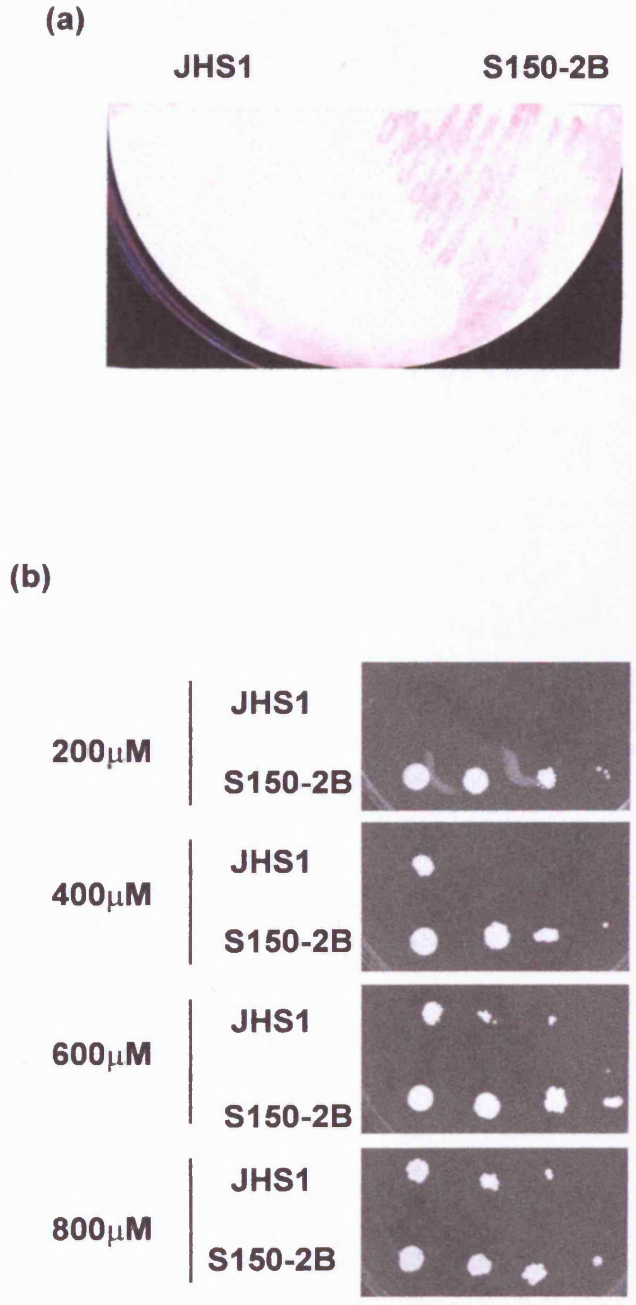
Restriction enzyme	Predicted fragment sizes (kb)		Measured fragment sizes (bp)	
	S150-2B	JHS1	S150-2B	JHS1
*Cla*I	2.7	2.9, 0.9	2.8	2.9, 0.9
*Eco*RI	5.8	6.7	6.0	7.8
*Kpn*I	7.2	4.8, 3.0	7.6	5.6, 3.8

Figure 3.3 Phenotypic analysis of JHS1

(a) The reductase activities of JHS1 and S150-2B were compared using a solid phase ferric reductase assay. Cells grown on SD media were replica plated on to nylon filters placed on the surface of MD-dipyridyl plates containing 300 μM $FeCl_3$. The plates were incubated at 30 °C for 5 hours and then the filters removed and incubated in assay buffer (50 mM sodium citrate, pH 6.5; 5 % glucose) for 5 minutes, followed by a 5 minute incubation in assay buffer containing $FeCl_3$ and BPS. Reductase activity is indicated by the staining of the filter red due to the formation of a $[Fe^{2+}(BPS)_3]$ complex.

(b) The ability of *S. cerevisiae* strains S150-2B and JHS1 to grow in low iron conditions was compared. Overnight cultures were grown to saturation in YNB media and their cell densities determined by counting. The cultures were then harvested and resuspended at 1×10^7 cells.ml^{-1}. A series of 1/10 dilutions were then made to 1×10^4 cells.ml^{-1} and 5μl of the suspensions spotted onto a MD-EDTA plates containing 200 μM to 800 μM added $FeCl_3$.

Figure 3.3 Phenotypic analysis of JHS1



strains constructed by other groups (Dancis *et al.*, 1992). The results obtained from the solid phase ferric reductase assay show clearly that ferric reductase activity is significantly reduced in JHS1 as compared to its parental strain. This makes it an ideal mutant to use for the identification of *C. albicans* clones capable of rescuing ferric reductase activity.

Chapter 4

Isolation of *Candida albicans* clones capable of rescuing a *Saccharomyces cerevisiae fre1* mutant

4.1 Introduction

The aim of the work described in this chapter was to isolate *C. albicans* clones capable of rescuing the *S. cerevisiae fre1* null mutant described in the previous chapter. Work carried out prior to this study had established that no clear signal was obtained from Southern blotting of *C. albicans* genomic DNA with a *S. cerevisiae FRE1* probe (Dickens, 1996) and thus functional complementation seemed the most feasible approach for isolating *C. albicans* ferric reductase genes.

Previous studies from our laboratory have shown that *C. albicans* possesses a cell surface ferric reductase activity that is similar to the ferric reductase of *S. cerevisiae*. The reductase is negatively regulated in response to iron and copper and is also regulated in response to growth, with maximum reductase activity observed during logarithmic growth (Morrissey *et al.*, 1996). Similar patterns of regulation are found in the *S. cerevisiae* system (Dancis *et al.*, 1990). It therefore seems likely that *C. albicans* may use an analogous mechanism of iron acquisition.

4.2 Cloning of *Candida albicans* ferric reductase genes

The *C. albicans* library used in this study was generated by the ligation of *Sau*3AI partially digested genomic DNA fragments from strain S/01 into the unique *Bam*HI site of YEp213 (P. Meacock, personal communication). YEp213 is a multicopy vector with approximately 20-30 copies per cell and the average insert size in this library is 7-8kb. The following equation was used to calculate the number of colonies required to cover the whole genome:

$$N = \frac{\ln (1-p)}{\ln (1-a/b)}$$

where: N = the number of colonies screened

p = probability that any given gene is present in the library (0.95)

a = average size of DNA inserts in the library (7-8 kb)

b = total size of genome (13.5-18.5 $\times$ 10^3 kb per haploid genome; in this calculation the highest value was used)

Therefore:

$$N = \frac{\ln(1-0.95)}{\ln(1-[8/18.5\times 10^3])}$$

N= **6739 colonies**

Therefore approximately 7,000 colonies need to be screened to give a probability of 95% of having screened the complete genome.

The C. *albicans* genomic library was transformed into the *S. cerevisiae fre1* mutant strain, JHS1 (efficiency 2 x 10^4 colonies. μg DNA^{-1}) resulting in approximately 36, 000 colonies spread over 80 plates. This should have represented approximately 5-times coverage of the *C. albicans* genome. The colonies were screened for reductase activity using the solid phase reductase assay (see Chapter 2) to identify clones that restored ferric reductase activity to the mutant. Twenty colonies were identified that appeared to have restored reductase activity and these were picked and re-screened. It was found that 8 no longer showed rescuing activity and these were discarded. The plasmids were rescued from the remaining 12 positives and transformed into *E. coli*. Plasmid preparations were then made from the *E. coli* stocks and re-transformed into JHS1. Again, the transformants were assayed for reductase activity and 5 clones were identified that were still capable of restoring reductase activity (Fig 4.1a). These 5 clones were named pJDF1.2, pJDF1.3, pJDF2.3, pJDF3.4 and JDF3.5.

Since one of the phenotypes associated with JHS1 is poor growth in low iron conditions, the rescuing clones were tested for their ability to rescue this phenotype. Two clones, pJDF1.3 and pJDF2.3, were used for this assay since the others were shown to be either identical or overlapping with these clones (see below). Growth in low iron conditions was restored to a level approximately intermediate between the *fre1* mutant and wild type by the presence of either of these plasmids in JHS1 (Fig 4.1b).

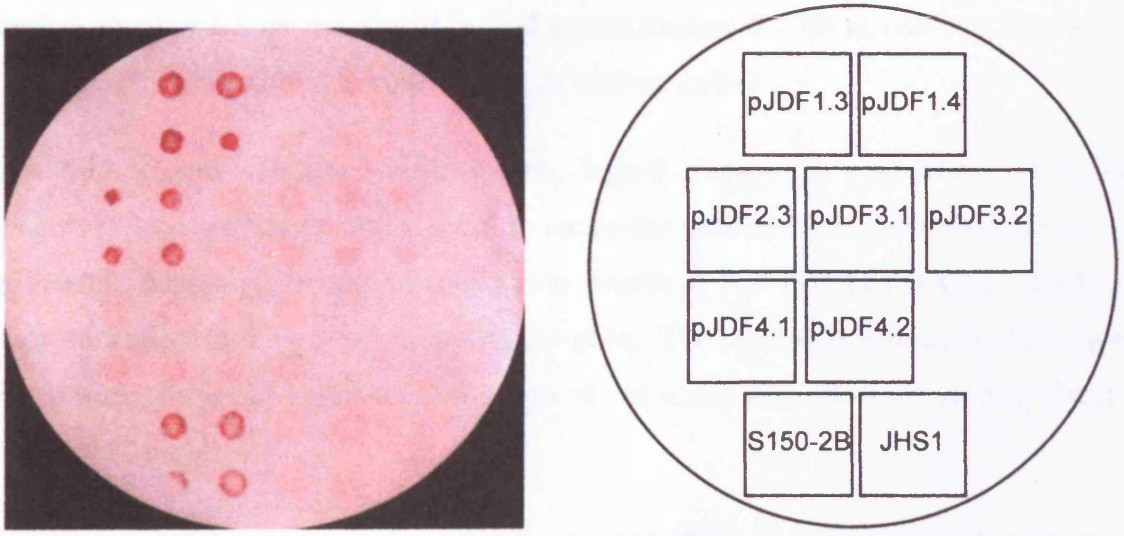
Figure 4.1 *C. albicans* genomic clones pJDF1.3 and pJDF2.3 rescue ferric reductase activity and growth on low iron in JHS1

(a) Reductase activity of rescuing clones was assessed using a solid phase reductase assay (Dancis *et al.*, 1990). Cells grown on SD media were replica plated on to nylon filters placed on the surface of MD-dipyridyl plates containing 300μM $FeCl_3$. The plates were incubated at 30°C for 5 hours and then filters removed and incubated in assay buffer (5 mM sodium citrate, pH 6.5; 5 % glucose) for 5 minutes, followed by a 5 minute incubation in assay buffer containing $FeCl_3$ and BPS (an Fe^{2+} chelator). Reductase activity is indicated by the staining of the filter red due to the formation of a $[Fe^{2+}(BPS)_3]$ complex. The controls on the bottom row of the filter show that ferric reductase activity of JHS1 is lost relative to the parental strain (S150-2B). Two *C. albicans* genomic clones, pJDF1.3 and pJDF2.3, restore ferric reductase activity to the *fre1* mutant. Other clones shown did not rescue ferric reductase activity.

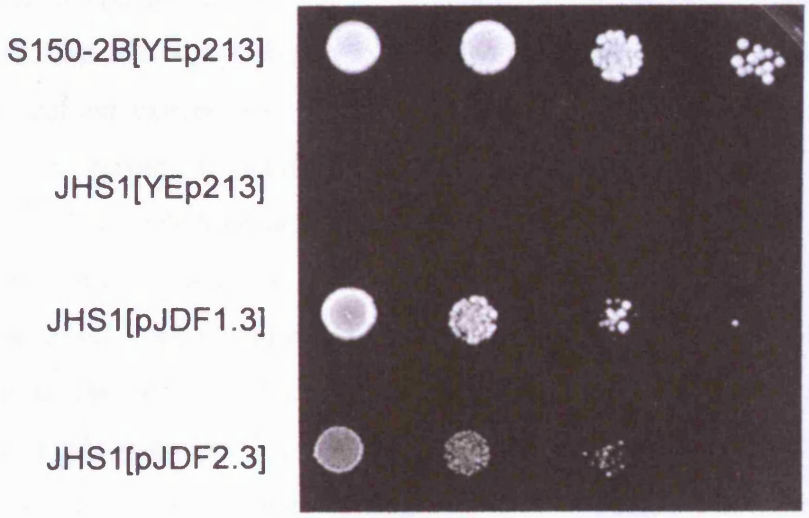
(b) Cells growing in YPD medium were harvested at mid-log phase, washed and resuspended in water at 1×10^7 cells.ml^{-1} A series of 10 fold dilutions were made and 5μl of cells at concentrations ranging from 1×10^7 cells.ml^{-1} to 1×10^4 cells.ml^{-1} were spotted on to agar plates made up of MD-EDTA media. The plates were incubated at 37°C for 5 days.

Figure 4.1 *C. albicans* genomic clones pJDF1.3 and pJDF2.3 rescue ferric reductase activity and growth on low iron in JHS1

(a)



(b)



4.3 Identification of rescuing open-reading frames and sequence analysis of ferric reductase genes

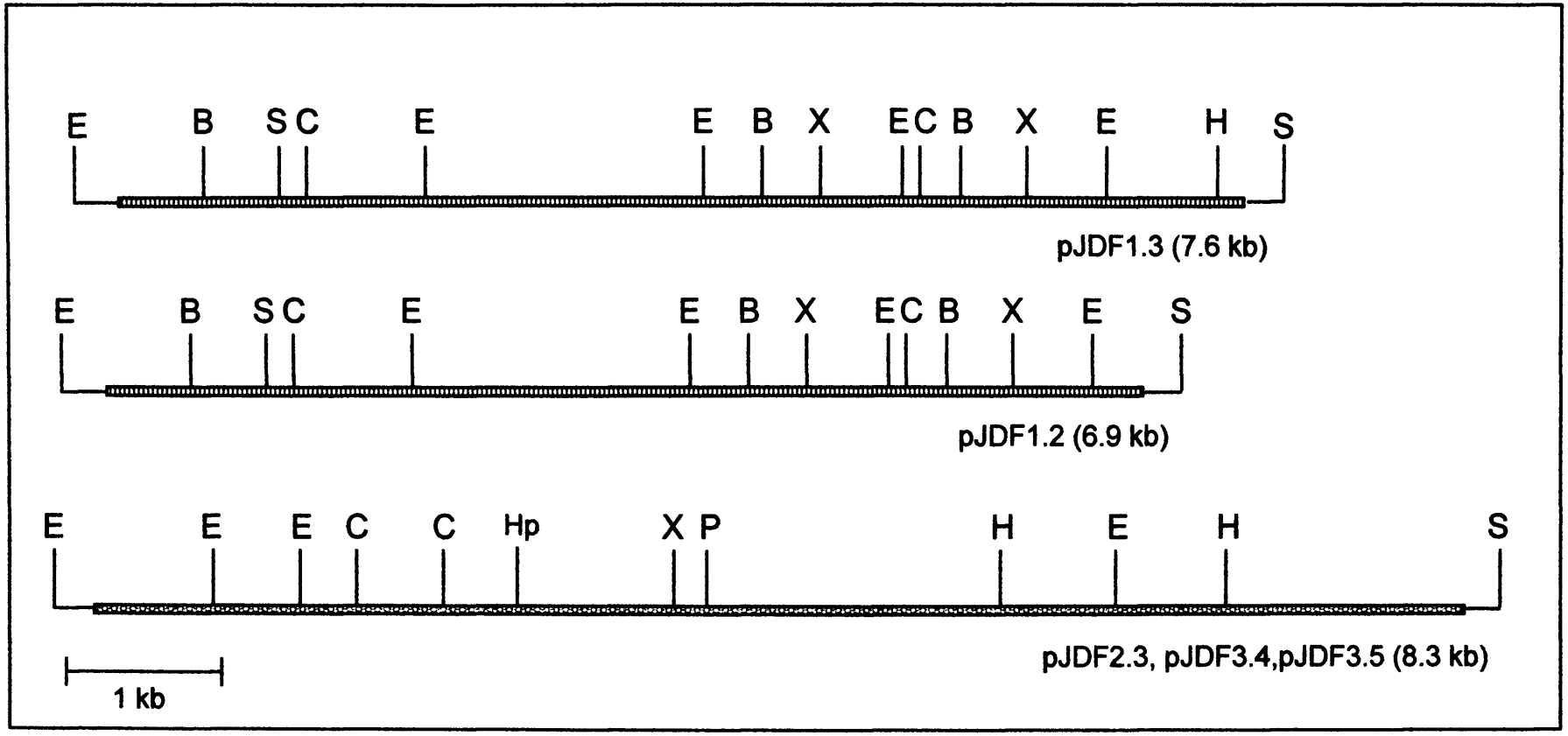
The restriction maps of all 5 rescuing clones were determined and it was found that pJDF2.3, pJDF3.4 and pJDF3.5 were identical, containing inserts of 8.3 kb, whilst pJDF1.2 (6.9 kb) and pJDF1.3 (7.6 kb) showed a large region of overlap with each other. In fact, pJDF1.2 was identical to pJDF1.3 except that it lacked approximately 0.7 kb at one end (Fig 4.2). The clones pJDF1.3 and pJDF2.3 were used in all further studies.

Since both clones contained large inserts, tagged transposon mutagenesis (Sedgwick & Morgan, 1994) was carried out in order to locate the gene conferring rescuing activity within the inserts. Mapping the site of transposon insertions that disrupt rescuing activity should locate the region of the insert containing the gene. The method is also useful for sequencing since primers designed to anneal to the ends of the transposon allow sequencing directly into the gene of interest.

Accordingly, both rescuing clones were used to transform *E. coli* strain DH1 harbouring the conjugative plasmid R388::*Tn1000(HIS3)*. This plasmid was used to introduce the transposon into the clones by cointegrate formation during conjugal mating with the streptomycin resistant strain MH1578, with selection for transfer of the ampicillin marker of YEp213 and the streptomycin resistance of the recipient strain. *E. coli* colonies showing ampicillin and streptomycin resistance were scraped off the agar plates and bulk plasmid preparations carried out. The resulting mixed plasmid preparation was transformed back into JHS1, selecting for leucine prototrophy conferred by YEp213. The transformants were again screened for reductase activity. In the case of pJDF1.3, 1, 249 colonies were screened and 56 were found to have lost rescuing activity and for pJDF2.3, 35 reductase-negative colonies were found from a total of 289 screened. Four rescuing and four non-rescuing derivatives of each of the original plasmid types were chosen for further analysis. Plasmid preparations were made from the *S. cerevisiae* colonies carrying the plasmids and were transformed into *E. coli*. The plasmids were then prepared from the *E. coli* and retransformed into JHS1 and the reductase assay repeated. It was thus confirmed that all plasmids maintained their originally observed phenotype.

Restriction analysis of the transposon-mutagenised plasmids with no reductase activity

Figure 4.2 Restriction maps of rescuing clones



Restriction analysis of pJDF2.3, pJDF3.4 and pJDF3.5 showed them to be identical, whilst pJDF1.3 and pJDF1.2 showed a large region of overlap with each other. The restriction enzymes used were: B = *Bgl*II; *C* = *Cla*I; E = *Eco*RI; H = *Hin*dIII; Hp = *Hpa*I; P = *Pst*I; S = *Sal*I; X = *Xba*I.

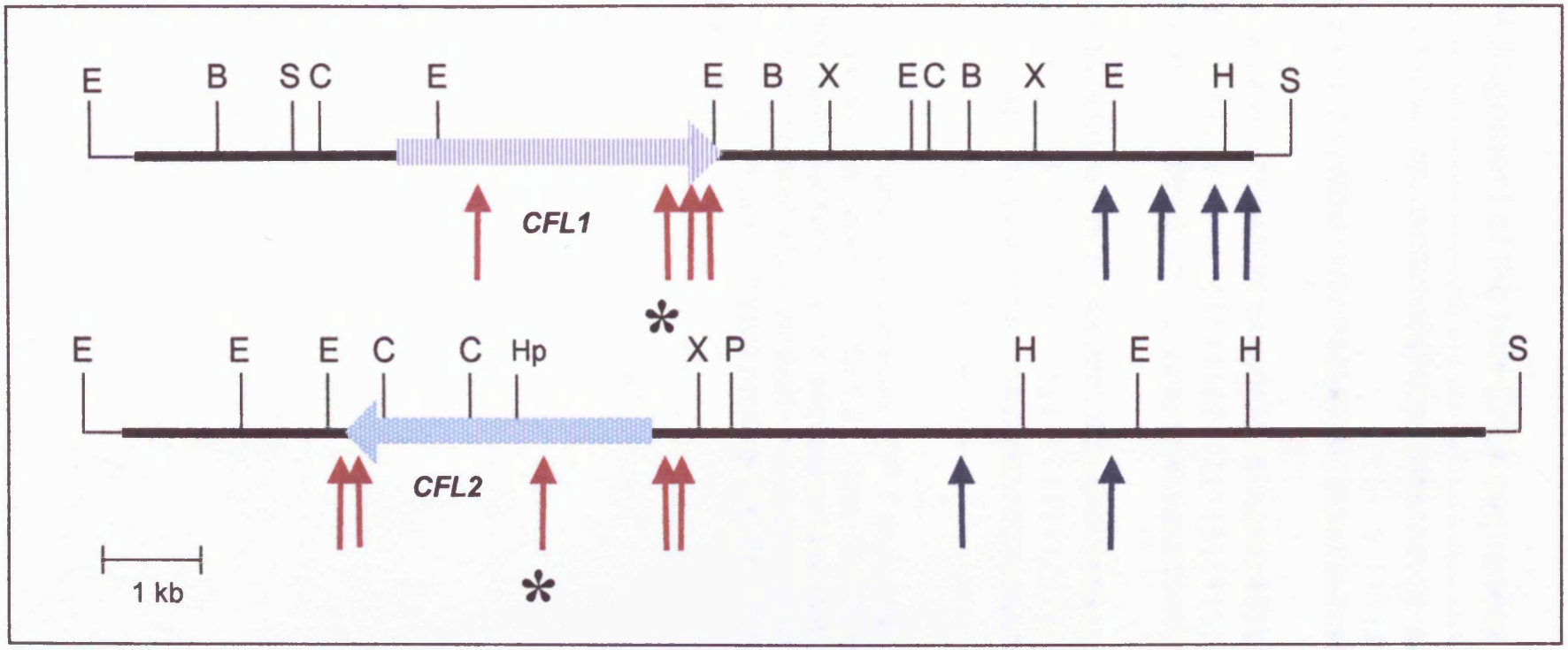
derived from pJDF1.3 revealed that all the transposons had inserted into the 1.8 kb *Eco*RI fragment (Fig 4.3) indicating that the gene of interest lay in this region. The pJDF1.3 derived transposon mutagenised plasmids that still maintained rescuing activity had transposons inserted in different regions. Similarly, restriction analysis of the clones derived from pJDF2.3 that no longer rescued, showed that these transposon insertions were clustered in a 2.6 kb *Eco*RI- *Xba*I fragment (Fig 4.3) whilst those that still rescued had insertions elsewhere, including two which were found to have inserts in the original vector sequence.

One of the non-rescuing plasmids from each clone type (designated pJDF1.3a and pJDF2.3a) was chosen for sequence analysis using primers to sequence outwards from the transposon (Fig 4.3). Searches of the *C. albicans* information pages (http://alces.med.umn.edu/Candida.html) revealed that the rescuing gene on pJDF1.3 had previously been sequenced and named *CFL1* (for *Candida* ferric reductase-like gene) (Yamada Okabe *et al.*, 1996). However, these authors were unable to demonstrate rescue by *CFL1* either of the ferric reductase deficiency of a *S. cerevisiae fre1* mutant or of the characteristic poor growth of a *S. cerevisiae fre1* mutant in low iron conditions. The sequences obtained from pJDF2.3a did not show similarity with any sequences in the *C. albicans* database.

Sequence analysis of pJDF1.3

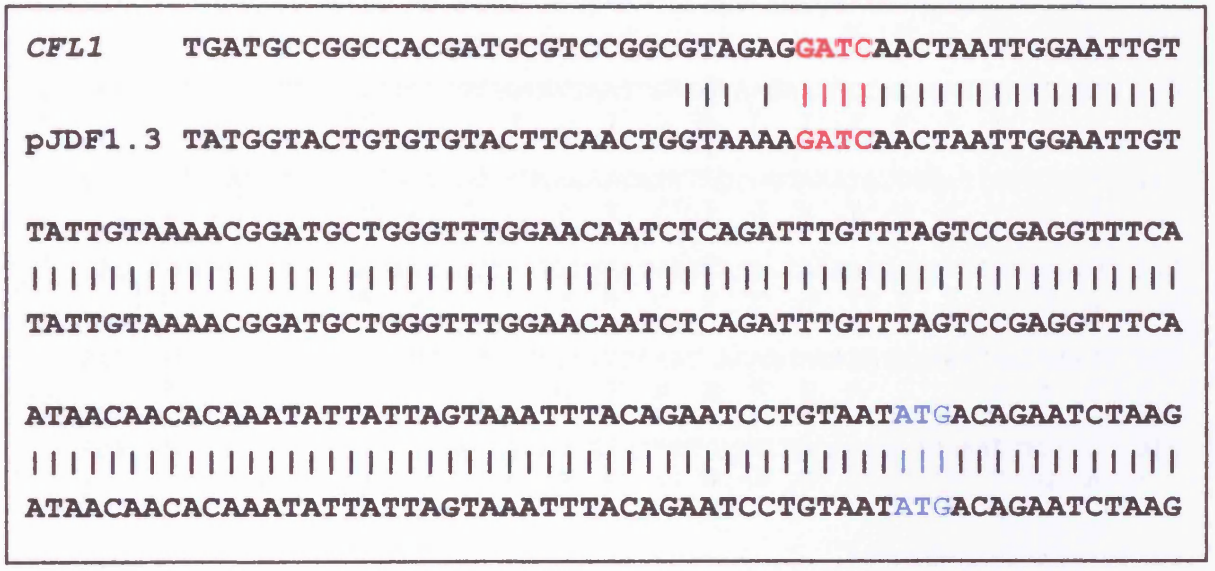
More extensive sequencing of pJDF1.3, using different primers to walk along the insert, showed 99% identity with the sequence within the *CFL1* ORF predicted by Yamada Okabe and co-workers (1996). This level of sequence identity also continued 269 base pairs downstream of the *CFL1* ORF, extending into the adjacent gene *CGT1* (encoding an mRNA capping protein, identified by Yamada Okabe *et al.*, 1996). Homology broke down abruptly, however, 122 base pairs upstream of the predicted ATG start site (Fig 4.4). The discrepancy arises at a *Sau*3A1 restriction site, suggesting that the sequence published by Yamada Okabe *et al.* (1996) may, in fact, have been derived from the incorrect juxtaposition of two non-contiguous genomic DNA fragments, thus explaining the observed inability of their plasmid to rescue a ferric reductase mutant. Our sequencing data predicted that the *CFL1* open-reading frame extends 276 base pairs upstream of the published ATG (Fig 4.5), giving a predicted protein product of 761 amino acids, with a putative cleavable N-terminal signal sequence of 18 amino acids, consistent with Cfl1p being a cell surface integral membrane

Figure 4.3 Restriction maps of pJDF1.3 and pJDF2.3 showing probable positions of the rescuing genes and the sites of transposon insertions



The red arrows indicate transposon insertions which abolished rescuing activity, whilst the blue arrows indicate transposon insertions where rescuing activity was retained. Two of the pJDF2.3 'rescuing' transposon insertions examined had inserted in vector sequences and are not shown on this diagram. The asterisks indicate the transposon insertions used for sequencing. The thick black line shows the *C. albicans* genomic insert and the thin black line represents flanking vector sequence. The restriction enzymes used were: B = *Bgl*II; *C* = *Cla*I; E = *Eco*RI; H = *Hin*dIII; Hp = *Hpa*I P = *Pst*I; S = *Sal*I; X = *Xba*I.

Figure 4.4 Alignment of the two *CFL1* sequences



FASTA alignment of the originally published *CFL1* sequence against the *CFL1*-like sequence found on the plasmid pJDF1.3. The *Sau*3AI site where sequence similarity breaks down between the two sequences is shown in red. The ATG start site predicted from the originally published sequence (Yamada Okabe *et al*., 1996) is shown in blue. Our sequencing data predicts that the *CFL1* ORF extends 276 bp upstream of this ATG.

Figure 4.5 The 5' end of *CFL1* and upstream flanking sequence

```
   1 ACCGTGGGTCGCTTCCAACATATAATCAAACCCCTCGCAAACAATGAAGATTCAACAATT  60
                                                 M  K  I  Q  Q  L  -

  61 GATAGTATTTTTGTTTGCGGTAGTATTAATCGATGCAAGAACACCGAAAAGATATTCCGA 120
      I  V  F  L  F  A  V  V  L  I  D  A  R  T  P  K  R  Y  S  E  -

 121 ATTGGATATCGTGATGCTGACTTGTACAACATTTATTGGGAAATATGGTACTGTGTGTAC 180
      L  D  I  V  M  L  T  C  T  T  F  I  G  K  Y  G  T  V  C  T  -

 181 TTCAACTGGTAAAAGATCAACTAATTGGAATTGTTATTGTAAAACGGATGCTGGGTTTGG 240
      S  T  G  K  R  S  T  N  W  N  C  Y  C  K  T  D  A  G  F  G  -

 241 AACAATCTCAGATTGTTTAGTCCGAGGTTTCAATAACAACACAAATATTATTAGTAAATT 300
      T  I  S  D  C  L  V  R  G  F  N  N  N  T  N  I  I  S  K  F  -

 301 TACAGAATCCTGTAATATGACAGAATCTAAGTTTCATGCTAAATATGATAAAATTCAGGC 360
      T  E  S  C  N  M  T  E  S  K  F  H  A  K  Y  D  K  I  Q  A  -
```

The 5' end of the *CFL1* gene and upstream flanking sequence described in this work is shown. The *Sau*3AI site where the sequence divergence between the sequence identified in this work and the previously published sequence (Yamada Okabe *et al.*, 1996) is shown in blue. The extended amino acid sequence is shown and the new ATG start codon is shown in red. The putative hydrophobic signal sequence is shown in green and the putative TATA box is shown in bold. The ATG start codon predicted from the original sequence is shown in purple.

protein (PSORT; http://psort.nibb.ac.jp:8800/; Von Heijne, 1983). The *CFL1* sequence found in this study has been deposited in the EMBL database (accession number: AJ387722)

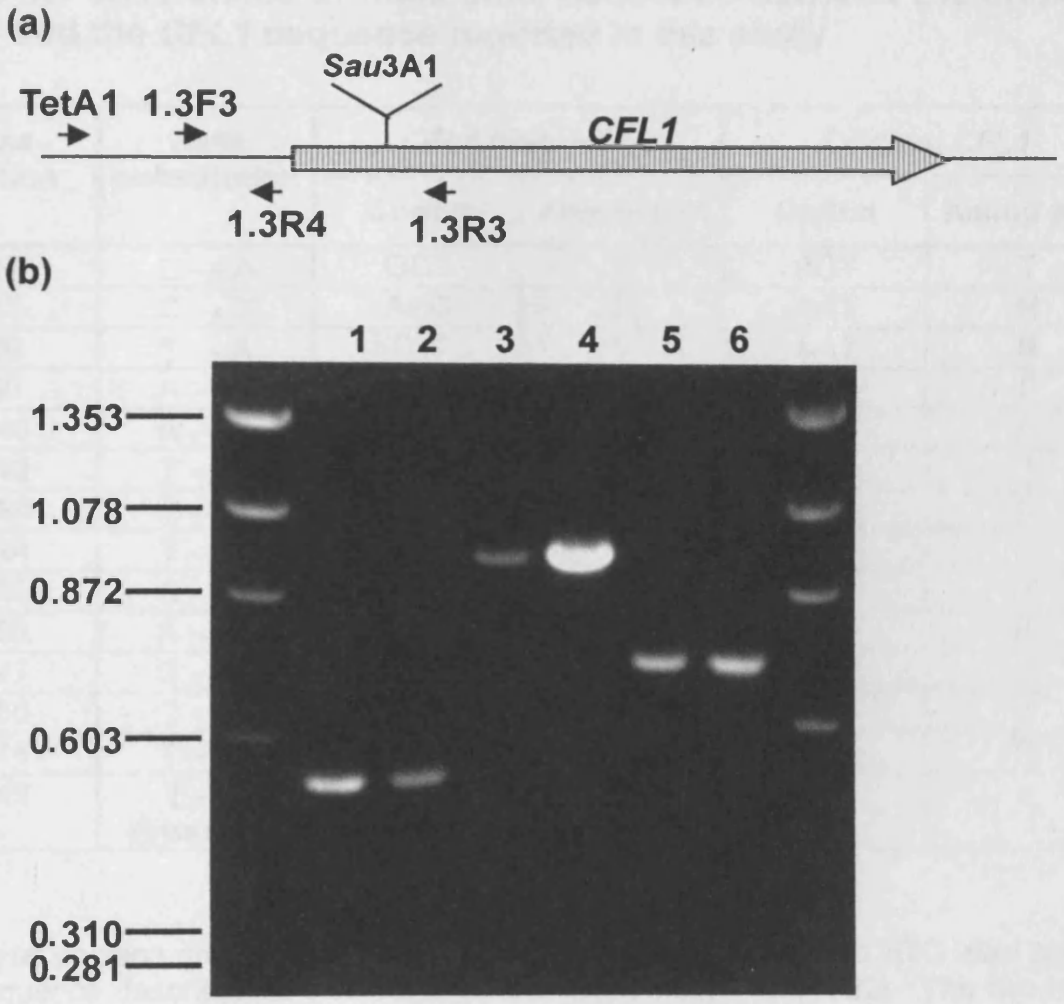
Comparative PCR analysis of the pJDF1.3 plasmid and genomic DNA from strain S/01 was performed using primers designed to amplify the 5' terminus of *CFL1* and the untranslated region upstream of *CFL1* (Fig 4.6). PCR products of identical sizes were obtained from both templates with each combination of primers used, including some that spanned the *Sau*3A1 site at which homology with the previously published sequence broke down. These data confirm that the DNA cloned in plasmid pJDF1.3 correctly represents contiguous sequence in the *C. albicans* strain S/01. Moreover, a sequence deposited in the *C. albicans* information pages (http://alces.med.umn.edu/Candida.html) during the course of the sequencing project (384170E06.s1.seq; *CFL99*) corresponds to the region spanning the *Sau*3A1 site and is identical to our *CFL1* sequence and not that of the previously reported sequence (Yamada Okabe *et al.*, 1996).

Analysis of the previously reported *CFL1* sequence and the sequence described here showed that in the regions of overlap there is 99 % identity at the nucleotide level. More exact analysis found that there were 14 base changes over approximately 2 kb, but only two of these resulted in amino acid changes (Table 4.1). Two of these base changes (at positions 1847 and 1850) resulted in the loss of a *Cla*I restriction site in our sequence which was present in the originally reported sequence. Using Southern blot analysis this RFLP was found to be heterozygous in strain CAI4, a *ura3Δ* strain used for constructing *C. albicans* mutant strains which is derived from the clinical isolate SC5314 (Fonzi & Irwin, 1993), but the site was not present in either chromosomal copy of *CFL1* in S/01. Southern blot analysis also showed that band sizes predicted for other restriction enzymes were consistent with the restriction map in both strains (Fig 4.7; Table 4.2). It therefore seems likely that the sequence identified in this study is present in CAI4 as well as S/01 and that the originally published sequence is indeed incorrect.

Sequence analysis of pJDF2.3

Further sequencing of the second clone, pJDF2.3, using different primers to walk along the insert, showed that this too encoded a ferric reductase-like gene. The complete sequence of this gene had not been previously noted and was named *CFL2*. However, a short region of

Figure 4.6 PCR analysis of *CFL1*



(a) *CFL1* and flanking genomic DNA are shown. The *CFL1* gene is shown as a purple arrow. The primers used are shown as horizontal arrows in their relative positions to the *CFL1* gene. The *Sau*3AI site where sequence homology broke down with the previously published sequence is shown

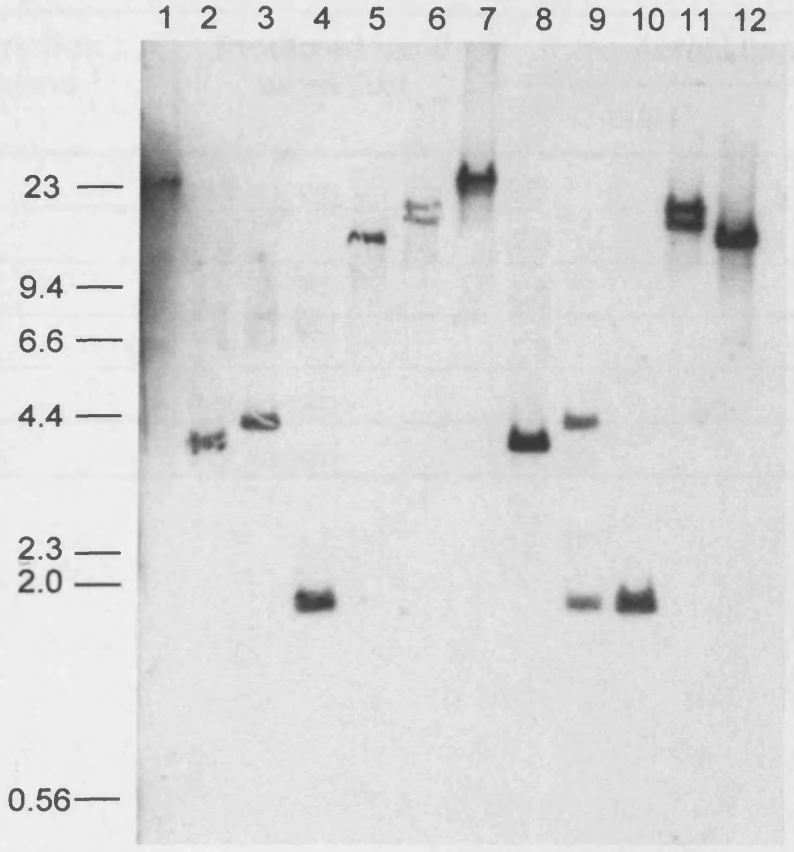
(b) PCR products following amplification using primers shown in **(a)**, were run on a 1 % agarose gel and stained using ethidium bromide. Lanes 1,3 and 5, *C. albicans* genomic DNA; lanes 2,4 and 6, pJDF1.3 DNA. Lanes 1 & 2, primers TetA1 + 1.3R4; lanes 3 + 4, primers TetA1 + 1.3R3; lanes 5 + 6, primers 1.3F3 + 1.3R3.

Table 4.1 Differences in nucleotide sequence between the original *CFL1* and the *CFL1* sequence reported in this study

Base position	Base substitution	*CFL1* (this work)		Original *CFL1*	
		Codon	Amino acid	Codon	Amino acid
387	G → A	**G**CT	A	**A**CT	T
515	C → T	AA**C**	N	AA**T**	N
726	T → A	**T**AT	Y	**A**AT	N
740	A → G	TC**A**	S	TC**G**	S
1448	A → C	GC**A**	A	GC**C**	A
1493	T → C	A**T**T	I	A**T**C	I
1784	G → T	GG**G**	G	GG**T**	G
1824	T → C	**T**TA	L	**C**TA	L
1829	C → T	GC**C**	A	GC**T**	A
1832	A → G	**A**AA	K	**A**AG	K
1847	G → A	CA**G**	Q	CA**A**	Q
1850	C → G	TC**C**	S	TC**G**	S
2114	T → C	GA**T**	D	GA**C**	D
2349	T → - (insertion)	-	-	-	-

The base position given is the number of bases from the predicted ATG start site in the sequence described in this work (accession number: AJ387722). The two base changes that result in the introduction of a *Cla*I site into the original *CFL1* sequence (accession number D83181) are indicated in purple. Bold type indicates the base changes between the two sequences and the resultant amino acid changes.

Figure 4.7 Southern blot of *C. albicans* genomic DNA using *CFL1* as a probe



Genomic DNA prepared from the *C. albicans* strains S/01 and CAI4 was digested with *Bam*HI, *Bgl*II, *Cla*I, *Eco*RI, *Hin*dIII and *Pst*I and electrophoresed in a 0.8 % agarose gel. The gel was then blotted onto Hybond-N filter paper and probed with a 1.8 kb *Eco*RI fragment from the *CFL1* encoding region of pJDF1.3. After overnight hybridisation the filter was washed using the standard stringency washes described in Chapter 2 and exposed to X-ray film which was developed after an overnight exposure at -80 °C. The predicted and actual band sizes for each of these enzymes is shown in Table 4.2. Lanes 1-6, S/01 genomic DNA; lanes 7-12, CAI4 genomic DNA. Lanes 1 & 7, *Bam*HI; lanes 2 & 8, *Bgl*II; lanes 3 & 9, *Cla*I; lanes 4 & 10, *Eco*RI; lanes 5 & 11, *Hin*dIII; lanes 6 & 12, *Pst*I

Table 4.2 Predicted and actual band sizes produced by Southern blot analysis of *C. albicans* strains S/01 and CAI4 using *CFL1* as a probe

Band sizes were predicted from the restriction mapping of pJDF1.3

Restriction enzyme	Predicted band sizes (kb)	Actual band sizes (kb)	
		S/01	CAI4
*Bam*HI	not known	28	29
*Bgl*I	3.8	4.0	4.2
*Cla*I	4.3	4.6	4.6, 1.9
*Eco*RI	1.8	1.8	1.9
*Hin*dIII	not known	16	16
*Pst*I	not known	21, 19	23, 18

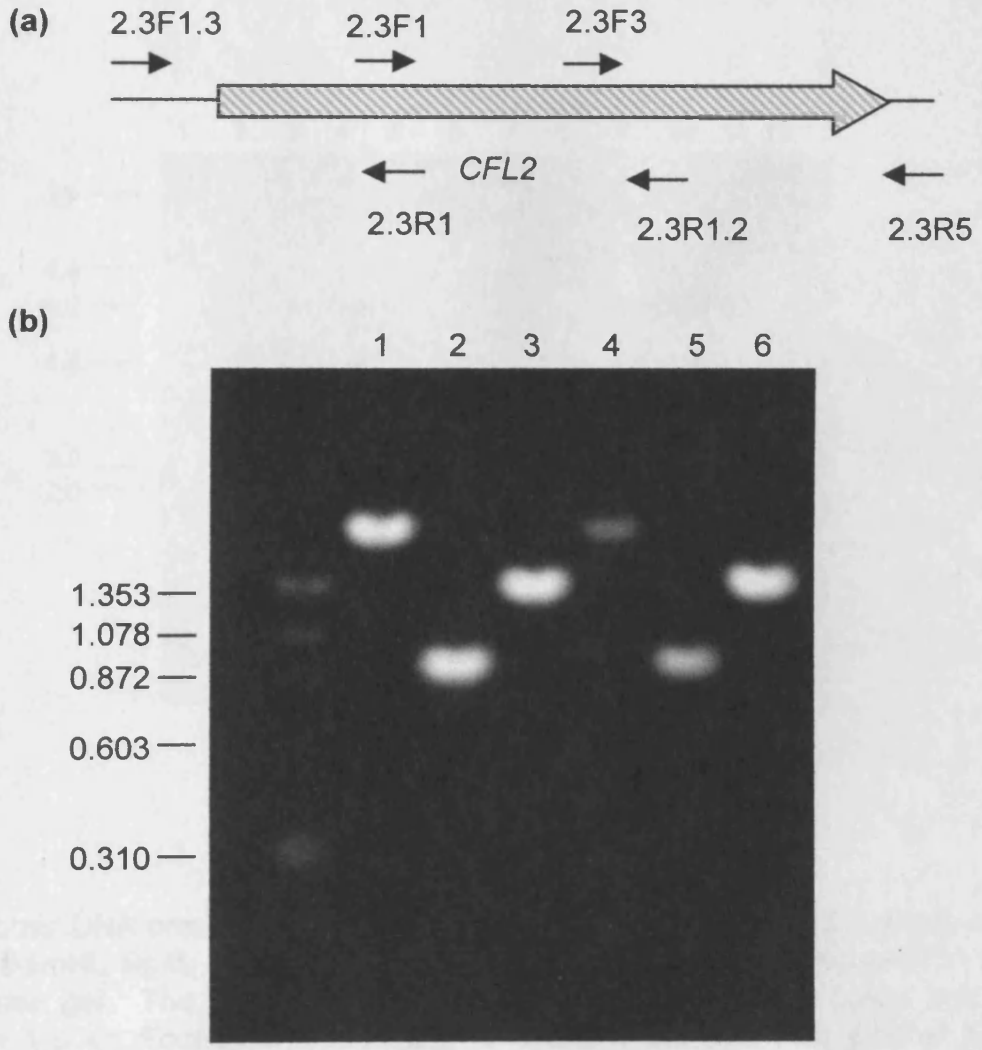
identical sequence was present on the *C. albicans* information pages corresponding to the extreme 5' end of the gene (265156D10.y1.seq; *CFL99*). This sequence fitted into a contig of 1672 nucleotides when a FASTA search was run against a database of incomplete microbial genomes provided by GenBank (http://wwwncbi.nlm.nih.gov/BLAST/unfinishedgenome.html). The *CFL2* sequence identified in this work showed 98.9% identity to this fragment of sequence over 1672 nucleotides. Comparison of *CFL2* to *CFL1* using the FASTA program (Pearson & Lipman, 1988) of the GCG package (Wisconsin Package Version 9.1, Genetics Computer Group, Madison, Wisconsin) showed that the two genes are 76.7% identical over 1022 nucleotides, which is a higher level of identity than is found between *FRE1* and *FRE2* of *S. cerevisiae* (53.1 % over 273 nt), but similar to the level of identity between *FRE2* and *FRE3* (76.9 % over 2134 nt).

Comparative PCR was used to check that the insert found in pJDF2.3 represented a contiguous piece of DNA in the *C. albicans* genome. Primers that spanned the whole of the gene including the 5' upstream region were used to PCR the gene in three fragments of approximately 1 kb. Identical fragments were obtained for pJDF2.3 and the *C. albicans* strain, S/01, thus indicating that pJDF2.3 does indeed represent contiguous genomic DNA (Fig 4.8). Southern blot analysis using genomic DNA from both S/01 and CAI4 showed that the banding patterns obtained with most enzymes were the same for the two strains. An extra band was present in the *Bgl*II digest of S/01 and an extra band was present in the *Cla*I digest of CAI4, possibly suggesting the presence of heterozygous RFLPs for these two enzymes (Fig 4.9).

4.4 Analysis of predicted protein products of *CFL1* and *CFL2*

Comparison of the predicted Cfl1p and Cfl2p amino acid sequences with the *S. cerevisiae* Genome Database (http://genome-www.stanford.edu/Saccharomyces/) using the FASTA program (Pearson & Lipman, 1988) revealed similarities with two *S. cerevisiae* proteins, Fre1p and Fre2p, both of which are structural components of the cell surface ferric reductase complex. Direct comparison using the GAP program from the GCG package (Wisconsin Package Version 9.1, Genetics Computer Group, Madison, Wisconsin) showed Cfl1p to have 37.7% similarity and 27.5% identity with Fre2p and 37.4% similarity and 26.8% identity with

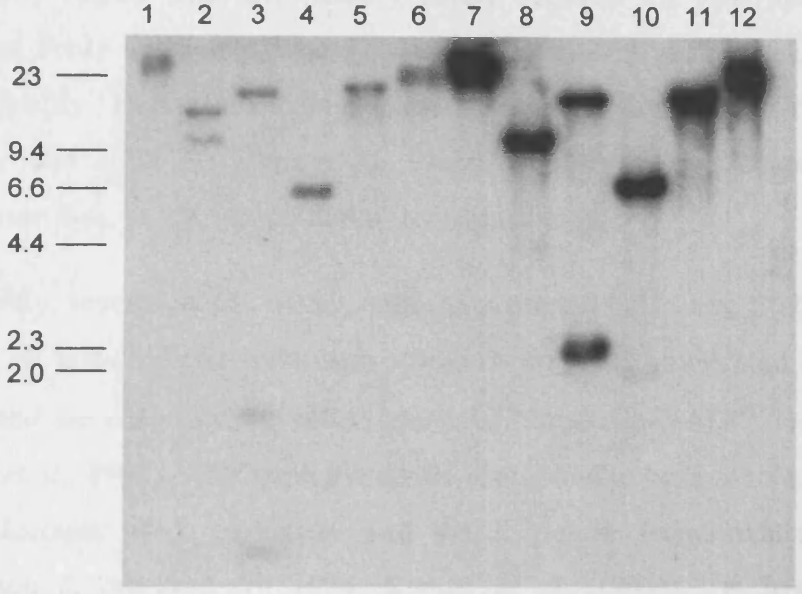
Figure 4.8 PCR analysis of *CFL2*



(a) *CFL2* and flanking genomic regions are shown. The *CFL2* gene is shown as a thick arrow. The primers used are shown as horizontal arrows in their relative positions to the *CFL2* gene.

(b) PCR products, following PCR amplification using primers shown in **(a)**, were run on a 1 % agarose gel and stained using ethidium bromide. Lanes 1,2 and 3, pJDF2.3 DNA; lanes 4,5 and 6, *C. albicans* genomic DNA. Lanes 1 & 4, primers 2.3F1.3 + 2.3R1; lanes 2 & 5, primers 2.3F1 + 2.3R1.2; lanes 3 & 6, primers 2.3F3 + 2.3R5.

Figure 4.9 Southern blot of *C. albicans* genomic DNA using *CFL2* probe



Genomic DNA prepared from the *C. albicans* strains S/01 and CAI4 was digested with *Bam*HI, *Bgl*II, *Cla*I, *Eco*RI, *Hin*dIII and *Pst*I and electrophoresed in a 0.8 % agarose gel. The gel was then blotted onto Hybond-N filter paper and probed witha 1.6 kb *Eco*RI/*Hpa*I fragment from the *CFL2* encoding part of pJDF2.3. After over-night hybridisation the filter was washed using the standard stringency washes described in Chapter 2 and exposed to X-ray film which was developed after over-night exposure at -80 °C. Lanes 1-6 contain S/01 genomic DNA, lanes 7-12 contain CAI4 genomic DNA. Lanes 1 & 7 digested with *Bam*HI; lanes 2& 8, *Bgl*II; lanes 3 & 9, *Cla*I; lanes 4 & 10, *Eco*RI; lanes 5 & 11, *Hin*dIII; lanes 6 & 12, *Pst*I.

Fre1p. Cfl2p shows 42.6 % similarity and 31.1 % identity to Fre1p 40.8 % similarity and 30.6 % identity and to Fre2p.

Cfl1p and Cfl2p are found to have a high level of identity at the amino acid level as well as at the nucleotide level (as discussed above). Alignment using the GAP program of the GCG package shows that Cfl1p is 83.2 % similar and 78.1 % identical to Cfl2p. This is significantly higher than the identity found between the two main *S. cerevisiae* ferric reductases, Fre1p and Fre2p, where a similar alignment gives values of 37.6 % similarity and 27.1 % identity. However, this high level of identity is not unique since Fre2p shows 81.7 % similarity and 75.9 % identity to Fre3p, another ferric reductase-like protein from *S. cerevisiae* that, as yet, has no function assigned to it.

Significantly, several motifs within both the predicted Cfl1p and Cfl2p protein sequences are found in all known ferric reductases; three of these are implicated in FAD and NAD(P)H binding and are found in the wider family of ferredoxin-$NADP^+$ reductase (FNR) proteins (Karplus *et al.*, 1991). The same motifs are also found to be conserved in the two cell surface ferric reductases of *S. cerevisiae* and the *S. pombe* ferric reductase, Frp1p (Fig 4.10; (Georgatsou & Alexandraki, 1994; Roman *et al.*, 1993)). A hydropathy plot (Kyte & Doolittle, 1982) of both proteins shows that they both have multiple hydrophobic regions (Fig 4.11a) consistent with their being multi-spanning membrane proteins like both Fre1p and Fre2p (Georgatsou & Alexandraki, 1994). The number of predicted transmembrane regions in each of these proteins depends on the program used to carry out the prediction. For example, PSORTII (http://psort. nibb.ac.jp:8800/) predicts 6 transmembrane regions for Cfl1p whilst TopPred 2 (http://www.biokemi.su.se/~server/toppred2/) predicts between 9 and 5, and JPRED (http:// jpred.ebi.ac.uk/) predicts 7. Similar figures are found for Cfl2p. Thus, the prediction of transmembrane regions is by no means certain since it depends on identifying stretches of mostly hydrophobic residues which are long enough to span a membrane. Predictions can vary in the proportion of hydrophobic residues required to call a region 'hydrophobic' and also the length of the hydrophobic region required to span the membrane. Therefore all that can be said with certainty is that both Cfl1p and Cfl2p are likely to contain multiple transmembrane domains. Both are likely to contain an odd number of transmembrane regions (excluding the initial hydrophobic signal sequence which is normally cleaved off from the mature protein) since this would place the C-terminal end of the protein containing the putative FAD and NAD(P)H binding sites inside the cell. Finegold and co-

Figure 4.10 Alignment of Cfl1p and CFl2p against known ferric reductase proteins showing consrved motifs

Motif 1

```
Cfl1p  345  G G R N N F L  351
Cfl2p  387  G G R N N F L  393
Fre2p  295  A G R N N F L  300
Fre1p  273  G I R N N P F  277
Frp1p  136  S I K N N P F  142
```

Motif 2: FAD binding

```
Cfl1p  526  Q S H P F T  530
Cfl2p  568  Q S H P F T  573
Fre2p  477  Q S H P F T  482
Fre1p  460  Q S H P F T  465
Frp1p  315  Q I H P F T  320
```

Motif 3

```
Cfl1p  574  E G P Y G  578
Cfl2p  616  E G P Y G  620
Fre2p  525  E G P Y G  529
Fre1p  513  E G P Y G  517
Frp1p  401  D G P Y G  405
```

Motif 4: NAD(P)H binding

```
Cfl1p  588  K N V V F V A G G N G I P G I Y S E C V D L A K K  612
Cfl2p  630  K N V V F I A G G N G I P G I Y S E C V D L A K K  654
Fre2p  538  N N V L L L T G G T G L P G P I A H A I K L G K T  562
Fre1p  528  R N L V G V A A G L G V A A I Y P H F V E C L R L  552
Frp1p  415  S Y L F L F A G G V G V S Y I L P I I L D T I K K  439
```

Motif 5: NAD(P)H binding

```
Cfl1p  726  C G H P  729
Cfl2p  768  C G H P  771
Fre2p  677  C G P P  780
Fre1p  652  C G P A  655
Frp1p  534  C G S D  537
```

The predicted amino acid sequences of *CFL1*, *CFL2*, *FRE2*, *FRE1* and *Frp1* were aligned using PILEUP from the GCG package and examined for motifs shown to be conserved throughout the FNR family of proteins. Three conserved motifs implicated in FAD and NAD(P)H binding are shown. Two other motifs of unknown function are also shown.

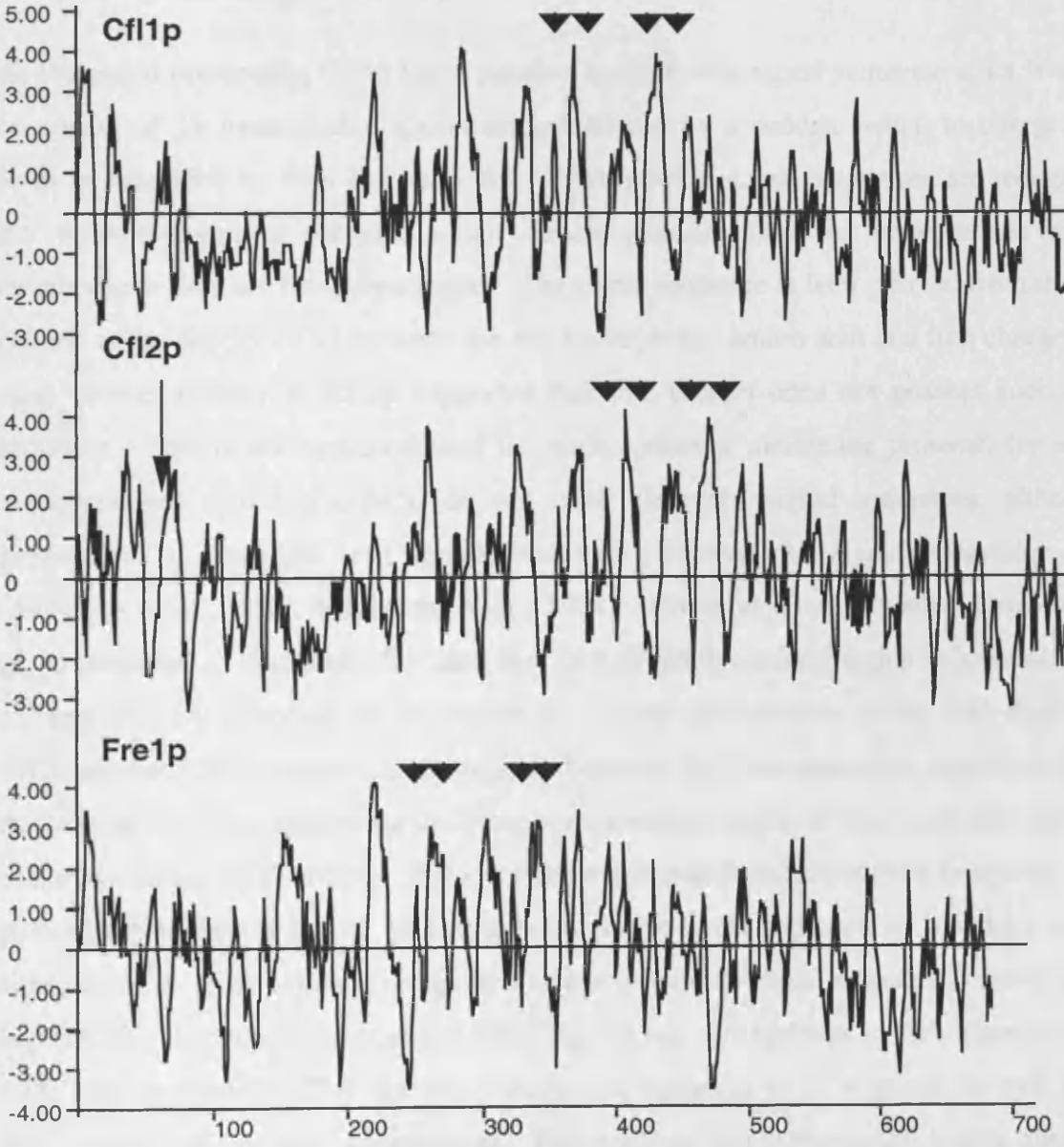
Figure 4.11 Hydrophobic nature of Cfl1p, Cfl2p and Fre1p and conservation of haem binding histidine residues in Cfl1p, Cfl2p, Fre2p, Fre1p and Frp1p

(a) Hydropathy plots of Cfl1p and Fre1p performed using the Kyte-Doolittle algorithm (1982). The four conserved histidine residues are shown as black triangles above the plots. The large arrow over Cfl2p shows the third methionine in the sequence which may be the actual translational start site.

(b) The predicted amino acid sequences of Cfl1p, Cfl2p, Fre2p, Fre1p and Frp1p were examined for conservation of the four histidine residues thought to play a role in haem binding. The four residues were conserved in all five proteins.

Figure 4.11 Hydrophobic nature of Cfl1p, Cfl2p and Fre1p and conserved haem binding histidine residues in Cfl1p, Cfl2p, Fre2p, Fre1p and Frp1p

(a)



(b)

Cfl1p	366	HRWISRVDVLLIIVH	380
Cfl2p	408	HRWISRIDVLLIIVH	422
Fre2p	316	HKWLGRMMFLDAMIH	330
Fre1p	294	HKWSAYVCFMLAVVH	308
Frp1p	157	HRRLSQYAIMIGAIH	171
Cfl1p	436	HIVLVVFFVVGGYYH	450
Cfl2p	478	HIVLVVFFVVGGFHH	492
Fre2p	386	HIVLGAMFFYACWEH	400
Fre1p	364	HKAMNIMFIIAMYYH	378
Frp1p	225	HHMCSIGFLITIWLH	239

workers (1996) demonstrated that there are four critical histidine residues in the transmembrane domains of the Fre1 protein which are important for binding of two haem co-factors. Four histidine residues are also found at comparable locations with the same spacing in both Cfl1p and Cfl2p (Fig 4.11b).

As discussed previously, Cfl1p has a putative hydrophobic signal sequence at its N-terminus consisting of 18 hydrophobic amino acids followed by a sudden switch to charged amino acids as described by Von Heijne (1983). Hydrophobic signal sequences are recognised by the signal-recognition particle, which directs peptides into the endoplasmic reticulum membrane as they are being synthesised. The signal sequence is later cleaved from the mature peptide at the peptide bond between the last hydrophobic amino acid and first charged amino acid. Initial analysis of Cfl2p suggested that this protein does not possess such a signal sequence. This is not unprecedented for multi-spanning membrane proteins, for example, rhodopsin and cytochrome-p450 do not have cleavable signal sequences, although the mechanism of insertion into membranes still involves the signal-recognition particle (Anderson *et al.*, 1983; Sakaguchi *et al.*, 1984). However, since all other ferric reductase genes identified to date and other members of this family possess signal sequences, it seems unlikely that Cfl2p should be an exception. Closer examination of the GAP alignment of Cfl2p against Cfl1p reveals that similarity between the two sequences begins at the third methionine of Cfl2p, suggesting that possibly translation begins at this point and not at either of the two earlier ATG codons. If it is assumed that translation starts from this point, then the protein begins with a run of 18 hydrophobic amino acids followed by a sudden switch to charged amino acids which is recognised as a hydrophobic signal sequence in programs such as PSORTII (http://psort. nibb.ac.jp:8800/; Fig 4.11a). In addition to this observation, this ATG has a putative TATA box and CAAT box upstream of it, whereas the two previous ATG codons have no such signal boxes. This suggests that transcription begins downstream of both of these ATG sequences, again suggesting that the third ATG is the one used *in vivo* (Fig 4.12).

Figure 4.12 5' sequence of *CFL2* showing possible ATG start sites and CAAT and TATA elements

The three possible ATG start sites and their corresponding methionine translations are shown in blue, purple and red respectively. The third one is considered the most likely to be used *in vivo*, since this is followed by a stretch of hydrophobic amino acids likely to constitute a signal sequence (coloured green). The TATA and CAAT elements likely to be associated with the transcription of this gene are shown on a blue background and an alternative possible TATA box is indicated on a green background.

4.5 Discussion

This chapter describes the isolation of two ferric reductase-like genes from *C. albicans* by functional complementation of a *S. cerevisiae* ferric reductase mutant. One of the genes, *CFL1*, had previously been noted but no ferric reductase had been shown to be associated with it (Yamada Okabe *et al.*, 1996). In contrast to this previously published report, it has been shown here that *CFL1* can rescue both the ferric reductase deficiency and slow growth on low iron phenotypes of a *S. cerevisiae fre1* mutant. The contradiction between the findings reported here and those of Yamada Okabe *et al.* (1996) is due to a divergence of sequence at the 5' end of the gene which is believed to have arisen by the joining of two non-contiguous fragments of genomic DNA in the previously published sequence. Our results indicate that the ORF is 276 base pairs longer than previously thought and the predicted amino acid sequence contains a putative hydrophobic signal sequence at the N-terminus. The second gene identified, *CFL2*, had not been previously identified but shows a high degree of similarity to *CFL1*. It is unusual in that it appears not to contain a N-terminal signal sequence, but this could be due to translation initiating at the third possible methionine residue in the putative peptide sequence. Analysis of possible TATA and CAAT boxes in the sequence also supports this hypothesis.

The predicted amino acid sequences of both Cfl1p and Cfl2p show homology with ferric reductase genes from *S. cerevisiae*. In particular a high level of conservation is found in several specific domains such as those implicated in FAD and NAD(P)H binding, which are also found to be conserved between the wider family of FNR proteins, as well as other domains with no known function. The conservation of the positioning of four histidine residues, essential for haem binding in Fre1p is also interesting and indicates that Cfl1p and Cfl2p may be haem-dependent.

It was not surprising to find 2 different clones capable of rescuing the *S. cerevisiae fre1* mutant. *S. cerevisiae* itself has at least two characterised genes encoding structural components of the reductase and 5 other similar genes whose function has not yet been elucidated; therefore it would not be unexpected that C. *albicans* too should have several genes encoding ferric reductase activities. Analysis of the *C. albicans* information pages (http://alces.med.umn.edu/Candida.html) shows that there are several other candidate ferric reductase genes currently present in the database which have been identified during the course

of the genome sequencing project. These sequences were not, however, present when this work was initiated.

CFL1 and *CFL2* also rescue the slow growth on iron demonstrated by JHS1, the *S. cerevisiae fre1* mutant. Slow growth on low iron can be seen as a reflection of intracellular iron concentrations of a cell. A cell that is unable to acquire iron by a high affinity mechanism is unable to grow in low iron conditions, explaining the observed phenotype of JHS1. The fact that *CFL1* and *CFL2* can rescue this phenotype suggests that these genes must be restoring the high affinity iron uptake mechanism of the cell, suggesting that they may be playing a direct role in iron accumulation by the cell. This would imply that Cfl1p and Cfl2p are being directed to the cell surface in *S. cerevisiae* and are not rescuing ferric reductase activity by an alternative mechanism.

Since the *S. cerevisiae* ferric reductase genes, *FRE1* and *FRE2*, are transcriptionally regulated in response to iron and copper (*FRE1*) or iron (*FRE2*) it might be expected that the *C. albicans* ferric reductase genes might be similarly regulated. If such a prediction could be confirmed this would suggest that the proteins encoded by *CFL1* and *CFL2* play a role in iron and/or copper uptake in *C. albicans*. Experiments to determine whether *CFL1* and *CFL2* are regulated by iron and copper are described in the next chapter.

Chapter 5

Expression of the *Candida albicans* ferric reductase genes, *CFL1* and *CFL2*, in *Saccharomyces cerevisiae* and *Candida albicans*

5.1 Introduction

The previous chapter described the isolation of two *C. albicans* ferric reductase-like genes by functional complementation of a *S. cerevisiae* ferric reductase mutant. Sequence analysis of these genes, *CFL1* and *CFL2*, showed that they resembled other known ferric reductases, suggesting that they may play a role in iron acquisition in *C. albicans*. However, it was not possible to ascertain the roles of these genes in iron acquisition without further study, and, indeed, it was not even known if these genes were expressed in *C. albicans*. Therefore, the aim of the work described in this chapter was to study the expression of *CFL1* and *CFL2*.

Expression of the cell surface ferric reductase activities of both *S. cerevisiae* and *C. albicans* are negatively regulated by iron and copper and so it was postulated that the reductase activities encoded by *CFL1* and *CFL2* might, likewise, be regulated by these metals. It was assumed that both *CFL1* and *CFL2* were expressed in *S. cerevisiae* since they were both capable of rescuing a ferric reductase mutant. However, it was not known whether these genes were regulated by iron in *S. cerevisiae* and this question is addressed in this chapter. Furthermore, the question of whether these genes are expressed in *C. albicans* is also investigated by Northern blot analysis, in conjunction with investigations into whether expression is regulated by iron or copper.

In *S. cerevisiae*, which possesses 7 ferric reductase-like genes, the only ferric reductase gene that is regulated by both iron and copper is *FRE1*, which encodes a component of the cell surface ferric reductase activity (Martins *et al.*, 1998). *FRE2-6* are regulated by iron and *FRE7* is regulated by copper (Martins *et al.*, 1998). It is unlikely that all of these genes encode components of the cell surface ferric reductase activity as the deletion of two ferric reductase genes, *FRE1* and *FRE2*, results in the loss of 98 % of cell surface ferric reductase activity. It therefore seems likely that the other ferric reductase genes encode intracellular ferric reductases responsible for intracellular iron trafficking. The different regulation

patterns of the ferric reductase genes might be a reflection of their differing roles in iron and copper metabolism. *C. albicans* too, possesses multiple ferric reductase-like genes (see Chapter 7; http://alces.med.umn.edu/ Candida.html), and it is also unlikely that all of these genes contribute to the cell surface ferric reductase activity. The analysis of the expression of *CFL1* and *CFL2* in *S. cerevisiae* and *C. albicans* may provide insights in to the possible roles of these genes in iron metabolism in *C. albicans*.

5.2 Construction of *Saccharomyces cerevisiae fre1/fre2* mutant defective in ferric reductase activity

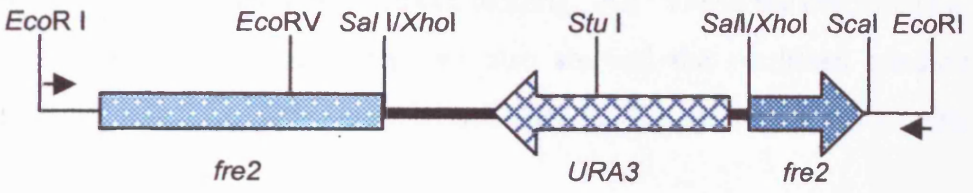
The mutant strain, JHS1, described in Chapter 3 carries a defective *fre1* gene, and was used for isolating the rescuing clones. However, quantitative analysis of the reductase activity of this mutant during culture growth showed that it retained some reductase activity during late log phase. Reports in the literature have shown that the ferric reductase activity encoded by another gene, *FRE2*, peaks during late log (Georgatsou & Alexandraki, 1994), thus suggesting that the reductase activity observed in JHS1 was due to Fre2p activity. In order to quantitatively analyse the reductase activity of the *C. albicans* rescuing clones a *fre1/fre2* double knock-out was constructed as it was thought that this would eliminate the remaining ferric reductase activity observed in JHS1.

Several attempts were made to construct a *fre1/fre2* double mutant from JHS1, the *fre1* mutant strain described in Chapter 3, whereby JHS1 was transformed with a *fre2* null allele containing *URA3* as a marker (Fig 5.1a). However, these attempts were unsuccessful and all transformants analysed had wild type copies of *FRE2*. One possible reason for this was that the null allele was being mis-directed to the *URA3* locus instead of the *FRE2* locus. The strain JHS1 carries the *ura3-52* allele of the *URA3* gene. This allele is non-functional due to a Ty1 insertion (Rose & Winston, 1984) but therefore still contains *URA3* sequences. An alternative 'designer deletion' strain, BY4733 (Brachmann *et al.*, 1998), which carries complete deletions of both the *URA3* gene and the *HIS3* gene was therefore used, excluding any possibility of homologous recombination at these sites.

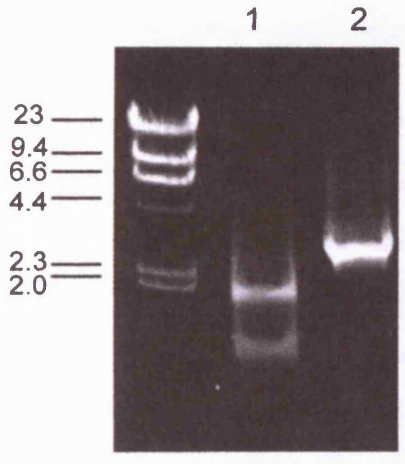
Strain BY4733 was transformed with the *fre2*Δ disruption cassette (transformation efficiency 1.8×10^5 colonies.μg DNA). Colonies were analysed using a PCR approach: PCR of the wild type *FRE2* allele gave a product of 2.4kb whilst that of the deletion allele gave a PCR product

Figure 5.1 *FRE2* disruption strategy

(a)



(b)



(a) The *FRE2* gene was amplified by PCR and cloned into the vector pUC13 on *Eco*RI ends. Disruption was achieved by the insertion of a *URA3* gene on *Sal*I ends into *Xho*I sites within the *FRE2* gene. This resulted in the removal of 846 bp from the middle of the *FRE2* gene. The thin black line represents genomic DNA flanking the *FRE2* gene, the *FRE2* gene is shown as a shaded box. The thick black line represents DNA flanking the *URA3* gene, and the *URA3* gene is shown as a lightly shaded box. The small arrows shown at the ends of the cassette represent the PCR primers used

(b) Genomic DNA was isolated from colonies transformed with the *fre2* disruption cassette and from the parental strain, BY4733, and the *FRE2* primers were used in PCR amplification. The resulting products were cut with the restriction enzyme *Stu*I, which cuts in the middle of the disruption cassette, but not in the wild-type allele. Lane1: *fre2Δ* mutant; lane 2: BY4733

of 2.6 kb, furthermore, the deletion allele contained a *Stu*I restriction site not present in the wild type allele. Restriction analysis of the PCR products with *Stu*I therefore made it easy to distinguish between mutant and wild type alleles since the mutant allele yielded two bands of 1.0 kb and another of 1.7 kb, whilst the wild type allele was not cut (Fig 5.1b). One of the colonies that showed the correct restriction pattern was picked, grown overnight and then transformed with the *fre1*Δ disruption cassette. Four colonies from the resulting transformation were analysed by Southern blotting. All showed the predicted banding pattern for the *fre2*Δ allele, however, only one also showed the predicted banding pattern for *fre1*Δ(Fig 5.2; Table 5.1). This strain, named JHS2.2, was used in further studies.

5.3 Quantitative analysis of ferric reductase activities associated with *CFL1* and *CFL2* in *Saccharomyces cerevisiae*

Strain JHS2.2, the *fre1/fre2* mutant, was transformed with the *C. albicans* genomic clones, pJDF1.3 and pJDF2.3, which carry the two ferric reductase genes, *CFL1* and *CFL2*, respectively. Quantitative reductase assays were carried out whilst cultures were grown in both high and low iron conditions in order to determine whether the reductase activities conferred by the two genes were regulated by iron. The parental strain, BY4733, transformed with YEp213 and JHS2.2 transformed with YEp213 were also used as controls. The growth patterns of the strains were monitored over a period of 24 hours in either MD containing 1 mM EDTA (low iron) or MD containing 1 mM EDTA and 2mM $FeCl_3$ (high iron) and reductase activity was assessed periodically (Fig 5.3).

It was found that the reductase activity of the parental strain, BY4733, was regulated in response to the iron levels in the growth medium, with reductase activity being reduced to a basal level in high iron conditions and being elevated approximately 5-fold in low iron media. As expected, the mutant strain, JHS2.2 showed basal levels of reductase activity in both high and low iron conditions. Growth in low iron conditions, which resulted in increased ferric reductase activity in the wild-type strain, BY4733, did not induce any increase in the ferric reductase activity of JHS2.2. The presence of either of the two rescuing clones, however, restored ferric reductase activity to JHS2.2. A similar increase in ferric reductase activity was observed for both high and low iron conditions. Some differences were observed between the

Figure 5.2 Southern blot analysis of *fre1/fre2* mutants.

Genomic DNA from BY4733 candidate *fre1/fre2* mutant strains was digested with either *Stu*I **(a)** or *Kpn*I **(b)** and electrophoresed in 0.8% agarose gels. The gels were then blotted onto Hybond-N filter paper and probed with a 1.4 kb *Eco*RV/*Sca*I fragment from the cloned *FRE2* gene **(a)** or the 1.8 kb *Eco*RI fragment from the cloned *FRE1* gene **(b)**. After overnight hybridisation the filters were washed using the standard stringency conditions described in Chapter 2 and exposed to X-ray film which was developed after one weeks exposure at –80 °C. Lane 1: BY4733; lane 2-5: candidate *fre1/fre2* mutants. The strain shown in lane 4 carries deletions in both *fre1* and *fre2*.

Table 5.1 Actual and predicted band sizes produced by Southern blot analysis of BY4733 and fre1/fre2 mutant.

Probe	Restriction enzyme	Predicted band size (bp)		Actual band size (bp)	
		BY4733	JHS2.2	BY4733	JHS2.2
FRE2	*Stu*I	8337	5471, 3184	8400	5500, 3200
FRE1	*Kpn*I	7152	4813, 3034	7800	5000, 3400

Figure 5.2 Southern blot analysis of *fre1/fre2* mutant

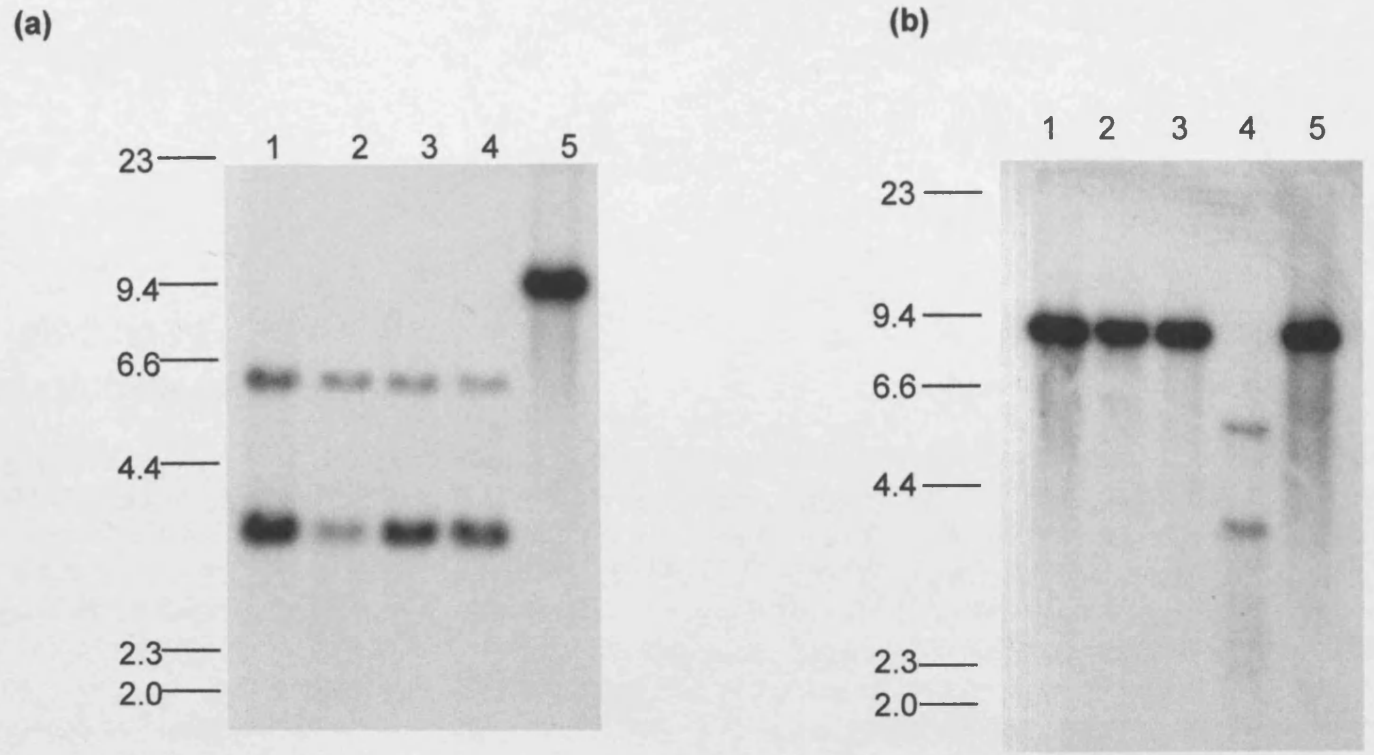


Figure 5.3 Quantitative assessment of reductase activities conferred by pJDF1.3 and pJDF2.3 on *S. cerevisiae* strain JHS2.2

Cells growing exponentially in MD medium (without EDTA) were transferred into fresh pre-warmed MD-EDTA media at a cell titre of 1×10^6 cells.ml^{-1}. The MD-EDTA media contained either no added iron (unfilled symbols) or 2mM $FeCl_3$ (filled symbols). The cultures were then incubated at 30 °C, with shaking, and at various time points samples were removed and cell density determined by counting as described in Chapter 2. Samples of 1×10^7 cells were then removed and washed in sterile water. The cells were then resuspended in 800 µl of assay buffer (see Chapter 2), 20 µl of 5 mM $FeCl_3$ and 200 µl of BPS. The cell suspension was incubated at 30 °C for 10 minutes and then the cells were harvested. The supernatant was transferred to a cuvette and the OD measured at 520 nm. This measures the formation of the [Fe^{2+}. BPS] complex. The experiment was repeated three times on three separate days. The results shown here are the results of one representative experiment.

(a) BY4733[YEp213], parental strain

(b) JHS2.2[YEp213], *fre1/fre2* mutant

(c) JHS2.2[pJDF1.3]

(d) JHS2.2[pJDF2.3]

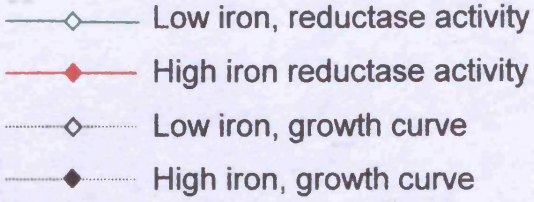
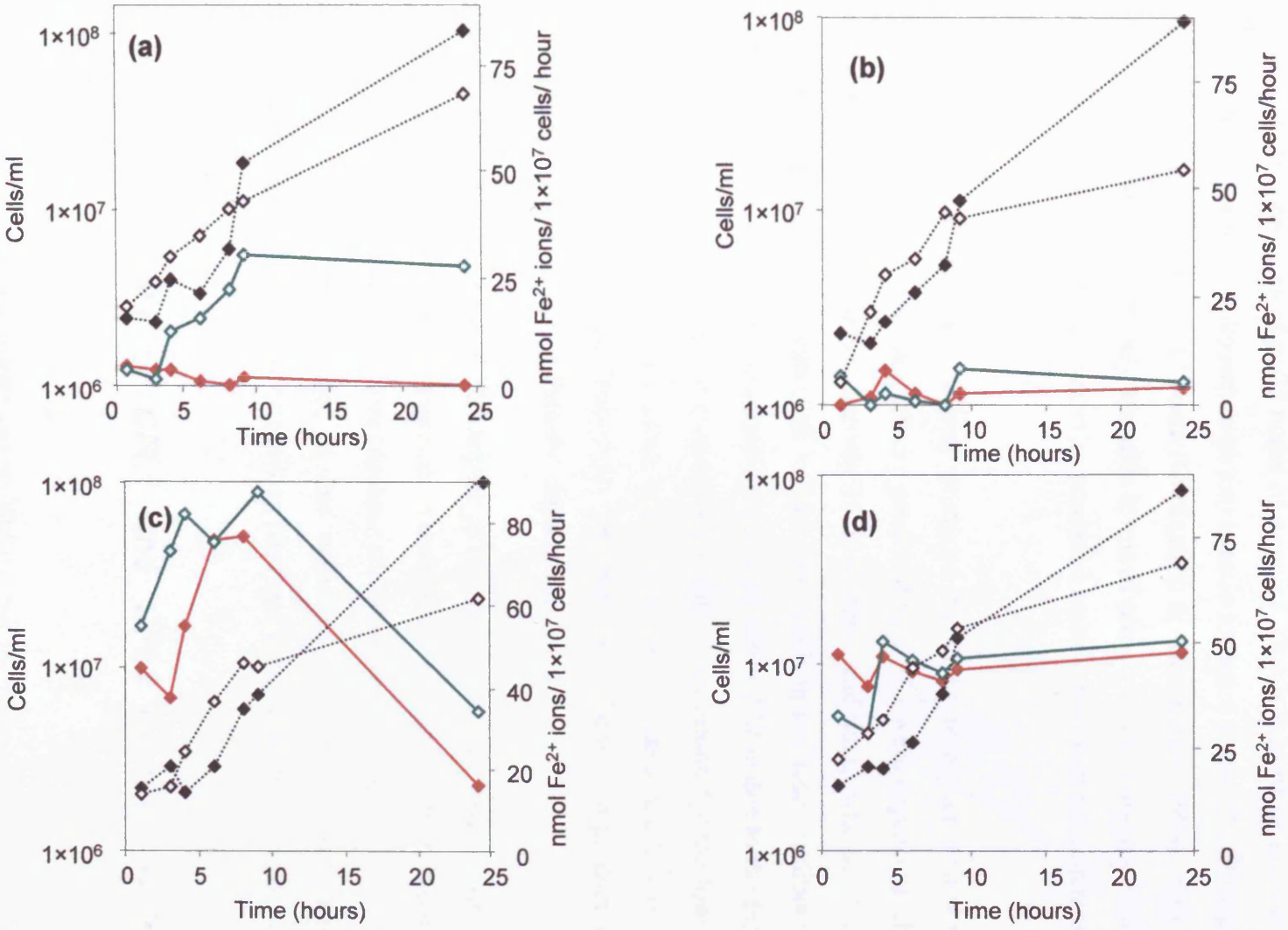


Figure 5.3 Quantitative assessment of reductase activities conferred by pJDF1.3 and pJDF2.3 on *S. cerevisiae* strain JHS2.2



ferric reductase activity produced by *CFL1* in low and high iron conditions, suggesting that it might be negatively regulated by iron to some extent. However, since the level of ferric reductase activity was increased only approximately 1.5-fold in low iron conditions, it was difficult to assess whether these differences were significant. Interestingly, the ferric reductase activity conferred by *CFL1* appeared to be regulated in response to growth phase, and reduced levels of reductase activity were seen after 23 hours of growth. This is interesting since the *S. cerevisiae* ferric reductase activity is known to be regulated in response to growth, although the mechanism by which this occurs is not known (Georgatsou & Alexandraki, 1994). *CFL2* produced reductase activity at approximately the same levels of activity as the wild-type strain grown in low iron, and the ferric reductase activity produced by this gene was not regulated by iron.

It is interesting that the ferric reductase activity produced by *CFL1* is higher than that produced by *CFL2*. The levels of reductase activity produced by *CFL1* were approximately 3-times that of wild-type ferric reductase activity in low iron conditions, whereas that produced by *CFL2* was approximately equivalent to wild type levels in low iron conditions. It is possible that the high level of reductase activity associated with *CFL1* is due to the fact that the library vector, YEp213, is a high copy number plasmid. The reason for the lower levels of activity associated with *CFL2* is not clear, however, it may be due to the lack of a hydrophobic signal sequence in Cfl2p (see Chapter 4). This may cause the protein product of *CFL2* to be directed to sites in the cell other than the cell surface.

It is not surprising that neither *CFL1* nor *CFL2* appear to be regulated in *S. cerevisiae* since neither of the two genes have Aft1p-like binding sites in their promoters. However, this does not suggest that they are not regulated in *C. albicans* since different transcription factors with different recognition sequences may be used in this organism. The transcription and regulation of these genes in *C. albicans* was tested using Northern blotting as described in the following section.

5.4 Northern blot analysis of *CFL1* and *CFL2* expression in *Candida albicans*

Northern blot analysis was used to investigate the possibility that the transcription of *CFL1* and *CFL2* may be regulated in response to iron and/or copper levels, as is found for the *FRE*

genes of *S. cerevisiae* (Dancis *et al.*, 1992; Hassett & Kosman, 1995; Martins *et al.*, 1998). RNA was prepared from *C. albicans* S/01 cultures grown in MD medium containing high and low iron and high and low copper concentrations. RNA samples were analysed for *CFL1* mRNA using the 1.8 kb fragment obtained by *Eco*RI digestion of pJDF1.3 as a probe (Fig 4.3), and for *CFL2* mRNA using the 1.6 kb *Eco*RI/*Hpa*I fragment from the *CFL2* encoding region of pJDF2.3 as a probe (Fig 4.3). Samples were also probed with the housekeeping gene *CaURA3* to check that samples had been equally loading. A transcript of approximately 3.45-kb was detected using the *CFL1* probe after 4 weeks exposure. This transcript was negatively regulated in response to iron and copper levels in the media (Fig 5.4). The length of exposure required to detect the transcript suggests that it is expressed at low levels. No transcript was detected using the *CFL2* probe despite the fact that the *URA3* loading control was consistently observed after an overnight exposure. This suggests that the *CFL2* transcript may not be abundant enough to be detected by Northern blotting or that it is expressed under different conditions. Alternatively, *CFL2* may be a pseudogene.

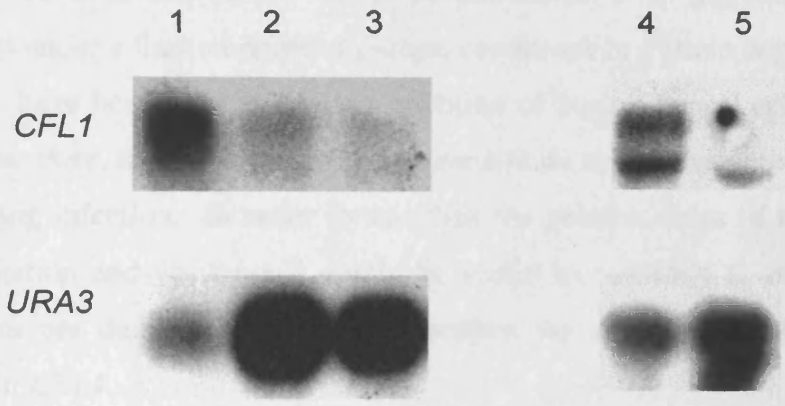
5.5 Discussion

CFL1 is expressed and regulated by iron and copper in *C. albicans*. This is interesting since only one of the *S. cerevisiae FRE* genes, *FRE1*, is regulated in response to both iron and copper. All of the other *FRE* genes are regulated in response to either iron or copper: *FRE2-6* are regulated in response to iron and *FRE7* is regulated in response to copper (Martins *et al.*, 1998). The fact that *CFL1* is regulated by both iron and copper may suggest that it plays an important role in iron and copper metabolism.

Interestingly, neither *CFL1* nor *CFL2* were found to be regulated by iron when expressed in *S. cerevisiae*. This is not entirely surprising since the promoters of these genes do not contain Aft1p-like binding sites. Aft1p is the transcription factor responsible for mediating iron responsive gene regulation in *S. cerevisiae*. This suggests that the *C. albicans* transcription factor responsible for iron-regulated gene expression is not closely related to the Aft1p transcription factor of *S. cerevisiae*.

No transcript was detected for *CFL2*. As discussed in Chapter 4, *CFL2* does not have a TATA-box located upstream of the most 5' ATG in the ORF. It was postulated that the third

Figure 5.4 Northern blot analysis of *CFL1* in *C. albicans*



Total RNA was extracted from exponentially growing cultures of *C. albicans* grown in MD-BPS media containing 0 μM (lane 1), 100 μM (lane 2) or 250 μM (lane 3) $FeCl_3$ or from cultures grown in MD-BCS media containing 0 μM (lane 4) or 100 μM (lane 5) $CuCl_2$. Following electrophoresis and transfer to a nylon membrane, duplicate sets of the three RNA samples were probed with either a ^{32}P-labelled 1.8 kb *Eco*RI fragment of *CFL1* (see Figure 3) or with the *C. albicans URA3* gene as a loading control.

ATG might be the actual translational start site in this sequence since this has a TATA-box upstream of it. However, given that no transcript was detected it is possible that this gene is not transcribed. Alternatively, it is possible that *CFL2* is transcribed under conditions not tested in this study. In the case of the *C. albicans* secreted aspartyl proteinase family (*SAP* genes) which possesses 9 members, the majority of transcription detected in culture conditions is accounted for by *SAP2*. However, other members of the gene family have been shown to be expressed under different conditions and, moreover, *SAP4-6*, which are transcribed under a limited range of culture conditions in a strain dependent manner (Hube *et al.*, 1994), have been detected during infection of human buccal epithelium (Schaller *et al.*, 1999). Therefore, the lack of transcript under culture conditions does not necessarily preclude a role during infection. In order to establish the possible roles of both *CFL1* and *CFL2* in iron acquisition and virulence it would be useful to construct *C. albicans* strains in which these genes are deleted. Chapter 6 describes the construction of a *C. albicans* mutant defective in *CFL1*.

Chapter 6
Disruption of *CFL1* in *Candida albicans*

6.1 Introduction

Two *C. albicans* ferric reductase genes, *CFL1* and *CFL2*, have been identified during the course of this work. In the previous chapter, *CFL1* was shown to be expressed in an iron and copper responsive manner. This implies that the product of the *CFL1* gene plays a role in iron and copper metabolism. It is possible that *CFL1* encodes a cell surface ferric reductase and plays an important role in iron and copper acquisition. It may, alternatively, be involved in facilitating the movement of iron or copper between different intracellular compartments. To investigate the possible roles of Cfl1p, it is necessary to construct a mutant strain in which *CFL1* is no longer functional. This strain can be used to determine the effects of the gene deletion on ferric reductase activity and iron acquisition, and ultimately, to determine the effects of the deletion on the virulence of *C. albicans* in appropriate animal models. The work in this chapter describes the construction of a *cfl1* mutant.

6.2 'URA-blasting'

C. albicans is an obligate diploid with no sexual cycle. This means that gene disruption is difficult since at least two chromosomal copies of most genes need to be deleted, and in some cases three copies of genes have been found (Gow *et al.*, 1994; Ramanan & Wang, 2000). It is important that the strains used for gene disruption are derived from clinical isolates since the ultimate aim of most gene deletions in *C. albicans* is to assess their impact on virulence. It is also preferable if the strains used in gene disruption studies contain directed mutations, since random mutagenesis techniques may generate multiple mutations, making it difficult to assign phenotypes to particular gene deletions.

C. albicans strains have therefore been developed for carrying out gene disruptions. The strains that are commonly used are derived from a *C. albicans* clinical isolate, SC5314 (Gillum *et al.*, 1984) and are deleted in either one (strain CAF2), or both copies (strain CAI4) of the *URA3* gene, which is replaced by immunity region of bacteriophage lambda *λimm434* (Fonzi & Irwin, 1993). Since these strains contain directed deletions, they are otherwise genetically identical to SC5314, and any phenotypic changes observed between the strains

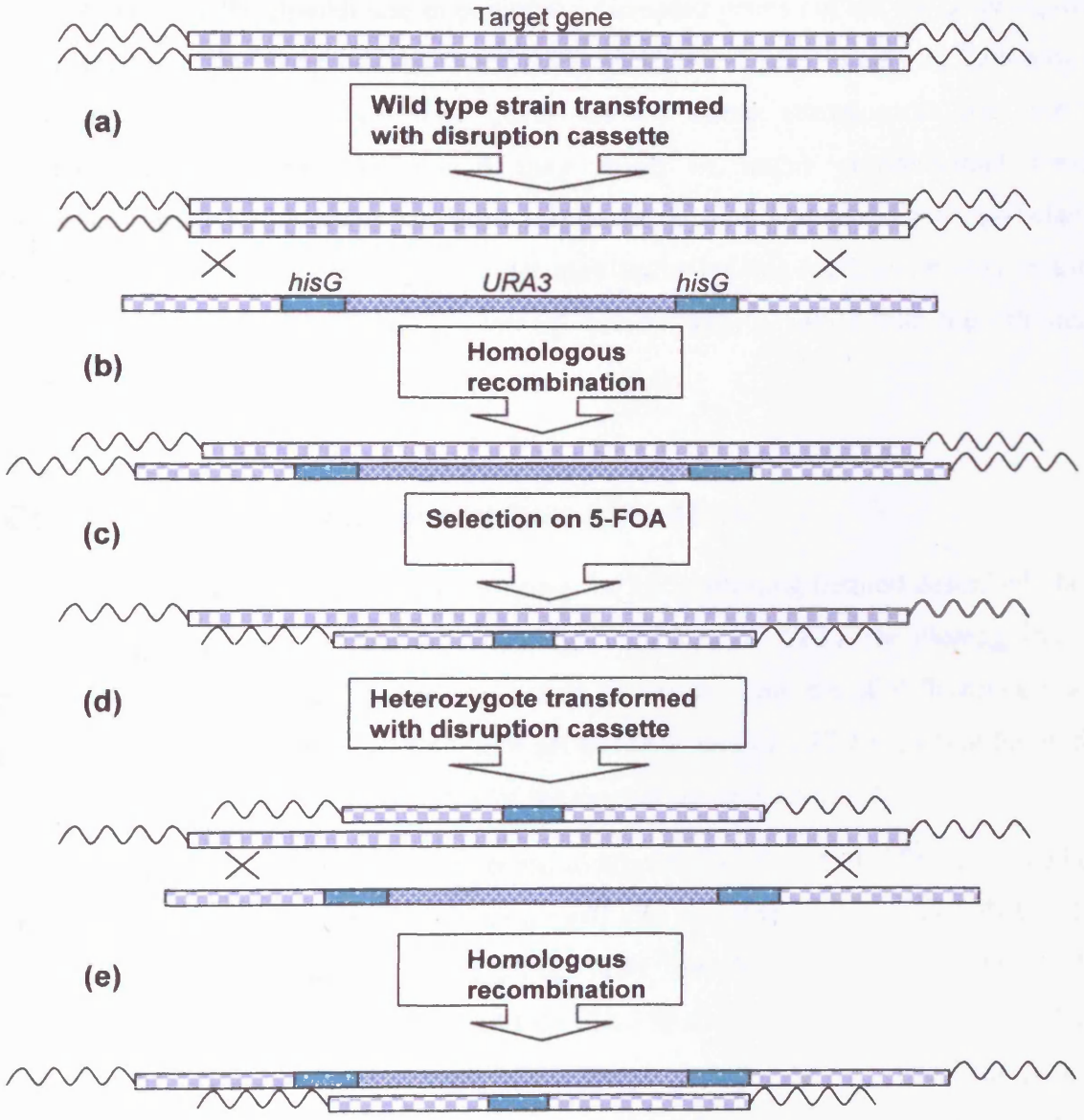
can be attributed to known deletions. In fact, CAI4 is found to be less virulent than either CAF2 or SC5314, a phenotype that is attributable to the loss of both copies of the *URA3* gene (Gow *et al.*, 1999). It is therefore important that strains derived from CAI4 which are used in virulence studies carry an intact *URA3* gene.

The strain CAI4 carries deletions in only the *URA3* gene, making this the only suitable marker gene for use in gene disruptions. Therefore, this marker gene has to be used in at least two rounds of transformations to delete both chromosomal copies of the target gene. The URA-blasting technique, originally developed for use in *S. cerevisiae* (Alani *et al.*, 1987), allows the *URA3* gene to be used multiple times as a selectable marker in gene disruptions. The method relies on the fact that strains carrying the *URA3* gene can be selected against by growth on 5-fluororotic acid (5-FOA) (Boeke *et al.*, 1984). 5-FOA is an analogue of orotic acid, which is an intermediate in the pyrimidine biosynthetic pathway. In the presence of orotidine-5' phosphate decarboxylase, the product of the *URA3* gene, 5-FOA is metabolised and enters into the pyrimidine biosynthetic pathway where it is eventually converted into 5-fluorodeoxyuridylate, an analogue of dUMP. 5-fluorodeoxyuridylate is a substrate of the thymidylate synthase enzyme, which catalyses the conversion of dUMP to dTMP. However, 5-fluorodeoxyuridylate irreversibly inhibits thymidylate synthase by becoming covalently linked to the enzyme. This leads to cell death, and therefore removes cells carrying the *URA3* from the population.

URA-blasting uses a cassette in which a *URA3* marker gene is flanked by the *Salmonella typhimurium hisG* gene on either side. This cassette is inserted into the target gene to construct a disruption cassette, which is then transformed into CAI4 with selection for uridine prototrophy. Colonies are checked to see that homologous recombination has taken place correctly and that one copy of the target gene is disrupted (see Fig 6.1 for an overview of *URA*-blasting; (Gow *et al.*, 1999). This is followed by selection on 5-FOA to allow the identification of colonies that have lost the *URA3* gene by recombination between the flanking *hisG* repeats. A second round of transformation of the disruption cassette should result in some uridine prototrophs in which the disruption cassette has inserted in the remaining intact allele of the target gene.

In this investigation two plasmids were used for *URA*-blasting: pMB7 and p5921, both of which contain the *URA*-blasting cassette (see Fig 2.3). The pMB7 plasmid differs from p5921 as it has a *Bam*HI site and a I-*Sce*I site incorporated into both copies of the *hisG* genes. This allows the chromosomal location of the gene to be determined since *C. albicans* contains

Figure 6.1 'URA-blasting'



(a) *C. albicans* strain CAI4 is transformed with a gene disruption cassette consisting of the target gene disrupted by a *hisG/URA3/hisG* cassette. **(b)** Homologous recombination occurs resulting in one chromosomal copy of the target gene being deleted. **(c)** Selection on 5-FOA leads to loss of the *URA3* gene by recombination between the flanking *hisG* regions. **(d)** The heterozygote may then be transformed with the same disruption cassette as was used in the first round of transformations with selection for uridine prototrophy. **(e)** Homologous recombination may then occur resulting in deletion of both chromosomal copies of the target gene.

no natural I-*Sce*I sites. Therefore, following disruption of the target gene, cutting with I-*Sce*I will cause one chromosome to be cut, allowing the chromosomal location of the gene to be determined. Also, if a second round of 5-FOA selection is carried out to remove the *URA3* gene, the presence of the *Bam*HI site in one of the disrupted genes but not the other allows it to be shown that the removal of the *URA3* gene has come about by homologous recombination between the two *hisG* genes on the same chromosome and not by interchromosomal recombination, which may result in larger chromosomal regions recombining. Since *C. albicans* has been shown to be highly heterozygous (Whelan & Magee, 1981), the loss of heterozygosity that may occur by this mechanism may result in phenotypes that are associated with the loss of heterozygosity, rather than the introduced mutation.

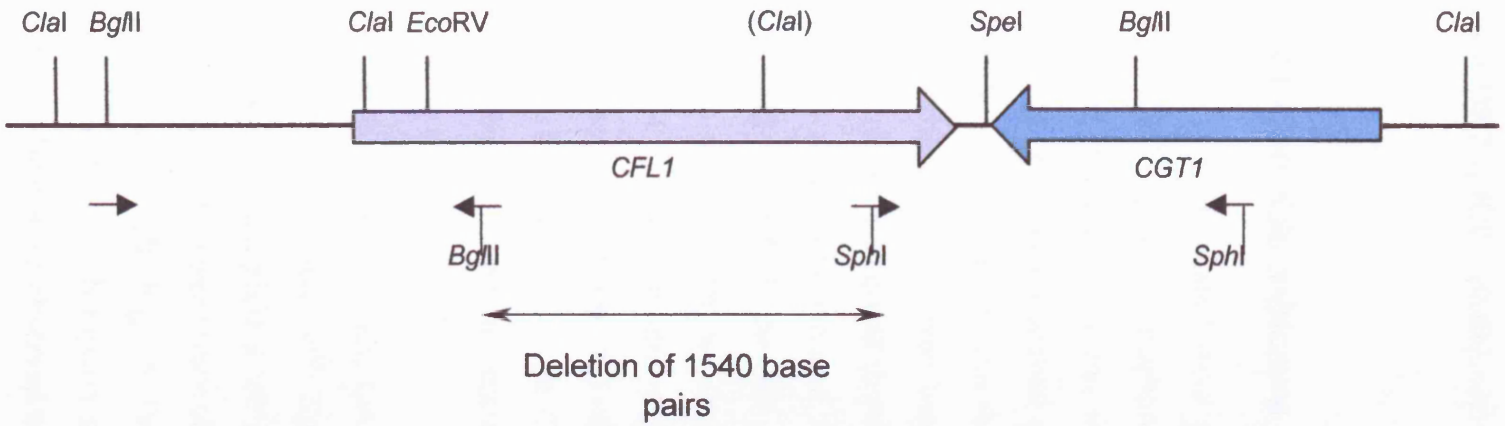
6.3 Construction of *CFL1* disruption cassettes

The *CFL1* gene disruption was carried out using the URA-blasting method described above. A PCR strategy was used to amplify flanking sequences of *CFL1* for cloning into the plasmids pMB7 and p5921 to produce disruption cassettes. One set of PCR primers were designed to the 5' end of *CFL1*, and another set to the 3' end of *CFL1* such that the middle section of *CFL1* was not covered by either of the two primer sets (Fig 6.2).

The first set of primers, which were engineered to amplify the 5' end of *CFL1* consisted of a forward primer (CFL376) designed to span a *Bgl*II site found in the genomic DNA 921 bp from the start of the ORF, and a reverse primer (CFL739) designed approximately 400 bp into the ORF. Mismatches were introduced into CFL739 such that a *Bgl*II site was produced, since no suitable restriction sites existed in the native genomic DNA. The PCR products (approximately 1300 bp in length) produced from these two primers were cloned into the *Bgl*II sites of pMB7 and p5921. The orientation of the cloned PCR products was checked using *Eco*RV. This enzyme produced different sized fragments depending on the orientation of the PCR product in the plasmid. Plasmids were identified that contained the *CFL1* genomic fragment in the correct orientation for both pMB7 and p5921 and these were called pJD8.1 and pJD9.1, respectively.

The second set of primers were engineered to contain *Sph*I sites to allow the cloning of the 3' end of *CFL1* into the *Sph*I sites of pJD8.1 and pJD9.1. The forward primer (CFL3278) was designed within the *CFL1* ORF, approximately 300 bp from the end of the gene, whilst the

Figure 6.2 *CFL1* disruption strategy



CFL1 and its flanking DNA is shown. The primers used for amplification of genomic DNA sequences for disrupting *CFL1* are shown as small arrows. Restriction sites shown on the primers are restriction sites that were introduced in the design of the primers. The one primer which has no restriction site marked on it contained a *Bgl*II site present in the genomic sequence. Restriction sites used for releasing the disruption cassette as well as sites used for Southern blot analysis of potential disruptants are shown. The *Cla*I site shown in brackets is present on one chromosomal copy of *CFL1* in the strain CAI4, but not the other.

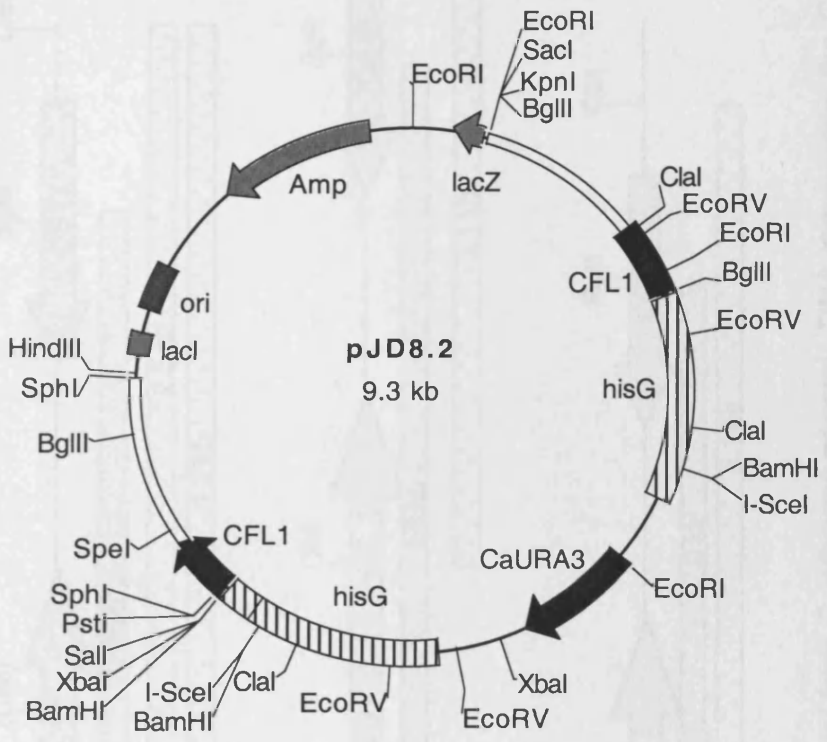
reverse primer was designed approximately 1000 bp upstream from the end of the ORF (CGT4219; Fig 6.2). This pair of primers produced PCR products of approximately 1300 bp. The PCR products were cloned into the plasmids pJD8.1 and pJD9.1 and their orientation checked using *Bgl*II. Plasmids carrying the second insertion in the correct orientation were identified were named pJD8.2 (pJD8.1/pMB7 derived) and pJD9.2 (pJD9.1/p5921 derived) (Fig 6.3).

6.4 Construction of *Candida albicans cfl1* mutant strain

The *cfl1* disruption cassette was separated from pJD8.2 by digestion with the restriction enzymes *Spe*I and *Kpn*I and subsequent electrophoresis on a 1 % agarose gel. The disruption cassette was purified by gel extraction and was transformed into CAI4 with selection for uridine prototrophy. Five colonies were obtained and all were analysed for the presence of the disruption cassette by Southern blotting. For the purpose of Southern blotting restriction enzymes were chosen for which the wild type band pattern was known from the sequence data available and mutant band patterns could therefore be predicted. *Bgl*II was chosen since digestion of genomic DNA with this enzyme was predicted to give band patterns that would distinguish between the wild type *CFL1*, the *cfl1* disruption cassette containing the *URA3* gene and the *cfl1* disruption cassette from which the *URA3* gene had been removed after selection on 5-FOA (Fig 6.4). *Cla*I was also used since it was known that this enzyme produced different band patterns for the two wild type chromosomal copies of *CFL1* in CAI4 due to the presence of an extra *Cla*I site in one *CFL1* allele (see Chapter 4; Section 4.3). Digestion with *Cla*I was also predicted to produce different band patterns between the parental strain and disruption strains (Fig 6.4).

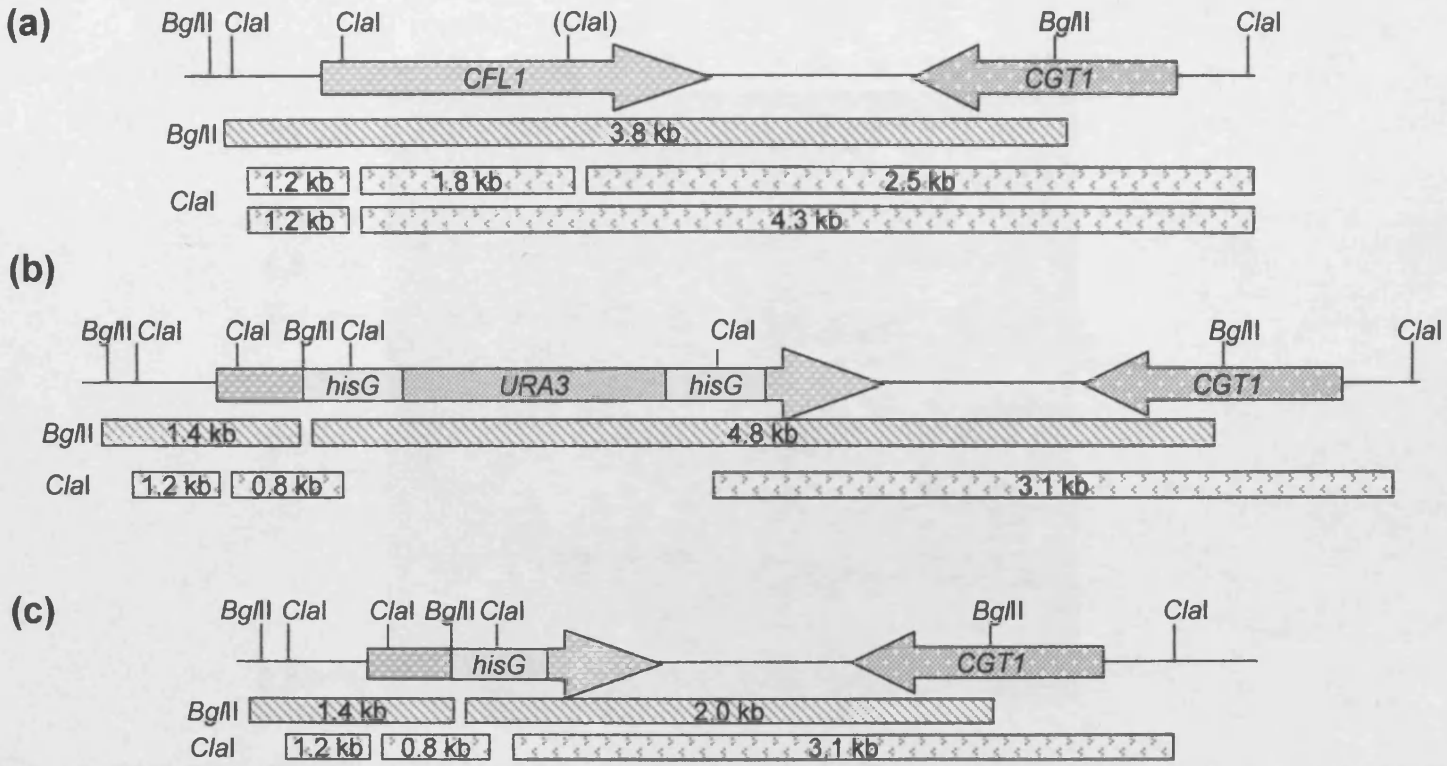
Genomic DNA from both the parental strain, CAI4, and the 5 candidate first round *cfl1* disruption strains were therefore digested with *Bgl*II and *Cla*I and analysed by Southern blotting. A 3.8 kb *Bgl*II fragment from pJD1.3, which spanned the *CFL1* ORF, was used as a probe (Fig 6.4). The expected band patterns were obtained for all five colonies, showing that all five carried one deleted copy of *cfl1* (Fig 6.5; Table 6.1). Interestingly, two distinct band patterns were observed when the *cfl1* disruption strains were digested with *Cla*I. Bands corresponding to the mutant allele were observed as expected in all 5 strains. However, in two of the strains the 4.3 kb wild type *Cla*I fragment was retained and the 1.8 kb and 2.5 kb wild type bands were lost. In the other three, the 4.3 kb *Cla*I fragment was lost and the 1.8 kb

Figure 6.3 *CFL1* disruption cassette



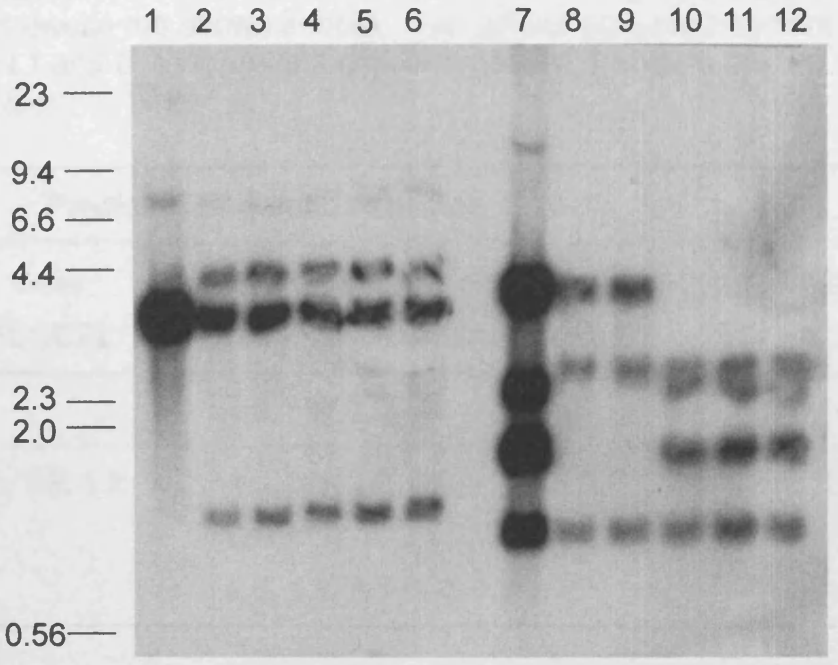
The *CFL1* disruption cassette carried on the vector pMB7 (see Fig 2.3). The *CFL1* gene is shown in black and the flanking chromosomal regions are shown as thin white lines. The *hisG/URA3/hisG* is inserted into the middle of the *CFL1* ORF, from which 1539 bp are deleted. The cassette was excised from the plasmid using the *Spe*I site and the *Kpn*I site.
This plasmid is identical to pJD9.2, which is derived from p5921 (see fig 2.3), except that pJD9.2 lacks the *Bam*HI and I-*Sce*I sites present in both copies of the *hisG* gene of pJD8.2

Figure 6.4 Banding patterns expected from *C. albicans* genomic DNA probed with *CFL1*



The restriction fragments produced by *Bgl*II and *Cla*I from *C. albicans* genomic DNA surrounding the *CFL1* locus are shown. **(a)** shows the fragment sizes expected from CAI4 wild type DNA. The *Cla*I site shown in brackets is present on only one chromosomal copy of *CFL1* in strain CAI4. The 3.8 kb *Bgl*II fragment was used as a probe for Southern blotting. **(b)** shows the expected fragment sizes from genomic DNA in which the *cfl1* disruption cassette has been inserted by homologous recombination. **(c)** shows the fragment sizes expected after loss of the *URA3* gene from the *cfl1* disruption cassette after selection on 5-FOA. This diagram is not to scale.

Figure 6.5 Southern blot of candidate *cfl1* disruptants after first round transformation



Genomic DNA from *C. albicans* strain CAI4 and the candidate *cfl1* disruptant strains were digested with *Bgl*II and *Cla*I and run on a 0.8 % agarose gel. The gel was blotted onto Hybond-N filter paper and the blot probed with a 3.8 kb *Bgl*II fragment from pJD1.3 that spans the *CFL1* ORF. After overnight hybridisation the blot was washed in standard stringency conditions as described in Chapter 2 and exposed to X-ray film, which was developed after an overnight exposure at -80 °C. Lane 1 and Lane 7 : CAI4 genomic DNA; lanes 2-6 and 8-12 genomic DNA from candidate *cfl1* disruption strains. Lanes 1-6 were digested with *Bgl*II; lanes 7-12 were digested with *Cla*I

Table 6.1 Fragment sizes produced from first round *cfl1* disruption Southern blot

The actual and predicted fragment sizes from the Southern blot shown in Figure 6.5 are shown. The bands arising from the correct insertion of the *cfl1* disruption cassette are shown in bold. Two sets of possible fragment sizes are shown for *Cla*I digestion since there is an RFLP for *Cla*I in *CFL1* and *C. albicans* is a diploid organism, therefore the first round of disruption deletes only one wild type copy of *CFL1* (see Figure 6.4).

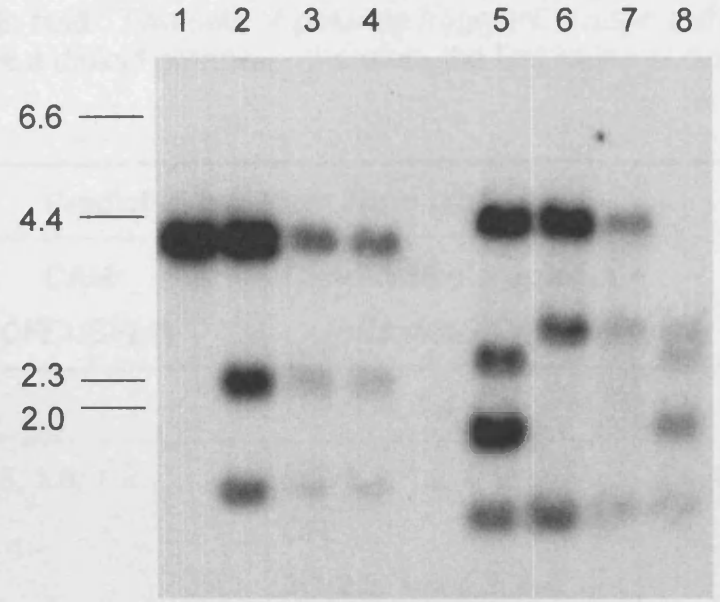
Restriction Enzyme	**Predicted fragment sizes (kb)**		**Actual fragment sizes (kb)**	
	CAI4 (*CFL1/CFL1*)	**Candidate *cfl1* disruptants (*cfl1::hisGURA3hisG/CFL1*)**	**CAI4 (*CFL1/CFL1*)**	**Candidate*cfl1* disruptants (*cfl1::hisGURA3hisG/CFL1*)**
*Bgl*II	3.8	**4.8**, 3.8, **1.4**	3.8	**4.8**, 3.8, **1.2**
*Cla*I	4.3, 2.5, 1.8, 1.2	4.3, **3.1**, 1.2, 0.8 OR: 2.5, 1.8, **3.1**, 1.2, 0.8	4.6, 2.5, 1.8, 1.1	4.6, **2.9**, 1.1 OR: **2.9**, 2.5, 1.8, 1.1

and 2.5 kb fragments were retained. The 1.8 kb and 2.5 kb fragments arise due to the fact that one allele of *CFL1* carries an extra *Cla*I site, which cleaves the 4.8 kb fragment yielding the two smaller fragments in its place. It was therefore shown that the *cfl1* disruption cassette was equally likely to insert into either chromosomal copy of *CFL1*.

Two strains, each showing different *Cla*I restriction patterns, were then chosen for further study and named JHC1 (retained 4.3 kb *Cla*I band) and JHC3 (retained 1.8 kb and 2.5 kb *Cla*I bands). JHC1 and JHC3 were grown overnight in YPD and 5 µl of each culture was streaked onto a SD plate containing 50 $\mu g.ml^{-1}$ uridine and 1 $mg.ml^{-1}$ 5-FOA. The plates were incubated at 30 °C for 5 days until colonies appeared. Colonies were picked and streaked onto fresh plates and then analysed for loss of the *URA3* gene. Genomic DNA, prepared from 2 colonies derived from JHC1 and 1 colony derived from JHC3, was digested with *Bgl*II and *Cla*I. Genomic DNA from CAI4, similarly digested with *Bgl*II and *Cla*I, was also used as a control. Southern blot analysis was carried out using the 3.8 kb *Bgl*II fragment from pJDF1.3 as a probe. The bands observed showed that the *URA3* gene had been lost from all three candidate strains analysed (Fig 6.6; Table 6.2). One strain derived from JHC1, and named JHC1.1 was used for further study.

JHC1.1 was transformed with the *cfl1* disruption cassette, which was isolated from pJD9.2 by digestion with *Kpn*I and *Spe*I. 482 colonies were obtained from 12 transformations. 30 colonies were analysed by colony PCR using 3 primers (Fig 6.7). One primer (CFL347) was designed to a region of genomic DNA upstream of the *CFL1* disruption cassette, whilst another (CFL2220) was designed to a region of *CFL1* that was deleted in the disruption cassette. These two primers should yield a 1.8 kb PCR product in both the wild type strain and in the heterozygous mutant, corresponding to the wild type *CFL1* allele. A third primer (hisG) was designed to the *hisG* region of the disruption cassette. This primer, in combination with the primer, CFL347, from the upstream region of *CFL1* should yield a 1.4 kb product in both the heterozygous *cfl1* mutant and the homozygous *cfl1* mutant, since both of these strains contain the mutant allele of *CFL1*. Five potential *cfl1/cfl1* homozygous mutants were identified by this method (Fig 6.8). Genomic DNA was prepared from these colonies and digested with *Bgl*II and *Cla*I. Southern blot analysis was then carried out. From the *Bgl*II digests it was clear that all the candidate *cfl1* mutant strains had lost the 3.8 kb wild type band and gained a 4.8 kb mutant band, which is produced by the *cfl1::hisGURA3hisG* allele. It also retained the 2 kb and 1.4 kb bands produced by the *cfl1::hisG* allele (Fig 6.4; Fig 6.9). The *Cla*I digests showed that the 1.8 kb and 2.5 kb wild type bands were lost whilst

Figure 6.6 Southern blot of first round of *cfl1* disruption following 5-FOA selection



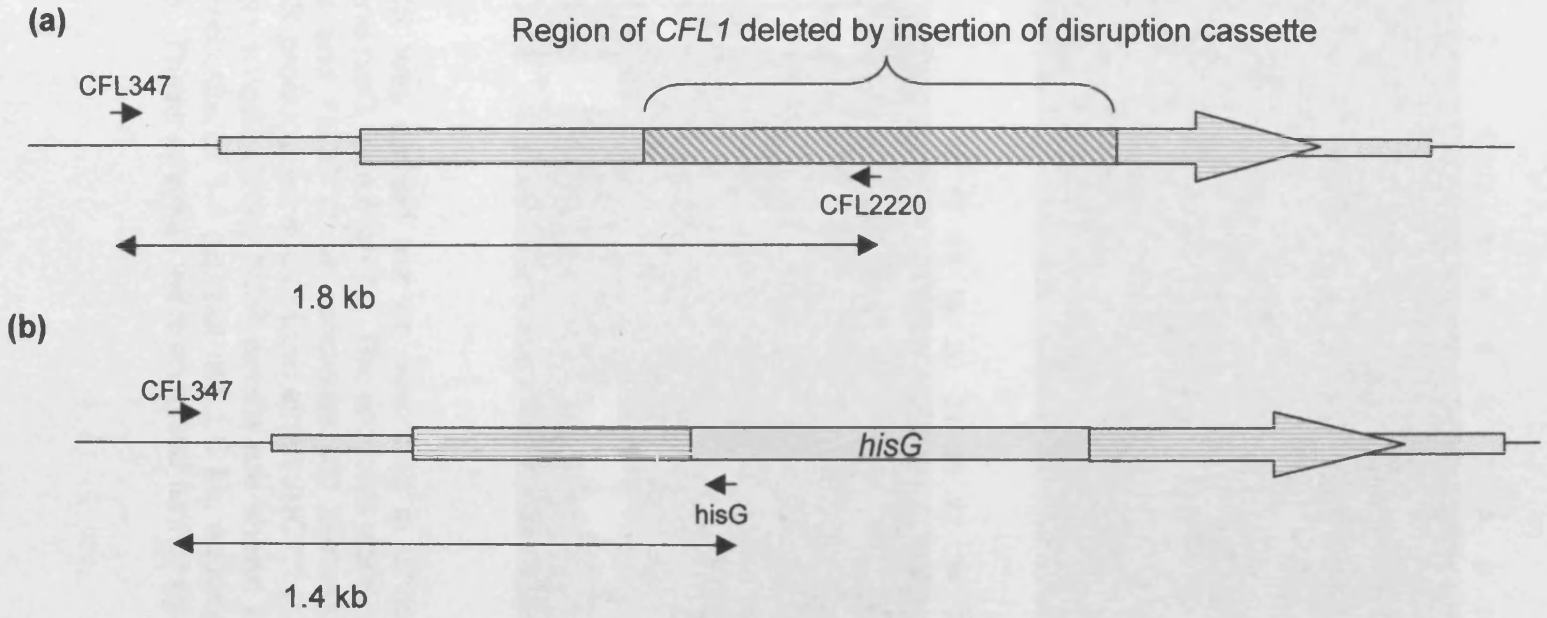
Genomic DNA from *C. albicans* strain CAI4 and three post 5-FOA selection *cfl1* disruption strains were digested with *Bgl*II and *Cla*I and run on a 0.8 % agarose gel. The gel was blotted onto Hybond-N filter paper and the blot probed with a 3.8 kb *Bgl*II fragment from pJD1.3 that spans the *CFL1* ORF. After over noght hybridisation the blot was washed in standard stringency conditions as described in Chapter 2 and exposed to X-ray film, which was developed after an over night exposure at -80 °C. Lane 1 and 5: CAI4 genomic DNA; lanes 2-4 and 6-8: *cfl1* disruption strains. Lanes1-4: DNA digested with *Bgl*II; lanes 5-8: DNA digested with *Cla*I

Table 6.2 Fragment sizes produced from Southern blot of the first round of *cfl1* disruption following 5-FOA selection

The actual and predicted fragment sizes from the Southern blot shown in Figure 6.6 are shown. The bands arising from the correct removal of the *URA3* are shown in bold. Two sets of possible fragment sizes are shown for *Cla*I digestion since there is an RFLP for *Cla*I in *CFL1* and *C. albicans* is a diploid organism, therefore the first round of disruption removes only one wild type allele (see Figure 6.4).

Restriction Enzyme	**Predicted fragment sizes (kb)**		**Actual fragment sizes (kb)**	
	CAI4 (*CFL1/CFL1*)	**Candidate disruptants (*cfl1::hisG/CFL1*)**	**CAI4 (*CFL1/CFL1*)**	**Candidate disruptants (*cfl1::hisG/CFL1*)**
*Bgl*II	3.8	3.8, **2.3**, **1.4**	3.8	4.0, **2.3**, **1.2**
*Cla*I	4.3, 2.5, 1.8, 1.2	4.3, **3.1**, 1.2, **0.8** OR: **3.1**,2.5, 1.8, 1.2, **0.8**	4.4, 2.6, 1.8, 1.05	4.4, **3.0**, 1.05 OR: **3.0**, 2.6, 1.8, 1.05

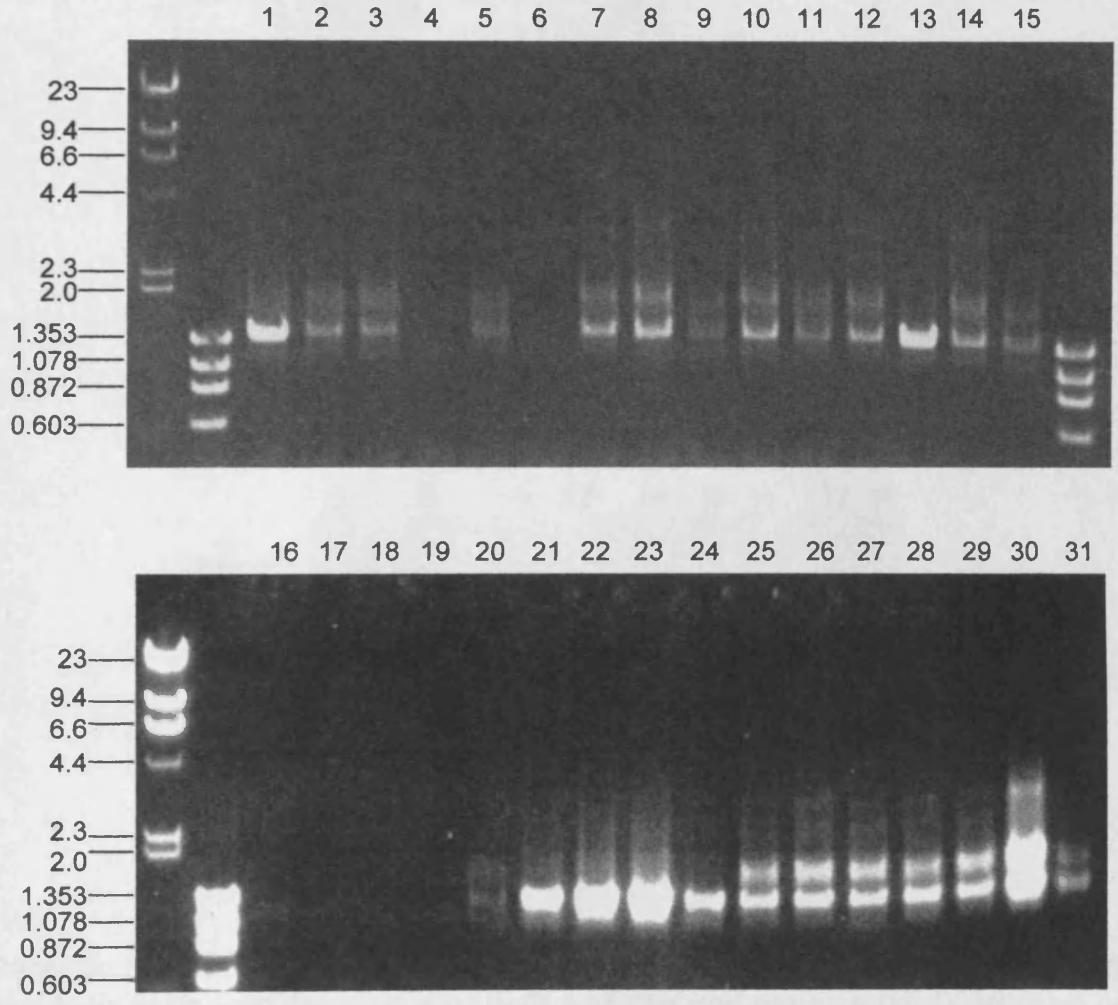
Figure 6.7 PCR strategy for identifying *cfl1* homozygous mutants.



(a) Shows binding sites of the two primers used to amplify the wild type allele. The region of *CFL1* shown in darker purple is deleted by the insertion of the disruption cassette. Therefore when both wild type alleles are lost a PCR product is no longer produced from these primers.
(b) Shows the binding sites of the two primers used to amplify the *cfl1* disruption allele. Note the 5' primer (CFL347) is used for both the wild type and deletion allele. This PCR product is only observed in *C. albicans* strains containing the *cfl1* disruption allele.

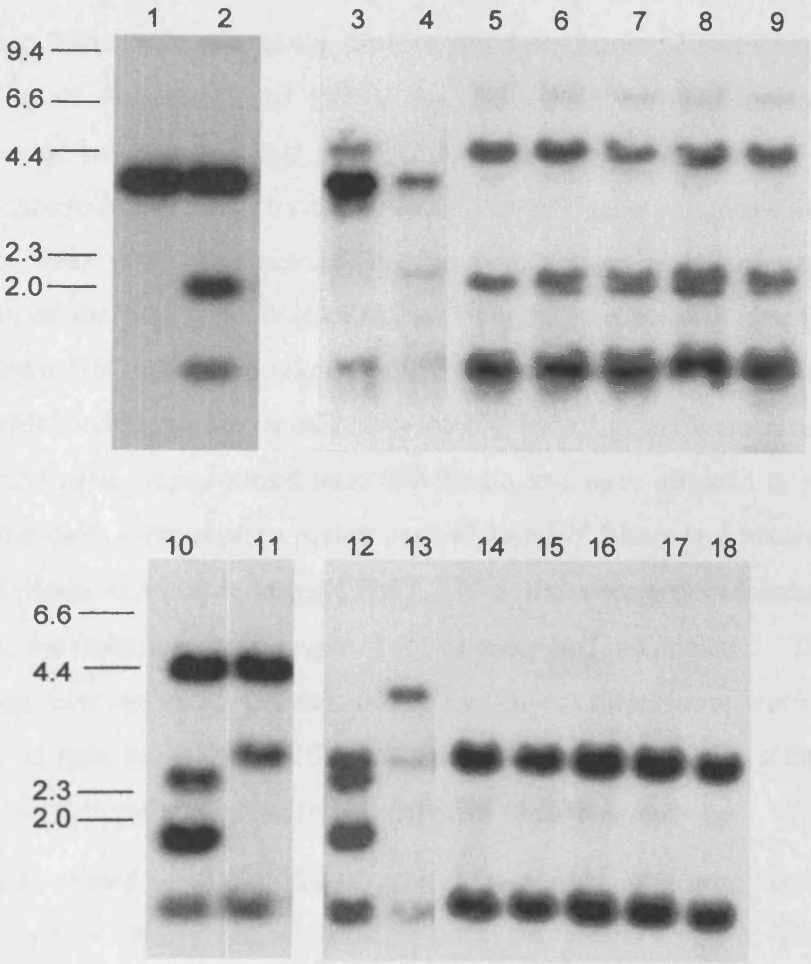
The thin black lines show flanking genomic DNA sequences. The white blocks show the extent of the cloned region of *CFL1* used in the construction of the disruption cassette. The purple blocks show the *CFL1* gene, and the yellow block shows the *hisG* insertion in the disrupted *cfl1*.

Figure 6.8 Colony PCR of second round transformants



Colony PCR was carried out as described in Chapter 2 using primers CFL347, CFL2220 and hisG (see Fig6.7). The products were run on a 1 % agarose gel.
Lanes 1-14 and 16-30 show candidate *cfl1* disruption strains. Lanes 15 and 31 contain PCR products produced from strain JHC1.1 (*CFL1/cfl1::hisG*).
The colonies whose colony PCR results are shown in lanes 13, 21, 22, 23 and 24 produced products of 1.4 kb, but not 1.8 kb, suggesting the loss of the wild type *CFL1* allele. These colonies were analysed further by Southern blotting.

Figure 6.9 Southern blot of candidate *cfl1* double mutants



Genomic DNA from *C. albicans* strains CAI4, JHC1, JHC1.1 and the 5 candidate *cfl1* double mutants was digested with *Bgl*II and *Cla*I and run on a 0.8 % agarose gel. The gel was blotted onto a nylon filter and probed with the 3.8 kb *Bgl*II fragment from pJDF1.3 that spans the *CFL1* ORF. After overnight hybridisation the blot was washed in standard stringency washes as described in Chapter 2 and exposed to X-ray film, which was developed after an overnight exposure. Genomic DNA in lanes 1-9 were digested with *Bgl*II; genomic DNA in lanes 10-18 were digested with *Cla*I. Lane 1: CAI4; lane 2: JHC1.1 *(cfl1::hisG/CFL1);* lane 3: JHC1 *(cfl1::hisGURA3hisG/CFL1);* lane 4: JHC3.1 *(CFL1/cfl1::hisG);* Lanes 5-9: candidate *cfl1* double mutants. Lane 10: CAI4; lane 11: JHC3 *(CFL1/cfl1::hisGURA3hisG);* lane 12 JHC1.1 (*cfl1::hisG/CFL1*; gives same banding pattern as JHC1); lane 13: JHC3.1 *(CFL1/cfl1::hisG);* lanes 13-18: candidate *cfl1* double mutants. Lanes 1-2 and 10-11 show blots carried out on a separate occasion, with all other conditions identical; lanes 3-4 and 12-13 were exposed for 2 days since these lanes contained less DNA.

the mutant bands were retained. One of these colonies was used for phenotypic analysis, and was named JHC1.2.

6.5 Phenotypic analysis

The solid phase ferric reductase assay (as described in Chapter 2 and Chapter 3) was used to assess the affect of the deletion of *cfl1* on the cell surface ferric reductase activity of strain JHC1.2. It would be expected that if *CFL1* encodes a major component of the *C. albicans* cell surface ferric reductase activity then deletion of this gene would result in significant loss of cell surface ferric reductase activity, as has been shown to be the case in *S. cerevisiae* where deletion of the *fre1* gene results in loss of reductase activity (see Chapter 3). Single colonies of strain JHC1.2 were picked and resuspended in water in a microtitre dish. JHC1 and CAF2 (which contains one intact copy of the *URA3* gene) were also used as controls. The resuspended cells were spotted onto SD media and were allowed to grow for two days. After 2 days the cells were replica plated onto Hybond-N filters and placed on the surface of MD-dipyridyl plates containing 80 μM $FeCl_3$. The plates were then incubated at 30 °C for 5 hours and then the reductase assay carried out as described in Chapter 2. The experiment was repeated in duplicate on three separate occasions but no differences were observed between mutant and wild type cells (Fig 6.10a). This suggests that the cell surface ferric reductase activity is not significantly affected by the deletion of *CFL1*.

JHC1.2 was also tested for its ability to grow in low iron conditions, since slow growth on low iron is another phenotype associated with the loss of ferric reductase activity in *S. cerevisiae* (see Chapter 3). The cell density of overnight cultures of JHC1.2, JHC1 and CAF2, grown to saturation in YPD was determined by counting and the cultures harvested. The cells were then resuspended at 1×10^7 cells.ml^{-1} and a series of 4 1/10 dilutions were made. 5 μl of each dilution was spotted onto MD-EDTA media containing no added iron and the plates were incubated at 30 °C for 5 days. The plates were then inspected for any differences in growth between the three strains. None was observed, so the colonies were replica plated onto MD-EDTA and grown for a further 5 days since the iron storing capabilities of *C. albicans* are not known. Again, this experiment was repeated on three separate occasions but no differences were observed between the mutant and wild type (Fig 6.10b). Similarly, no differences in growth rate were observed between JHC1.2 and CAF2 when cultured in either MD-EDTA or YPD. This suggests that JHC1.2 does not experience

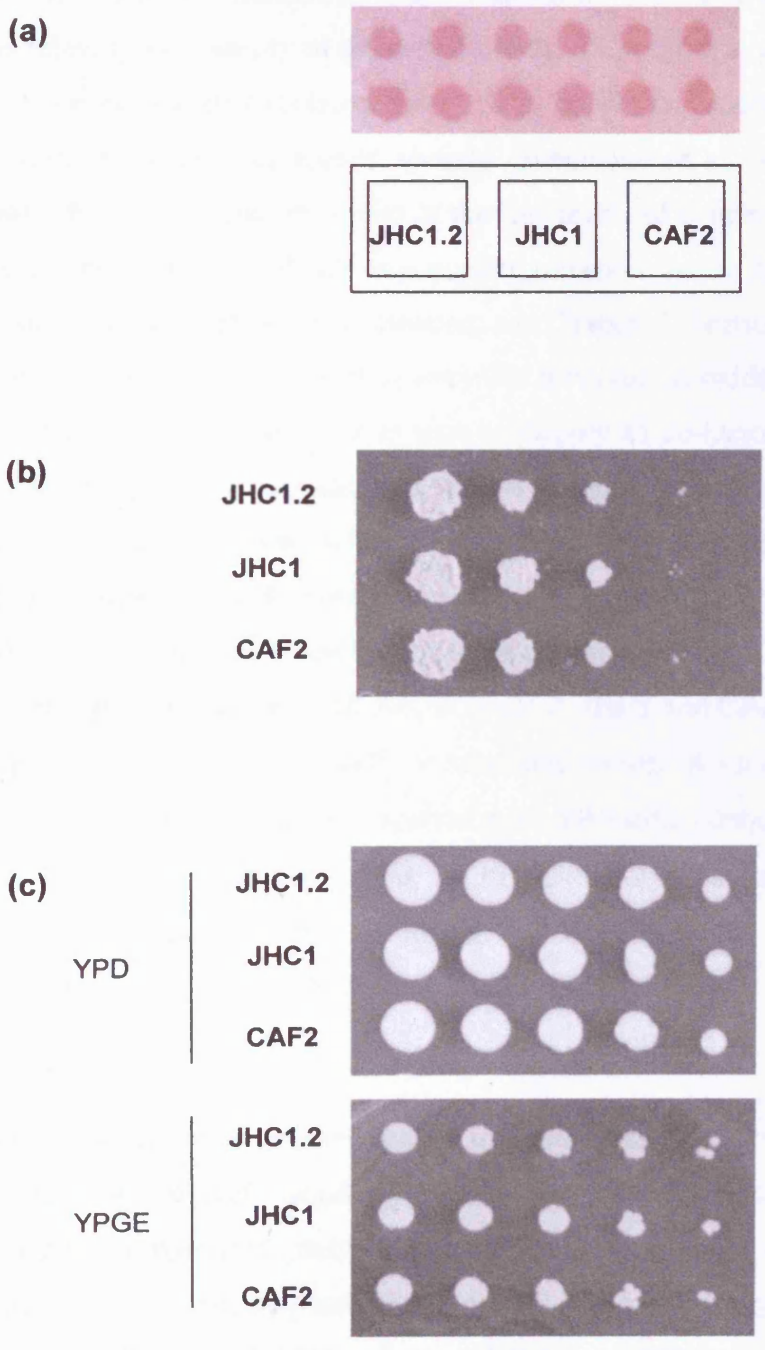
Figure 6.10 Phenotypic analysis of *C. albicans cfl1* mutant

(a) The reductase activities of JHC1.2 (*cfl1::hisG/cfl1::hisGURA3hisG*), JHC1 (*cfl1::hisGURA3hisG/CFL1*) and CAF2 (*CFL1/CFL1*) were compared using a solid phase ferric reductase assay. Cells grown on SD media were replica plated on to nylon filters placed on the surface of MD-dipyridyl plates containing 300 μM $FeCl_3$. The plates were incubated at 30 °C for 5 hours and then the filters removed and incubated in assay buffer (50 mM sodium citrate, pH 6.5; 5 % glucose) for 5 minutes, followed by a 5 minute incubation in assay buffer containing $FeCl_3$ and BPS. Reductase activity is indicated by the staining of the filter red due to the formation of a $[Fe^{2+}(BPS)_3]$ complex. No differences were observed between the wild type strain (CAF2) and the *cfl1* double mutant (JHC1.2).

(b) The ability of *C. albicans* strains CAF2, JHC1 and JHC1.2 to grow in low iron conditions was compared. Overnight cultures were grown to saturation in YNB media and their cell densities determined by counting. The cultures were then harvested and resuspended at 1×10^7 cells.ml^{-1}. A series of 4 1/10 dilutions were then made and 5 μl of the suspensions spotted onto a MD-EDTA plates containing no added iron.

(c) The ability of *C. albicans* strains JHC1.2, JHC1 and CAF2 to grow on media containing ethanol and glycerol as carbon sources was compared. The cell densities of overnight cultures grown to saturation in YPD media were determined and the cells harvested and resuspended at 1×10^7 cells.ml^{-1}. A series of 4 1/10 dilutions were then made and 5 μl of the suspensions were then spotted onto either YPD (containing glucose as a carbon source) or YPGE (containing 3 % glycerol and 3 % ethanol as carbon sources). The plates were then incubated for 3 days and then growth was compared.

Figure 6.10 Phenotypic analysis of *C. albicans cfl1* mutant



any difficulties in acquiring iron. It might also suggest that it has no difficulty in mobilising iron once it has been taken up by the cell since an inability to move iron to specific sites in the cell might result into an impaired ability to grow in low iron conditions.

A third phenotypic test was carried out to test the ability of JHC1.2 to grow on media containing glycerol and ethanol as carbon sources. The ability to grow on these carbon sources is related to the ability of the cell to use the TCA cycle to generate energy. It has been found in *S. cerevisiae* that mutants defective in copper transport are unable to grow using either ethanol or glycerol as carbon sources (Jungmann *et al.*, 1993; Knight *et al.*, 1996). This growth defect has been attributed to the low levels of copper and iron in the cell, which arise due to the lack of high affinity copper transport (since high affinity iron transport requires copper, iron levels are also affected; see Chapter 1, Section 1.5.3). This in turn leads to reduced activities of mitochondrial enzymes involved in oxidative phosphorylation since many of these enzymes require either iron or copper as co-factors. It has not been shown whether a similar phenotype exists for *S. cerevisiae* mutants defective in iron transport, but it has been shown that mutants defective in *yfh1*, a gene implicated in mitochondrial iron transport, are unable to grow using either ethanol or glycerol as carbon sources (Babcock *et al.*, 1997). This growth assay was therefore used to compare the *C. albicans cfl1* mutant with the wild type strain. Overnight cultures of JHC1.2, JHC1 and CAF2 were counted, harvested and resuspended at 1×10^7 cells.ml^{-1}, as described above. A series of 4 1/10 dilutions were again carried out and the cells were spotted onto YP media containing 3 % ethanol and 3 % glycerol. The plates were incubated at 30 °C for 2 days and then inspected for any differences in growth between the strains. The experiment was repeated three times, however, no difference in growth were observed (Fig 6.10c).

6.6 Discussion

This chapter describes the construction of a *C. albicans* strain in which both chromosomal copies of *cfl1* were deleted. Southern blotting confirmed that the strain was deleted in both copies of *CFL1*. Phenotypic analysis showed that no differences were observed between the mutant strain and either of its parental strains, JHC1 (*ura3/ura3*; *cfl1::hisGURA3hisG/CFL1*) and CAF2 (*ura3/URA3*), in terms of its cell surface ferric reductase activity, its ability to grow under low iron conditions, its ability to grow on different carbon sources or its growth rate.

It is not clear, therefore, what the role of *CFL1* might be in iron acquisition or metabolism. However, since *C. albicans* does possess at least 9 ferric reductase genes, it is possible that some of these genes may encode proteins with overlapping or redundant function. Another explanation of the lack of phenotype of the *cfl1* double mutant is that *CFL1* may encode a cell surface ferric reductase that has a minor function in the conditions tested in this study, but which becomes more important under other conditions. For instance, hyphal induction might induce the expression of specific ferric reductase genes, which play only a minor role under other conditions.

Given that no phenotype was found for the *cfl1* mutant, it seems likely that *C. albicans* possesses other genes that encode the cell surface ferric reductase activity. The work described in Chapter 7 analyses the sequences and expression patterns of 7 other *C. albicans* ferric reductase-like genes identified during the course of the *C. albicans* genome sequencing project(http://alces.med.umn.edu/Candida.html)

Chapter 7

Analysis of *CFL*-like genes identified during the *Candida albicans* genome sequencing project

7.1 Introduction

Two *C. albicans* ferric reductase genes have been identified during the course of this work. These were identified by their ability to rescue a *S. cerevisiae fre1* mutant strain, which is defective in ferric reductase activity. Inevitably, the functional complementation approach used to isolate these genes may not be appropriate for all potential ferric reductase genes, since the method relies on the presence of promoter sequences in the genomic clone that allow the gene of interest to be heterologously expressed in *S. cerevisiae*. The *C. albicans* genome sequencing project (http://alces.med.umn.edu/ Candida.html) has identified 7 more potential ferric reductases genes. The work described in this chapter describes the analysis of these sequences and expression studies on these genes.

7.2 Analysis of *CFL*-like gene sequences in the *Candida albicans* genome database

The *C. albicans* genome sequencing project, which is based at the Stanford Sequencing and Technology Center has used a shotgun approach to sequencing the entire genome of strain SC5314. This methodology generates numerous fragments of sequence approximately 500 bp in length. The sequences of these fragments were made available on a public database (http://alces.med.umn.edu/Candida.html) as they accumulated and could therefore be used to assemble contigs.

When the work described in this chapter was initiated several sequences, approximately 500 bp in length, showing similarity to ferric reductase genes had been identified by the Stanford sequencing project, and were designated *CFL90-CFL99*. In order to identify additional sequences on the database which overlapped with these short sequences I carried out BLAST searches against the database using the *CFL90-99* sequences as query sequences. This

method was used to build up contigs, varying in length from 2.7 kb to 0.7 kb, for 6 of the *CFL*-like genes (Fig 7.1). Three of the *CFL*-like sequences present in the database were not analysed (*CFL99* and *CFL90* and *CFL98*). The sequence designated *CFL99* was found to be identical to the 5'-end of the *CFL1* sequence isolated and sequenced during the course of this work (see Chapter 4). The *CFL99* sequence was not identified by the Stanford group as *CFL1* since it diverged from the then known *CFL1* sequence (Yamada Okabe *et al.*, 1996) at the same *Sau*3AI site as was found for the *CFL1* gene cloned in this work (Chapter 4). *CFL90* and *CFL98* were not used since no overlapping sequences were identified for *CFL98* and only one overlapping sequence was identified for *CFL90*, which resulted in a contig of 606 bp, of which 431 bp were overlapping. The sequencing traces were poor for both sequences and the contig contained multiple ambiguities. Therefore, this sequence was not used in further work. Thorough sequence analysis was not carried out at this stage since the sequences were of poor quality and did not cover the whole open reading frames, however, several small motifs known to be conserved within the ferric reductase family could be identified (Fig 7.1). Enough sequence was available to design PCR primers for each of the 6 contigs. Primers were designed bearing in mind the positions of the conserved motifs within the contigs, so that the PCR products would be predicted to span regions of the open-reading frame (Fig 7.1; Fig 7.2). These PCR products were then used as probes for Northern blotting experiments (see Section 7.3).

More recently, larger contigs have become available containing more accurate sequencing data (http://www-sequence.stanford.edu/group/candida). The whole of each of the open reading frames of *CFL91*, *CFL93*, *CFL94*, *CFL95*, *CFL96*, *CFL97* and *CFL98* are present on the Stanford database, in large contigs raging in size from 5 kb to 25 kb.

Multiple sequence alignments of the predicted protein sequences were carried out and it was shown that all of these genes conform to the generalised structure of a ferric reductase (Fig 7.3), and they are all approximately 700-750 amino acids in length (Table 7.1). They all possess conserved motifs that are associated with ferric reductase proteins, which are also found in the *S. cerevisiae* ferric reductase proteins, Fre1p and Fre2p, and the two *C. albicans* ferric reductases identified in this work, Cfl1p and Cfl2p. These motifs include those implicated in NADPH, FAD and haem binding (Finegold *et al.*, 1996; Karplus *et al.*, 1991) as well as those with, as yet, no defined role (Fig 7.4). As would be expected, the *C. albicans*

Figure 7.1 Contig assembly of *CFL*-like genes.

Sequences identified as *CFL*-like in the *C. albicans* genome database were used to scan the database using the BLAST program to identify overlapping sequences. The overlapping sequences were assembled into contigs. The red arrows indicate the sequences in the genome database identified as showing similarity to *CFL* sequences. The black arrows show sequences identified by scanning the database for overlapping sequences. The green arrow shows the consensus sequence and the direction of the arrow indicates the direction of the *CFL*-like gene, although it does not indicate the extent of the ORFs. The blue box on the consensus sequence shows the position of the FAD binding (HPFT) motif, the purple box shows the position of the EGYPG motif and the pale purple box shows the position of the GRNN motif (not to scale). The small black arrows beneath the consensus sequence show the binding sites of the primers used to amplify DNA for use as probes in Northern blot analysis (not to scale). The numbers on the bottom line indicate the length of the contig in nucleotides.

Figure 7.1 Contig assembly of *CFL*-like genes

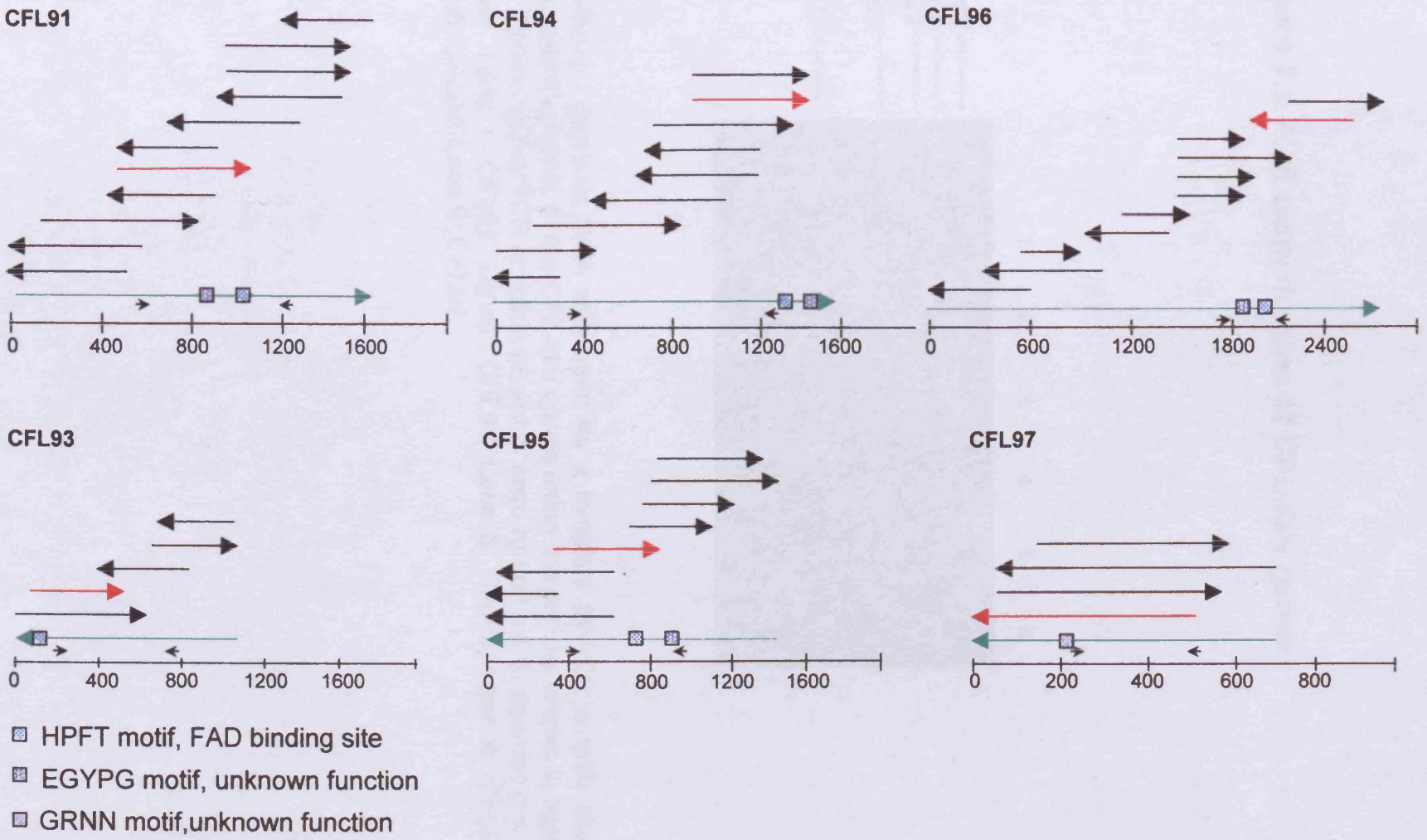
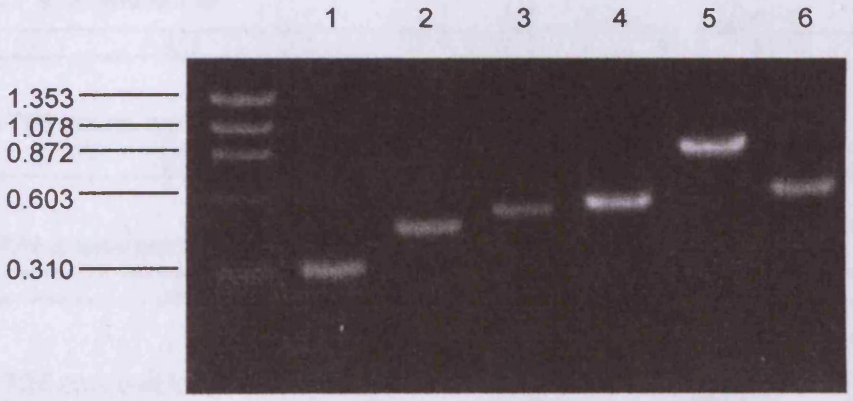
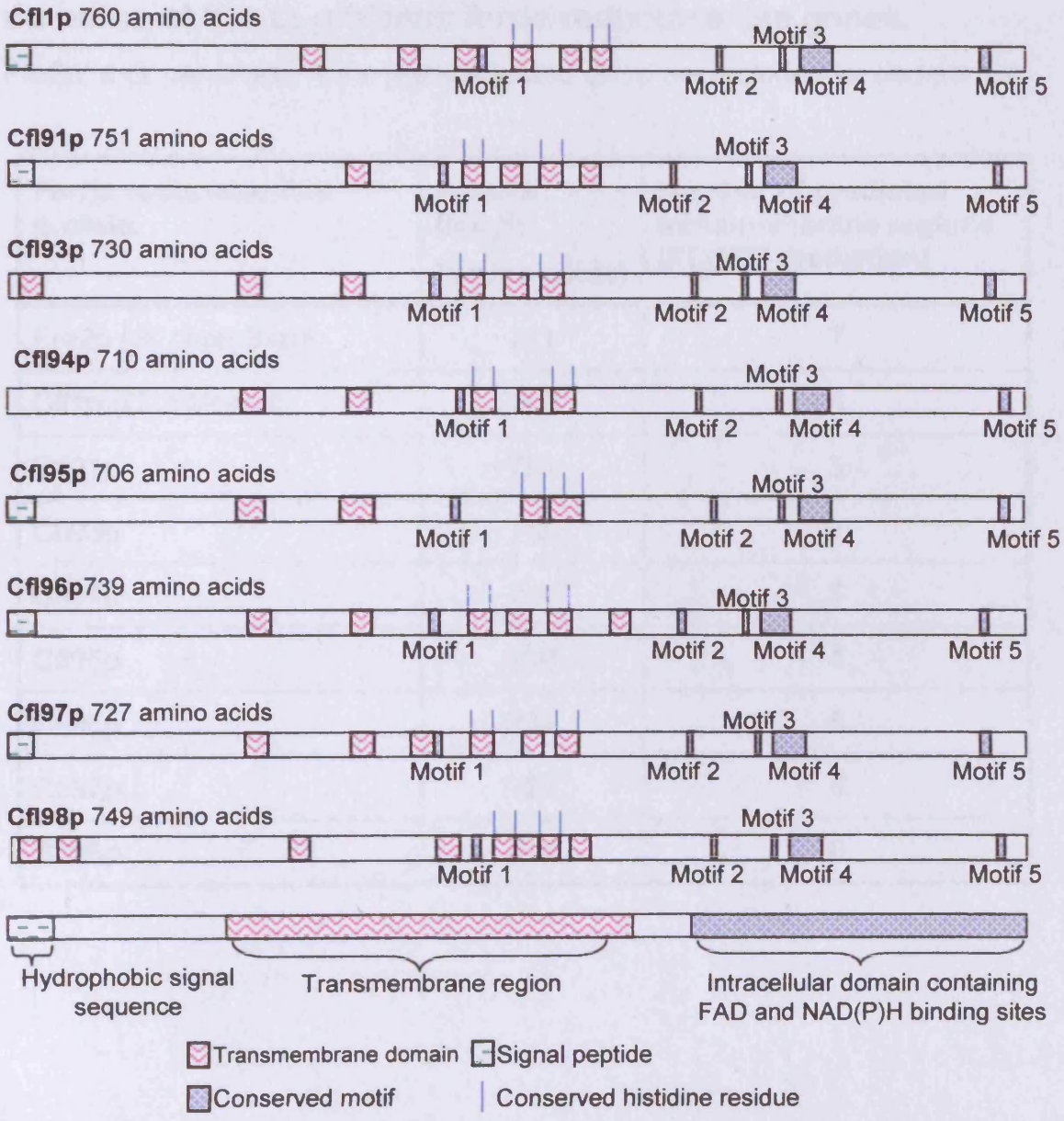


Figure 7.2 PCR amplification of *CFL*-like genes



C. albicans genomic DNA was used as a template to PCR amplify short intragenic fragments of the *CFL*-like genes using the primers shown in figure 7.1. Following the PCR reaction products were run on a 1 % agarose gel as shown. Lane 1: *CFL97*; Lane2: *CFL96*; Lane 3: *CFL95*; Lane 4: *CFL93*; Lane5: *CFL94*; Lane 6: *CFL91*

Figure 7.3 Structure of *C. albicans* ferric reductase proteins



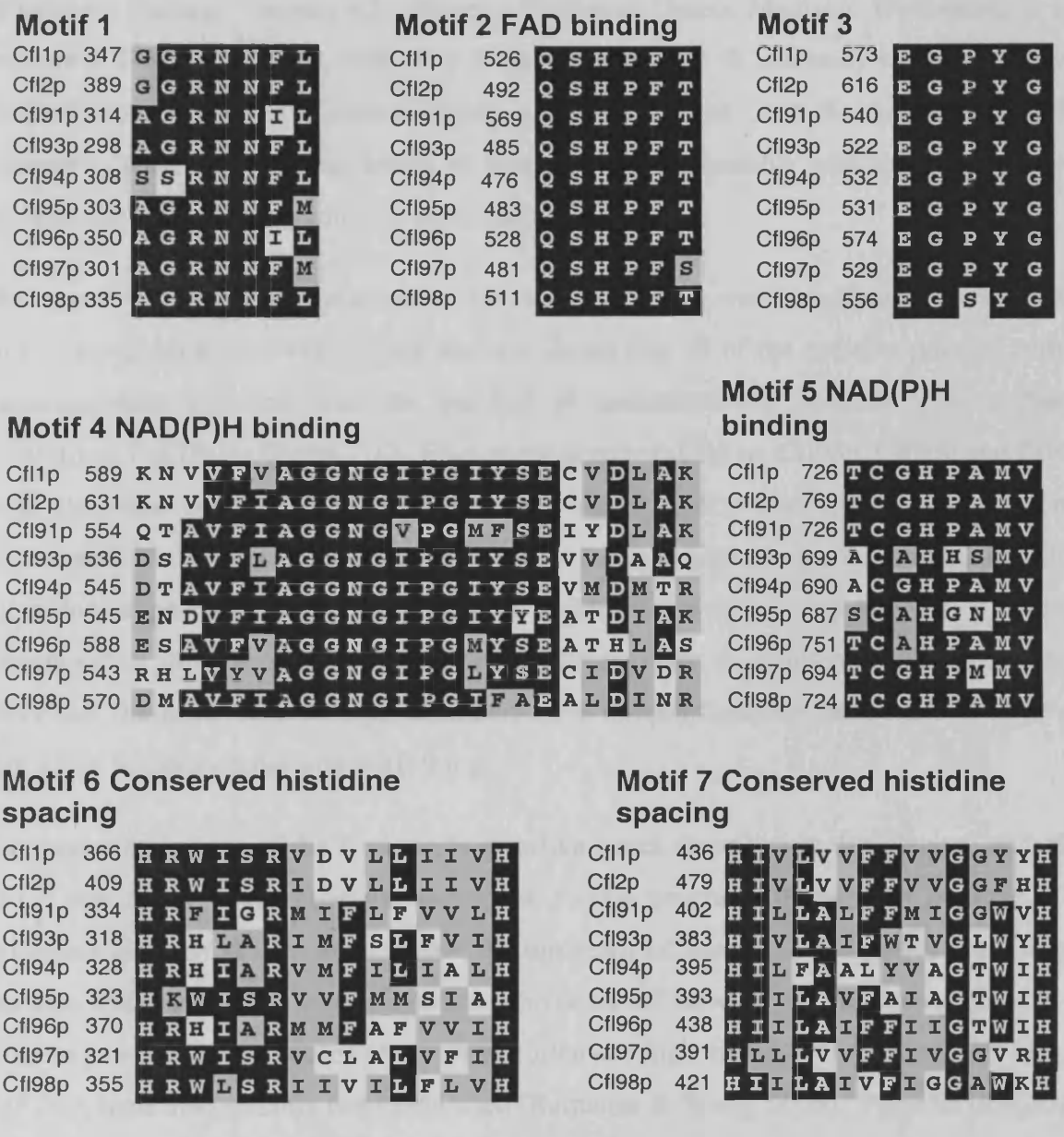
Proteins belonging to the ferric reductase family have a generalised structure (shown at the bottom of the figure). The *C. albicans* ferric reductases conform to this structure. The majority of proteins from this family have a hydrophobic signal sequence, which directs the peptide into the endoplasmic reticulum as it is synthesised. The middle section of the protein is hydrophobic and contains multiple transmembrane domains. The number of transmembrane domains can vary from protein to protein. The C-terminal region of the protein contains conserved motifs, some of which are thought to be important for FAD and NAD(P)H binding (see Fig 7.4 for alignments of these motifs).

Table 7.1 Protein length and number of transmembrane domains of the *C. albicans* ferric reductase like genes.

Fre2p, a *S. cerevisiae* ferric reductase and Cfl1p are included for comparison

Ferric reductase-like protein	Protein length (amino acids)	Number of predicted transmembrane regions (PSORT prediction)
Fre2p (*S. cerevisiae*)	711	7
Cfl1p (*C. albicans*)	760	6
Cfl91p	751	5
Cfl93p	730	7
Cfl94p	710	5
Cfl95p	706	4
Cfl96p	739	6
Cfl97p	727	6
Cfl98p	746	8

Figure 7.4 Motifs conserved between the *C. albicans* ferric reductase-like proteins



The predicted amino acid sequences the of the 9 *C. albicans* ferric reductase-like genes were aligned using the PILEUPprogram from the GCG package and examined for conserved motifs. Three motifs thought to be involved in FAD and NAD(P)H were identified as well as two other motifs of unknown function. Two sets of motifs containing conserved histidine residues, which are thought to be important for haem binding were also found.

ferric reductase proteins show a high degree of sequence similarity between each other. The highest level of sequence similarity, as assessed by the GAP program of the GCG package (Wisconsin Package Version 9.1, Genetics Computer Group, Madison, Wisconsin), is seen between Cfl93p and Cfl94p, with 50.6 % identity and 61.2 % similarity whereas the lowest level of identity seen is between Cfl95p and Cfl97p, with 36.3 % identity and 45.7 % similarity (Table 7.2). These levels of similarity are comparable with the levels observed between the *S. cerevisiae* family of ferric reductase proteins.

The predicted protein sequences of these genes were analysed using PSORT (http://psort.nibb.ac.jp:8800/). This analysis shows that all of the proteins possess multiple transmembrane domains, with the number of transmembrane domains varying from 4 (Cfl95p) to 7 (Cfl93p) (Table 7.1). Four of the proteins (Cfl91p, Cfl95p, Cfl96p and Cfl97p) have cleavable N-terminal signal sequences, whilst Cfl93p and Cfl98p appear to have uncleavable N-terminal signal sequences. No N-terminal signal sequence is found in Cfl94p, but it does appear to posses a mitochondrial targeting sequence, suggesting that it may be a mitochondrial protein (http://psort.nibb.ac.jp:8800/). This is of interest since it is thought likely that the ferric reductase proteins may have different cellular locations and some may play a role in intracellular iron trafficking.

The promoter regions of the ferric reductase-like genes described in this chapter, as well as *CFL1* and *CFL2*, were analysed using the FASTA program of the GCG package. The promoters of each of the genes (1000 bp upstream of the ATG start sites) were aligned pairwise with each other. No similarities were observed between the promoter regions of any of these genes. The sequences of the two *C. albicans* high affinity transporters, *CaFTR1* and *CaFTR2*, have also recently been published (Ramanan & Wang, 2000). Pairwise comparison of the promoters of these genes, likewise, did not reveal any similarities. The promoters were also analysed for the presence of Mac1p- and Aft1p-like binding sites, but none were found. Mac1p is the *S. cerevisiae* transcription factor responsible for copper responsive gene regulation, whilst Aft1p is responsible for iron responsive gene regulation. Putative Mac1p binding sites have been found in the promoter of a *C. albicans* copper transporter gene, which has recently been identified in our laboratory.

Table 7.2 Similarities between Cfl proteins

Alignments were performed using the GAP program of the GCG package. Figures in italics indicate % identities, whilst the other figures indicate % similarities.

	CFL91p	Cfl93p	Cfl94p	Cfl95p	Cfl96p	Cfl97p	Cfl98p	Cfl1p	Cfl2p	Fre2p
Cfl91p		*36.3*	*42.2*	*37.0*	*47.3*	*35.3*	*39.3*	*40.7*	*39.3*	*29.9*
Cfl93p	50.1		*50.6*	*36.5*	*40.7*	*35.3*	*42.2*	*38.8*	*40.5*	*29.5*
Cfl94p	53.8	61.2		*37.1*	*43.3*	*37.5*	*42.7*	*41.8*	*42.6*	*29.1*
Cfl95p	47.2	46.9	46.9		*36.7*	*36.3*	*36.5*	*37.1*	*38.4*	*29.5*
Cfl96p	59.1	51.0	54.7	46.9		*37.9*	*36.9*	*38.0*	*38.9*	*29.5*
Cfl97p	46.8	46.8	48.9	45.7	48.4		*35.6*	*46.5*	*47.9*	*30.9*
Cfl98p	49.9	52.5	53.7	45.9	49.3	48.9		*35.4*	*38.4*	*30.0*
Cfl1p	50.6	49.2	51.8	48.4	49.2	57.2	47.6		*78.3*	*31.1*
Cfl2p	49.0	52.4	52.9	49.6	50.4	57.5	50.2	83.3		*30.6*
Fre2p	40.0	39.3	40.5	41.2	39.9	41.3	42.6	41.5	40.8	

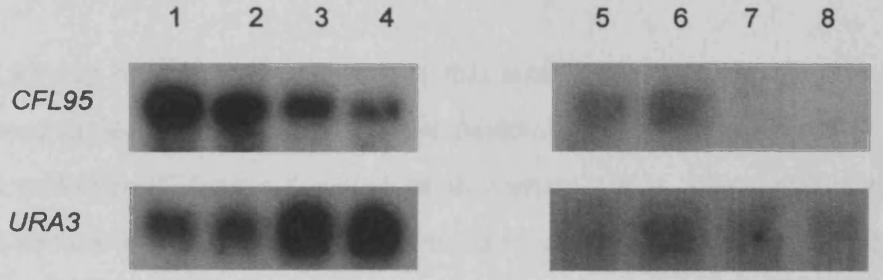
7.3 Northern blot analysis of *CFL*-like genes in *Candida albicans*

Northern blot analysis was used to investigate the expression patterns of the *CFL* genes described in this chapter. Cultures were grown in both high and low iron conditions and high and low copper conditions to investigate the possibility that the expression of these genes might be regulated in response to iron and/or copper, as was found to be the case for *CFL1* (see Chapter 5). RNA was prepared from *C. albicans* S/01 cultures grown in MD medium containing high and low iron and high and low copper concentrations. RNA samples were analysed for *CFL91*, *CFL93*, *CFL94*, *CFL95*, *CFL96*, and *CFL97* using the PCR products covering regions of the open-reading frames of these genes as described in section 7.2. Samples were also probed with the housekeeping gene *CaURA3* as a loading control. A transcript of approximately 2.4 kb was detected using the *CFL95* probe, which was negatively regulated in response to iron and copper levels in the media (Fig 7.5). This transcript was detected after overnight exposure to X-ray film, suggesting that *CFL95* is expressed at high levels under the conditions described here. This is in contrast to *CFL1* where four weeks exposure was required to detect the transcript (see Chapter 5) and might suggest that *CFL95* encodes the main ferric reductase under these conditions and may therefore explain why no phenotype was observed for the *cfl1* homozygous strain. Under the conditions used in this study, no transcripts were detected for any of the other genes although the *URA3* loading control was observed consistently after an overnight exposure. This suggests that these genes are either expressed at a low level which is not detected by Northern blotting or that they are not expressed under the conditions investigated during this study.

7.4 Discussion

Analysis of the *C. albicans* genomic database identified seven novel ferric reductase-like genes. It was shown that their predicted protein products all conformed to the generalised structure of the ferric reductase family of proteins. They possessed the conserved motifs implicated in FAD and NAD(P)H binding, as well as the four histidine residues thought to be involved in haem binding (Finegold *et al.*, 1996; Karplus *et al.*, 1991) and multiple transmembrane domains.

Figure 7.5 Northern blots of *CFL95* in high and low iron conditions and high and low copper conditions



Total RNA was extracted from cultures *C. albicans* grown in either MD-BPS (100 μM) containing 0 μM $FeCl_3$ (lane 1), or MD-BPS (50 μM) containing 0 μM (lane 2), 50 μM (lane 3) or 100μM (lane 4) $FeCl_3$ or from cultures grown in MD-BCS (100 μM) containing 0 μM $CuCl_2$ (lane 5), or MD-BCS (50 μM) containing 0 μM (lane 6), 50 μM (lane 7) or 100 μM (lane 8) $CuCl_2$. Following electrophoresis and transfer to a nylon membrane duplicate sets of the four RNA samples were probed with either a ^{32}-P-labelled *CFL95* PCR product or with the *C. albicans URA3* gene as a loading control.

It is interesting that both *S. cerevisiae* and *C. albicans* possess multiple ferric reductase genes. *S. cerevisiae* possesses 7 ferric reductases, whilst *C. albicans* possesses at least 9 ferric reductases, and it is possible that still more may be identified during the course of the sequencing project. As was discussed in Chapter 1 (Section1.5.1), it is thought likely that some of the *S. cerevisiae* ferric reductase genes encode intracellular reductases that play a role in intracellular iron trafficking, and it is possible that the same is true in *C. albicans*. Interestingly, one of the *C. albicans* ferric reductase-like proteins discussed in this chapter possesses a mitochondrial targeting sequence and is predicted by PSORT to be located in the mitochondrial membrane.

Only *CFL95* was found to be expressed in this study. This suggests that the other genes may be expressed at low levels which are not detectable by Northern blotting or that they are expressed under conditions not tested in this study. It is also possible that they are not expressed and are pseudogenes. As discussed in Chapter 5 (Section 5.5), the *SAP* (secreted aspartyl proteinase) family of genes in *C. albicans* are expressed under different conditions, and even those which are expressed under a limited set of culture conditions are important for the disease causing process (Hube *et al.*, 1994; Schaller *et al.*, 1998). It is therefore possible that although no transcript was detected for 5 of the *CFL*-like genes under culture conditions, they may still play a role in infection.

From the promoter alignments carried out it was not possible to identify putative transcription factor binding sites in the promoters of these genes; nor were any sequences similar to the Aft1p and Mac1p binding sites found in *S. cerevisiae* identified. It is possible that any potential binding sites may not be conserved enough to be identified by this method. It is interesting that none of the promoters contain Mac1p-like binding sites, since these consensus sequences have been found in the promoter of a *C. albicans* copper transporter gene that has recently been identified in our lab (M. Marvin, personal communication). It is possible that another transcription factor exists in *C. albicans*, which is capable of mediating copper responsive gene regulation, or that Mac1p-like binding sites in *C. albicans* are not so highly conserved as seems to be the case in *S. cerevisiae*.

Chapter 8
General discussion

The work presented here has identified two ferric reductase genes through a functional complementation approach. One of these genes, *CFL1*, had previously been isolated but no ferric reductase activity had been shown to be associated with it (Yamada Okabe *et al.*, 1996). Our sequence differed from the previously identified sequence at the 5' end and it seems likely that the originally published sequence arose from the joining of two non-contiguous fragments of *C. albicans* genomic DNA. The second gene, *CFL2*, had not previously been described. Seven other *C. albicans* ferric reductase-like genes were identified through the analysis of the *Candida albicans* genome sequencing project web pages (http://alces.med.umn. edu/Candida.html). All of these genes encode putative proteins containing motifs found in all other known ferric reductases. The presence of multiple ferric reductase-like genes in *C. albicans* suggests that this organism uses a similar iron acquisition mechanism to that found in *S. cerevisiae*. The expression of *CFL1* and one of the genes identified through database analysis, *CFL95*, have been shown to be negatively regulated by both iron and copper in *C. albicans*. A *C. albicans* strain in which both copies of *CFL1* were deleted was constructed and analysed for loss of ferric reductase activity and ability to grow in low iron conditions. No difference was observed between the mutant and wild type.

The predicted amino acid sequence of all of the 9 ferric reductase genes discussed in this study show sequence similarity to known ferric reductase proteins. In particular, all predicted protein sequences show high conservation of domains implicated in FAD and NAD(P)H binding. These domains are also found to be conserved in the wider family of FNR proteins (Karplus *et al.*, 1991). Other domains with no known function are also found to be conserved. Significantly, the ordering of the conserved domains, which is identical in all previously described ferric reductases from *S. cerevisiae* and *S. pombe*, is also found to be the same in the *C. albicans* ferric reductase proteins. The conservation of the positioning of four histidine residues, which have been shown to be essential for haem binding in Fre1p (Finegold *et al.*, 1996) is also interesting and indicates that these proteins may be haem-dependent.

The presence of multiple ferric reductase-like genes in *C. albicans* is not surprising since *S. cerevisiae* is known to have 7 ferric reductase genes, all of which are expressed and are regulated in response to either iron and/or copper. Recent evidence suggests that some of the

S. cerevisiae ferric reductase proteins may have specific roles in releasing iron from siderophores at the cell surface (Yun & Philpott, 2000), but it also seems likely that some of these genes encode intracellular reductases which play a role in intracellular iron trafficking. Both of these possibilities may also apply to the *C. albicans* ferric reductase-like genes. Indeed, the *C. albicans* ferric reductase-like gene Cfl94p, is predicted to have a mitochondrial location, suggesting that it may play a role in transporting iron in or out of the mitochondria. None of the *S. cerevisiae* ferric reductases have mitochondrial targeting sequences, however, Lesuisse and co-workers (1990) have reported that isolated mitochondria do possess ferric reductase activity. It is also interesting to note that although no ferric reductase activity has been reported to be associated with the vacuole, a ferrous transporter complex consisting of two proteins encoded by *FET5* and *FTH1* has been associated with this organelle in *S. cerevisiae* (Urbanowski & Piper, 1999). This appears to be responsible for moving iron out of the vacuole. It is, therefore, possible that there is also an associated ferric reductase which plays a role in mobilising stored iron from the vacuole in *S. cerevisiae* and a similar scenario may be found in *C. albicans*.

Although the work presented here has not shown any ferric reductase activity to be associated with any of the 9 ferric reductase genes in *C. albicans*, *CFL1* and *CFL2*, were identified through their ability to restore ferric reductase activity to the *S. cerevisiae fre1* mutant. This indicates that *CFL1* and *CFL2* both encode functional ferric reductase proteins. Northern blot analysis of all 9 ferric reductase genes showed that both *CFL1* and *CFL95* are expressed in *C. albicans* and are negatively regulated by both iron and copper, although no transcript was found for *CFL2* and its role remains unclear. The expression patterns of *CFL1* and *CFL95* show interesting parallels with the *FRE1* gene of *S. cerevisiae*. The *S. cerevisiae FRE1* gene encodes a structural component of the cell surface ferric reductase of this organism and is the only ferric reductase gene to be regulated by both iron and copper. The other 6 *S. cerevisiae* ferric reductase genes are regulated by either iron or copper: *FRE2-6* are negatively regulated by iron and *FRE7* is negatively regulated by copper (Martins *et al.*, 1998).

The fact that there are multiple ferric reductase-like genes in *C. albicans* and that iron and copper regulate the expression of at least two of them suggests that *C. albicans* may use a similar mechanism to *S. cerevisiae* to acquire iron. This supports the previous biochemical evidence from our laboratory showing that *C. albicans* possesses a cell surface ferric reductase activity which is negatively regulated in response to iron and copper (Morrissey *et*

al., 1996). Other work in our laboratory has identified a *C. albicans CTR1*-like gene, giving further evidence that the iron and copper uptake mechanisms in *C. albicans* are similar to the *S. cerevisiae* mechanisms. Furthermore, a recent publication has reported the isolation of two ferrous transport-like genes (*CaFTR1* and *CaFTR2*) both of which are regulated in response to iron (Ramanan & Wang, 2000). A deletion mutant of *CaFTR1* was shown to have reduced virulence in the mouse systemic model of candidiasis (Ramanan & Wang, 2000). This suggests that the reductive mechanism of iron uptake is used when colonising host tissues and plays a crucial role in the virulence of this organism.

CFL1 and *CFL95* do not possess any motifs in their promoter regions that show any similarity to the Aft1p and Mac1p binding sites found in the promoters of *S. cerevisiae* ferric reductase genes. Since Aft1p and Mac1p respectively mediate the iron and copper responsive regulation of the *S. cerevisiae* ferric reductase genes, it would seem unlikely the iron and copper responsive transcription factors present in *C. albicans* are closely related to those of *S. cerevisiae*. However, a *C. albicans* copper transport gene, *CaCTR1*, has been identified in our laboratory, and this gene possesses several Mac1p-like binding sites in its promoter (M. Marvin, personal communication). This presents the intriguing possibility that *C. albicans* may possess more than one transcription factor mediating copper responsive gene regulation. Alternatively, the binding site may be less well conserved in *C. albicans* and for this reason may not be readily identifiable. No similarities were detected between the promoters of *CFL1* and *CFL95* or any of the other ferric reductase-like genes, and no putative transcription factor binding sites could be identified. It is, however, notoriously difficult to identify transcription factor binding sites through computer analysis. Currently, the best method of identifying putative binding sites is experimentally by synthesising nested deletions of promoters.

No transcripts were detected for any of the other 7 ferric reductase-like genes. This may indicate that they are expressed at a low level or may be expressed under different conditions. The members of other *C. albicans* gene families have been shown to be expressed under different conditions. For example, the secreted aspartyl proteinase family has 9 members, of which one, *SAP2*, is expressed at high levels under culture conditions. *SAP4-6* are found to be expressed only during hyphal growth, but this is highly strain specific, and sometimes no transcript is detected at all (Hube *et al.*, 1994). However, these genes are expressed *in vivo* during the course of experimental infections (Schaller *et al.*, 1998). A similar situation may exist for the ferric reductase genes and some may be hyphal specific and may be important

for iron acquisition during infection. Alternatively, different ferric reductases may have different specificities, which are suitable for different environments or iron chelators. It has recently been suggested that this may be one reason for the large family of ferric reductases present in *S. cerevisiae*, and it has been shown that a *fre3* mutant grows less well on hydroxamate siderophores, suggesting that Fre3p is responsible for releasing iron from this class of siderophores (Yun & Philpott, 2000). Therefore, it may be that the *S. cerevisiae* ferric reductases are more specific than has previously been postulated, and a similar situation may exist in the case of the *C. albicans* ferric reductase genes. An interesting example of differential expression of iron uptake genes is found in the bacterial pathogen, *Pseudomonas aeroginosa*, which produces two siderophores, pyoverdin and pyochelin. The siderophore receptors for these siderophores are differentially expressed, and expression is stimulated by the presence of the ferric-siderophore (Gensberg *et al.*, 1992). Precedent therefore exists for the differential expression of iron uptake genes under different environmental conditions. If the *C. albicans* ferric reductase genes are indeed expressed under different conditions, they may respond to different transcription factors, and this may explain why no similarities were found between the promoters of *CFL1* and *CFL95*, and also why no Mac1p-like binding sites were found in the promoters of these genes.

Two ferric reductase genes were identified through the functional complementation approach, whilst subsequent work established that *C. albicans* possesses at least 9 ferric reductase-like genes. The functional complementation approach to gene isolation from *C. albicans* relies on the gene of interest being transcribed heterologously in *S. cerevisiae*. This requires either a cDNA library in which a promoter is present in the vector or a genomic library in which the gene of interest retains its own promoter which is responsible for its transcription. The use of a genomic library may lead to genes possessing partial promoters which have lost functionality, or promoters which are not functional in the heterologous host. In addition, proteins may be misdirected and their potential rescuing activity may not be seen for this reason. Therefore, it may not be possible to isolate all potential complementing genes by this method. An alternative possible reason for not having identified more ferric reductase genes by this method is that the library may not have been completely representative. As was shown in Chapter 4, 5 rescuing clones were initially identified, of which 3 were identical and carried the *CFL2* gene. The final two clones were identical to each other except that one contained approximately 700 bp extra sequence at one end. The fact that one clone was identified three times and that the two clones carrying the *CFL1* gene were virtually identical

suggests that the library may have been over-representative of these clones, with the consequent presumed depletion of others. It is therefore possible that other ferric reductase-like genes were not identified during this screen since they were not present in the original library.

The phenotype of the *C. albicans cfl1* mutant suggests that *CFL1* may not play a direct role in iron acquisition. This brings into question the cellular location of Cfl1p and it would therefore be interesting to establish where Cfl1p is located in the cell. Although it is predicted to have a cell surface location by the program, PSORT (http://psort.nibb.ac.jp:8800/), this does not necessarily preclude an alternative cellular location, since the presence of a N-terminal hydrophobic signal sequence merely suggests that the protein is directed into the secretory pathway. A *CFL1-GST* fusion plasmid that expresses the fusion protein at high levels in *E. coli* has been constructed in our laboratory, with a view to initiating localisation studies. The purified protein would be used to raise antibodies against Cfl1p, and these antibodies could be used in immunofluorescence studies to localise Cfl1p.

Since it is possible that *CFL95* may encode an important component of the ferric reductase system it would be interesting to construct a *cfl95* disruption mutant to ascertain the effect of this mutation on iron acquisition and virulence. This work has been initiated in our laboratory. Strains carrying deletions of the other ferric reductase genes might lead to insights into their role in iron acquisition and therefore would be interesting to construct. It is of importance to determine the effects of all of the ferric reductase genes on virulence. The effects of ferric reductase gene deletions could be tested in animal models such as mice or guinea pigs and it might be interesting to compare the effects of ferric reductase gene deletions between systemic and mucosal infection since different iron acquisition mechanisms may be important for the two types of infection. An alternative method of estimating the role of the ferric reductase genes in infection would be to use RT-PCR to find if any transcript is detected under these conditions. This would have the advantage of not needing to knockout genes to carry out the experiments.

Since putative regulatory regions in the promoters of *CFL1* and *CFL95* could not be identified through searching for conserved blocks of sequence, an alternative approach to this problem would be to construct nested deletions in the promoter regions of these genes. Regulatory regions could then be identified through comparison of promoters which retained regulatory activity and those which had lost it. This method would identify regions of the promoter necessary for iron and copper responsive regulation. Comparison of these

sequences might allow a consensus sequence to be defined, and with the sequencing of the *C. albicans* genome nearly complete, this would allow the identification of other genes containing similar motifs in their promoters. It would then be possible to test the hypothesis that virulence genes unconnected with iron acquisition may be regulated by iron. The identification of regulatory sequences would also allow regulatory proteins to be identified through affinity binding studies.

All evidence available to date suggests that *C. albicans* possesses a reductive iron acquisition mechanism similar to that found in *S. cerevisiae*. Nine ferric reductase-like genes have been identified during the course of this work, two of which are negatively regulated by iron and copper. Recently published work (Ramanan & Wang, 2000) has reported the isolation of two *C. albicans* ferrous transporter genes, one of which was shown to be essential for the virulence of *C. albicans* in the systemic infection of mice. This suggests that the reductive iron acquisition mechanism may be a crucial virulence determinant. The future directions that this work might take will lead to fascinating insights into the iron acquisition and virulence mechanisms of *C. albicans*.

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