

Reductive iron uptake by *Candida albicans*: role of copper, iron and the *TUP1* regulator

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High-affinity iron uptake by a ferrous permease in the opportunistic pathogen *Candida albicans* is required for virulence. Here this iron uptake system has been characterized by investigating three distinct activities: an externally directed surface ferric reductase, a membrane-associated PPD (*p*-phenylenediamine) oxidase and a cellular ferrous iron transport activity. Copper was required for the PPD oxidase and ferrous transport activities. In contrast, copper was not required for iron uptake from siderophores. Addition of iron to the growth medium repressed ferric reductase and ferrous transport, indicating homeostatic regulation. To identify the genes involved, orthologous mutants of *Saccharomyces cerevisiae* were transformed with a genomic library of *C. albicans*. *CFL95*, a gene with sequence similarity to ferric reductases, restored reductase activity to the orthologous *S. cerevisiae* mutant. *CaFTR2* and *CaFTR1*, genes with homology to ferrous permeases, conferred ferrous transport activity to the orthologous *S. cerevisiae* mutant. However, neither a genomic library nor *CaFET99*, a multicopper oxidase homologue and candidate gene for the PPD oxidase, complemented the *S. cerevisiae* mutant, possibly because of problems with targeting or assembly. Transcripts for *CFL95*, *CaFTR1* and *CaFET99* were strongly repressed by iron, whereas the *CaFTR2* transcript was induced by iron. Deletion of the *TUP1* regulator perturbed the homeostatic control of reductive iron uptake. Incidentally, iron starvation was noted to induce flavin production and this was misregulated in the absence of *TUP1* control. The opposite regulation of two iron permease genes and the role of *TUP1* indicate that the process of iron acquisition by *C. albicans* may be more complex and potentially more adaptable than by *S. cerevisiae*.

Keywords: iron regulation, ferric reductase, multicopper oxidase, ferrous permease, siderophores

INTRODUCTION

Candida albicans is a fungal commensal that inhabits mucosal surfaces, such as the mucosa of the gastrointestinal tract. In certain clinical situations, *C. albicans* may become virulent, invading locally and disseminating via the bloodstream (Fridkin & Jarvis, 1996). When granulocyte counts are low, such as following bone marrow transplantation, chemotherapy or in patients with AIDS, the risk of infection is extremely high and the infections are difficult to eradicate (Warnock, 1998).

Abbreviations: BCS, bathocuproinedisulfonate; BPS, bathophenanthrolinedisulfonate; CSM, complete synthetic medium; PGK, phosphoglycerol kinase; PPD, *p*-phenylenediamine.

In infected tissue, *C. albicans* proliferates in both yeast and filamentous forms. The filamentous morphology is repressed by the *TUP1* regulator (Braun & Johnson, 1997) and may play a role in invasiveness.

Virulence of many pathogens requires expression of specific iron uptake systems, perhaps to counter iron-withholding host defences that operate in different environmental niches (Ratledge & Dover, 2000). A recent report implicates a plasma membrane component of a high-affinity iron uptake system in virulence of *C. albicans* (Ramanan & Wang, 2000). Here we characterize this iron acquisition system using the related organism, *Saccharomyces cerevisiae*. In this related yeast, the initial step in iron uptake is mediated by an externally directed reductase. (Dancis *et al.*, 1990). The

genes responsible for this activity, *FRE1* and *FRE2*, encode membrane-associated b-type haem proteins (Shatwell *et al.*, 1996). The ferrous iron produced by the reductase in turn is captured and transported across the plasma membrane by a protein complex. The components of this complex are a multicopper oxidase homologous to ceruloplasmin (encoded by *FET3*) (Askwith *et al.*, 1994) and a polytopic membrane permease (encoded by *FTR1*) (Stearman *et al.*, 1996). In *S. cerevisiae*, interference with copper delivery to the oxidase abrogates high-affinity iron uptake from ferric chelates (Dancis *et al.*, 1994). Each of these components, the reductase, the oxidase and the permease are individually required for iron acquisition from ferric chelates and the entire system is homeostatically regulated in response to available iron with induction occurring in response to iron starvation (Askwith *et al.*, 1996).

Here we show that in *C. albicans* similar iron acquisition activities are present – ferric reductase, PPD (*p*-phenylenediamine) oxidase and ferrous transport. We show that, as in *S. cerevisiae*, copper is needed for the oxidase and iron transport activities. Complementation of mutants of *S. cerevisiae* was used to identify orthologous genes from *C. albicans*, and regulation of reductive iron uptake was examined by biochemical assays and Northern blotting with candidate genes for ferric reductase, oxidase and ferrous permease. Iron exposure during growth has major effects, and *TUP1*, a global regulator of morphology and metabolism, also plays a major role in iron-dependent gene regulation. A separate copper-independent system for efficient iron uptake from siderophores exists in *C. albicans* (Lesuisse *et al.*, 2002). Overall our results suggest that the complexity of the *C. albicans* reductive iron uptake system is greater than in baker's yeast.

METHODS

Strains. *S. cerevisiae* strain 499Δ1Δ2 (*MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 Δfre1::LEU2 Δfre2::HIS3*) lacking ORFs for *FRE1* and *FRE2* (Finegold *et al.*, 1996) was used for complementation cloning of *CFL95*. To clone *CaFTR2*, *S. cerevisiae* strain 42-3C1 (*MATa ura3-52 lys2-801 ade2-101 his3-Δ200 leu2-Δ1 ftr1Δ1::TRP1*), in which the entire *FTR1* ORF was replaced with *TRP1*, was used (Stearman *et al.*, 1996). *C. albicans* strains used were SC5314 (wild-type), CBS5737 (wild-type, ATCC 32032) and CAI4 (*ura3Δ::imm434/ura3Δ::imm434*) (Fonzi & Irwin, 1993). BCa02-10 (*tup1Δ::bisG/tup1Δ::bisG::p405-URA3, ura3/ura3*) was derived from CAI4 and was a gift from B. Braun and A. Johnson (Braun & Johnson, 1997).

Media and growth conditions. *S. cerevisiae* and *C. albicans* were grown in YPD supplemented with 20 mg adenine l⁻¹ (YPAD) at 30 °C. Defined growth medium for growth of *C. albicans* consisted of complete synthetic medium (CSM)-uracil (Bio101) supplemented with 100 mg uridine l⁻¹, 6.7 g yeast nitrogen base without amino acids l⁻¹ (Difco) and 2% D-glucose. Amino acids and uridine were omitted from the defined medium as required for selecting plasmids. The iron-limited defined medium contained yeast nitrogen base lacking iron and copper (Bio101). Copper sulfate and ferric ammo-

nium sulfate were added as required. The iron chelator bathophenanthrolinedisulfonate (BPS) or the copper chelator bathocuproinedisulfonate (BCS) were added as noted to limit availability of iron or copper, respectively.

PCR and plasmid construction. The ORFs of *CFL95*, *CaFTR1*, *CaFTR2* and *CaFET99* were amplified by PCR with the following oligonucleotide pairs. Genomic sequence is shown in upper case letters and flanking linkers are shown in lower case letters.

CFL95: 5'-tagagctcATGGTAGCTATCAATTCATTATTA-TTGCTGCC-3' and 5'-taggatccCTACCAGCTTTGCAT-TTGTTCAAACAATTCAACTCTG-3'

CaFTR1: 5'-tagagctcATGGTTGACGTATTTAAACGTTCA-AATTTTC-3' and 5'-taggatccTTATTTGTTTTCTTTGG-ATTCGATCAATTTG-3'

CaFTR2: 5'-tagagctcATGGTTGATGTTTTCAATGTTCA-AGTC-3' and 5'-taggatccTTATTTATTTTCTTGAGTTT-CGACTAATTTG-3'

CaFET99: 5'-tagagctcATGCGGTTTATTGTATCATCAT-TTATATTTTTATCTC-3' and 5' taggatccCTAATGT-TGTTTAGAGTTACTTCCAGAAGAAGAGG-3'

Genomic DNA isolated from *C. albicans* SC5314 was used as the template and a combination of polymerases, Pfu-Turbo (Stratagene) and *Taq* (Promega) in a 4:1 ratio was added. The PCR products were cloned into pCRII-TOPO. The plasmids generated were termed pTOPO-CFL95, pTOPO-CaFTR1, pTOPO-CaFTR2 and pTOPO-CaFET99. The DNA sequences were compared to sequences of clones isolated from the genomic library, p1A2 (*CFL95*) and pBS1996 (*CaFTR2*), and to Contig 6 of the *C. albicans* genome from the Stanford DNA Sequencing and Technology Center (<http://www-sequence.stanford.edu/group/candida>). The ORFs isolated from the pCRII-TOPO vectors by digestion with *Sst*I and *Bam*HI were ligated into the corresponding sites of the *S. cerevisiae* expression vector YIpDCE1 (Stearman *et al.*, 1998), yielding plasmids pDC-CFL95, pDC-CaFTR1, pDC-CaFTR2 and pDC-CaFET99. Two CUG codons in the coding region of *CaFET99* in pDC-CaFET99 were converted by site-directed mutagenesis (Quick Change; Stratagene) to AGC and TCT, respectively, creating pDC-CaFET99-Ser. For co-expression of *CaFET99* with *CaFTR1*, the ORF for *CaFTR1* was subcloned from pTOPO-CaFTR1 into the second multiple cloning linker of pDC-CaFET99-Ser between *Aval* and *Avr*II. For co-expression of *CaFTR2* with *CaFET99*, the ORF for *CaFTR2* was inserted into the second multiple cloning site of pDC-CaFET99-Ser between *Avr*II and *Xho*I.

Transformation of *S. cerevisiae* with a *C. albicans* genomic DNA library. The *C. albicans* genomic library for complementation of *S. cerevisiae* was a gift from Gerald Fink, Whitehead Institute (Liu *et al.*, 1994). *S. cerevisiae* was transformed with the library using electroporation (Becker & Guarente, 1991). For transformation of individual plasmids, the lithium acetate method was used (Agatep *et al.*, 1998). p1A2 containing *CFL95* was cloned by selecting *S. cerevisiae* 499Δ1Δ2 transformants on specially designed medium. The medium included (l⁻¹): 6.7 g yeast nitrogen base without iron or copper (Bio101), 0.8 g CSM-ura (Bio101), 20 g D-glucose, 25 mM MES, pH 6.1 (Sigma), 15 g agarose (Fluka) and 1 mM ferrozine (Fluka). Strain 499Δ1Δ2 does not grow on this medium. Selected transformants were assayed for ferric reductase activity (see below). pBS1996 containing *CaFTR2* was cloned by selecting strain 42-3C1 (*Δftr1*) transformants on low-iron medium (Stearman *et al.*, 1996). The *Δftr1* *S. cerevisiae* strain grows slowly on this medium. Plasmids were

rescued from more rapidly growing transformants and retransformed into *E. coli* strain DH5 α for large-scale plasmid preparations.

Ferric reductase, high-affinity ferrous uptake, ferrichrome uptake and PPD oxidase assays. The assays for ferric reductase have been described previously (Dancis *et al.*, 1990) and rely on the formation of a coloured BPS-Fe(II) complex. High-affinity ferrous iron uptake was measured as described previously (Dancis *et al.*, 1994). Equal volumes of ferrichrome and ferric chloride (260 μ M each in water) were incubated for 5 min at room temperature after which 1 M Tris/HCl buffer, pH 7.4, was added to give a final iron-siderophore concentration of 100 μ M. The final concentration of 55 Fe-labelled siderophore used for iron uptake assays was 1 μ M. Ferric reductase and ferrous uptake activities were expressed per mg total cellular protein in reporting experiments involving filamentous cells. Protein levels in yeast or filamentous forms of *C. albicans* were determined by solubilizing in Y-PER reagent (Pierce) and quantifying the soluble protein (BCA; Pierce).

To assay multicopper oxidase activity, a cell membrane fraction was prepared from exponentially grown cells as described by Yuan *et al.* (1995). Oxidase activity was detected using a gel-based assay (Yuan *et al.*, 1995) or liquid assay (Spizzo *et al.*, 1997).

RNA analysis. Cells or hyphae from 100 ml cultures were washed in buffer (50 mM citrate, pH 6.6, 5% dextrose) and total RNA was isolated by extraction with hot acidic phenol. After separation of 30 μ g aliquots on formaldehyde gels, RNAs were transferred to nitrocellulose membranes (Schleicher & Schuell) by capillary blotting. Probes for *CaFTR1*, *CaFTR2*, *CFL1*, *CaFET3*, *CaFET99* were generated using primer pairs to amplify approximately 100 bp of region immediately 3' of the stop codon for each gene. We selected the non-coding regions because these provided unique probes for each of these genes. Genomic DNA from SC5314 was used as template for the PCR reactions and the products were isolated from polyacrylamide gels. The *CFL95* probe was a 1.1 kb *HindIII*-*BglII* fragment isolated from plasmid p1A2. The *ACT1* probe was a 1.1 kb fragment containing the *ACT1* ORF of *S. cerevisiae*. All probes were labelled with 32 P radionuclide by random priming (Gibco-BRL). Prehybridization and hybridization were carried out in 50% formamide at 42 $^{\circ}$ C. Blots were washed with 0.2 \times SSC/0.1% SDS at room temperature followed by washes at 42 $^{\circ}$ C. PhosphorImage screens were exposed for 72 h with the exception of the *CaFET99* blot which was exposed for 6 d. Images obtained from the PhosphorImager (Storm 860; Molecular Dynamics) were adjusted for contrast using the levels tool in Adobe Photoshop.

Flavin analysis. Supernatants from stationary-phase *C. albicans* cultures were obtained by centrifugation. Spectra for each supernatant were obtained between 550 and 300 nm using an Olis modified Cary 14 spectrophotometer. Flavins were quantified at the absorption maximum of 446 nm against a calibration curve constructed from a riboflavin standard.

RESULTS

Cell-surface ferric reductase activity

Cell-surface ferric reductase activities of two reference strains of *C. albicans*, SC5314 and CBS5736, were evaluated. These strains originated as clinical isolates and SC5314 has been widely used in biochemical, genetic

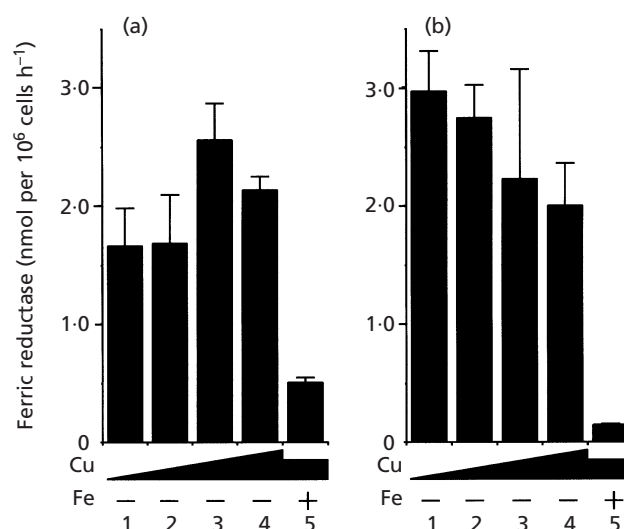


Fig. 1. Regulation of *C. albicans* cell-surface ferric reductase activity. Wild-type *C. albicans* strains SC5314 (a) and CBS5736 (b) were grown at 30 $^{\circ}$ C, in defined medium with different concentrations of copper or iron. The copper chelator BCS was added at 1 mM (bar 1) or 0.1 mM (bar 2). No addition (bar 3) or 10 μ M copper sulfate (bar 4) was added; finally 10 μ M ferric ammonium sulfate was added (bar 5). Cells were grown to exponential-growth phase, washed with 50 mM citrate buffer, pH 6.6, with 5% D-glucose and resuspended in the same. Cell-surface ferric reductase activity of the cell suspension was assayed. Results are presented as mean values \pm SD for triplicate determinations.

and genomic analyses (Fonzi & Irwin, 1993). After growth in iron-depleted medium, both strains showed equivalent levels of induced surface ferric reductase activity (Fig. 1a, b). Copper deprivation or copper loading during growth had no effect on ferric reductase activity. However, the addition of 10 μ M ferric ammonium sulfate decreased ferric reductase activity to 10–20% of the induced levels.

Copper-dependent high-affinity ferrous iron uptake and copper-independent ferrichrome uptake

Cells from the same cultures analysed for ferric reductase activity were examined for high-affinity ferrous uptake. Under iron-limiting and copper-replete conditions, ferrous iron uptake activity was 10-fold higher than that observed for *S. cerevisiae* (see Fig. 2a, b, bar 3 and data not shown). The effect of copper depletion on high-affinity ferrous iron uptake was examined by growing cells in defined medium containing the copper chelator BCS. The cells were washed free of chelator and ferrous iron uptake was measured (Fig. 2a, b, bars 1 and 2). High-affinity ferrous uptake was abrogated by cellular copper depletion, indicating the presence of a copper-dependent step in iron uptake. The addition of 10 μ M iron to copper-replete growth medium decreased high-affinity iron uptake to 10–15% of the induced level (Fig. 2a, b, bar 5). By contrast, iron uptake in SC5314 from the siderophore ferrichrome was not affected by

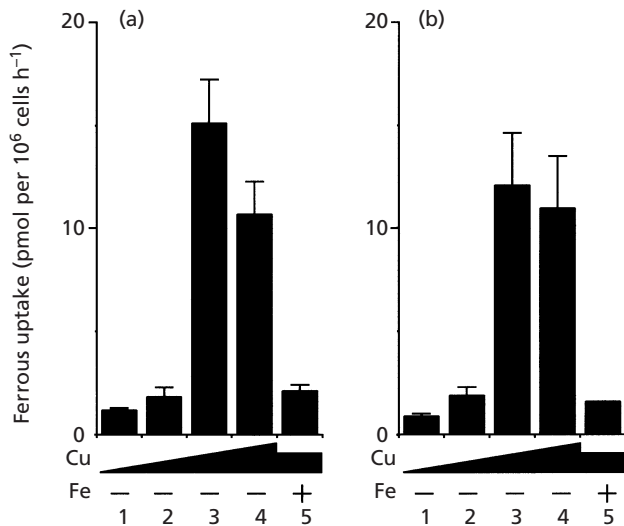


Fig. 2. Regulation of *C. albicans* ferrous iron uptake. Wild-type *C. albicans* strains SC5314 (a) and CBS5736 (b) were grown as described in the legend to Fig. 1 and cellular ⁵⁵Fe ferrous uptake was measured. Results are presented as mean values \pm SD for triplicate determinations.

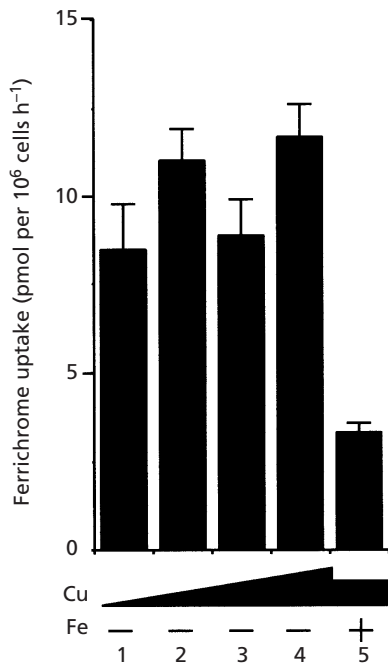


Fig. 3. Regulation of *C. albicans* ferrichrome iron uptake. Wild-type *C. albicans* strain SC5314 was grown as described in the legend to Fig. 1. The cell suspensions were assayed for cellular ⁵⁵Fe iron uptake from ferrichrome. Results are presented as mean values \pm SD for triplicate determinations.

copper deprivation (Fig. 3, bars 1 and 2). The addition of iron to the growth medium decreased iron acquisition from ferrichrome to 30–35% of the induced value (Fig. 3, bar 5). In summary, both ferrous iron and siderophore iron transport were homeostatically regulated by iron

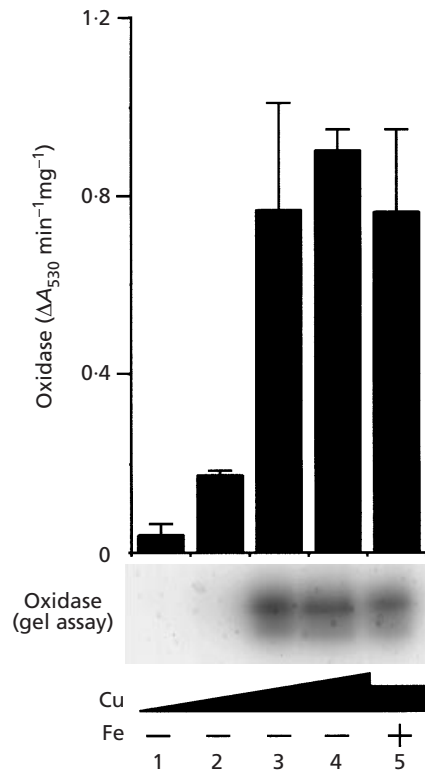


Fig. 4. *C. albicans* possesses copper-dependent oxidase activity. Wild-type *C. albicans* strain SC5314 was grown as described in the legend to Fig. 1. Membrane fractions were prepared and assayed for PPD oxidase activity. The mean values of two independent spectrophotometric assays are presented in the column graph and the error bars indicate the higher value. The activity gel assay for PPD oxidase activity of one set of samples is shown below the graph.

exposure during growth. Pathways for iron acquisition were distinguished by the copper requirement for ferrous, but not for siderophore iron transport.

Copper-dependent PPD oxidase activity

S. cerevisiae contains a plasma membrane multicopper oxidase, encoded by *FET3*, that is essential for high-affinity ferrous iron transport into cells (Askwith *et al.*, 1994). Activity of this oxidase, which can be assayed using PPD as an artificial substrate, is dependent on copper and delivery of copper into the secretory pathway (Yuan *et al.*, 1995). As noted above, we observed copper dependence of ferrous transport for *C. albicans*. We also investigated whether the PPD oxidase was copper-dependent. Cells were grown under varying metal conditions and membrane fractions were isolated and solubilized in Triton X-100. PPD oxidase activity was evaluated by gel electrophoresis and activity staining or by liquid phase assay. Oxidase activity was observed only in the cultures containing copper. Copper starvation produced by addition of copper chelator virtually abolished oxidase activity (Fig. 4). The parallel requirement of copper for oxidase activity and ferrous uptake suggests that a multicopper oxidase may be

involved in a reductive pathway of iron uptake in *C. albicans*, analogous to *S. cerevisiae*. Addition of iron had little effect on total membrane oxidase activity in contrast to the repressing effect on cellular iron uptake. However, the *C. albicans* genome contains five homologues of the *S. cerevisiae* *FET3* gene (see below). Therefore, the lack of iron regulation of oxidase activity in the biochemical assays may reflect lack of iron regulation of one or more of these homologous genes.

Cloning of *CFL95* by complementation of a ferric reductase mutant of *S. cerevisiae*

To identify genes involved in iron uptake in *C. albicans*, we sought to complement mutants of *S. cerevisiae* with a *C. albicans* genomic library, a strategy that has been utilized by others (Gillum *et al.*, 1984; Kohler & Fink, 1996; Prasad *et al.*, 1995). *C. albicans* is an obligate diploid, making identification of mutant phenotypes more difficult, whereas *S. cerevisiae*, a closely related yeast, can be maintained as a haploid. Many genes of *C. albicans* function in *S. cerevisiae* and mutants of the latter with clearly defined phenotypes exist for ferric reductase, multicopper oxidase and ferrous permease. A strain of *S. cerevisiae* lacking cell-surface ferric reductase activity was transformed with a genomic library from *C. albicans*. Five transformants were identified with enhanced growth on iron-chelated plates and recovery of reductase activity. Although the *C. albicans* genome contains multiple homologues with resemblance to ferric reductases, sequence analysis of the complementing clones revealed that they shared a single ORF of 2.1 kb. The corresponding gene, referred to as *CFL95* (*Candida* ferric reductase-like) by the *C. albicans* genomic sequencing project, exhibited some homology to the *S. cerevisiae* *FRE1* and *FRE2* ferric reductase genes. The level of amino acid identity was 25 and 28% with *FRE1* and *FRE2* proteins, respectively, and was confined to motifs predicted to mediate cofactor interactions. Histidines implicated in haem coordination at residues 309, 324, 379 and 393 were present, as was the HPFT sequence at 471–473 for FAD-isoalloxazine binding and conserved hydrophobic stretches (Shatwell *et al.*, 1996). The corresponding transcript, previously identified as a target of *TUP1* repression has been referred to as *RBT2* (repressed by *TUP1*) (Braun *et al.*, 2000). The *CFL95* ORF was placed behind the strong phosphoglycerol kinase (PGK) promoter and transformed into the $\Delta fre1\Delta fre2$ mutant of *S. cerevisiae* lacking reductase activity. The transformants expressed high levels of surface ferric reductase activity approximately 10-fold more than the wild-type *S. cerevisiae* strain (Fig. 5a), confirming that the *CFL95* ORF conferred ferric reductase activity.

Cloning of *CaFTR2* by complementation of a ferrous transport mutant of *S. cerevisiae*

S. cerevisiae lacking the iron permease gene *FTR1* grows extremely slowly on agar plates supplemented with the iron chelator ferrozine (Stearman *et al.*, 1996). This mutant strain was transformed with the *C. albicans*

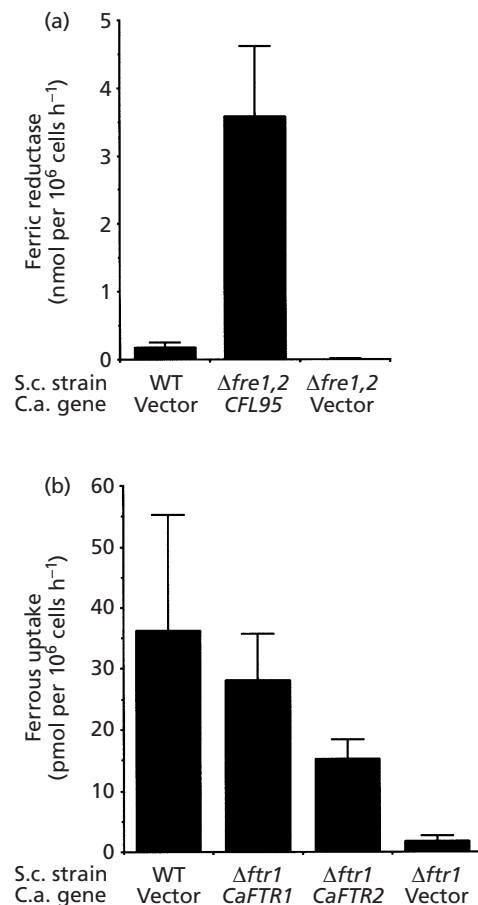


Fig. 5. *C. albicans* iron acquisition genes function in *S. cerevisiae*. (a) *CFL95* ORF under the control of the *S. cerevisiae* PGK promoter was transformed into the reductase-deficient mutant of *S. cerevisiae* YPH499 Δ 1 Δ 2 ($\Delta fre1,2$). For controls the empty vector was transformed into YPH499 (WT) or mutant ($\Delta fre1,2$). Transformants were assayed for ferric reductase activity. (b) *CaFTR1* or *CaFTR2* ORFs, each under the control of the *S. cerevisiae* PGK promoter, were transformed into a mutant of *S. cerevisiae* 42-3C1 lacking the iron permease ($\Delta ftr1$). For controls the empty vector was transformed into YPH499 (WT) or mutant ($\Delta ftr1$). Transformants were assayed for ferrous iron transport activity.

library and two transformants were selected that grew in the presence of chelator. The plasmids were rescued and found to contain a common 2.2 kb genomic fragment. Sequencing revealed a 1.1 kb ORF identical to *CaFTR2* (Ramanan & Wang, 2000) with the exception of a single silent nucleotide substitution of C for T at position 552 of the coding region. While analysing the flanking regions of *CaFTR2*, we noted the presence of a highly homologous ORF in the genome database located 18.2 kb away on the opposite DNA strand. The ORF for this gene, referred to as *CaFTR1* in a recent publication (Ramanan & Wang, 2000), was 83% identical with *CaFTR2* at the nucleotide level and 87% identical at the amino acid level. The sequence similarity was restricted to the coding regions and did not extend to the immediate 5' and 3' untranslated regions or flanking regions. In these flanking regions, no significant se-

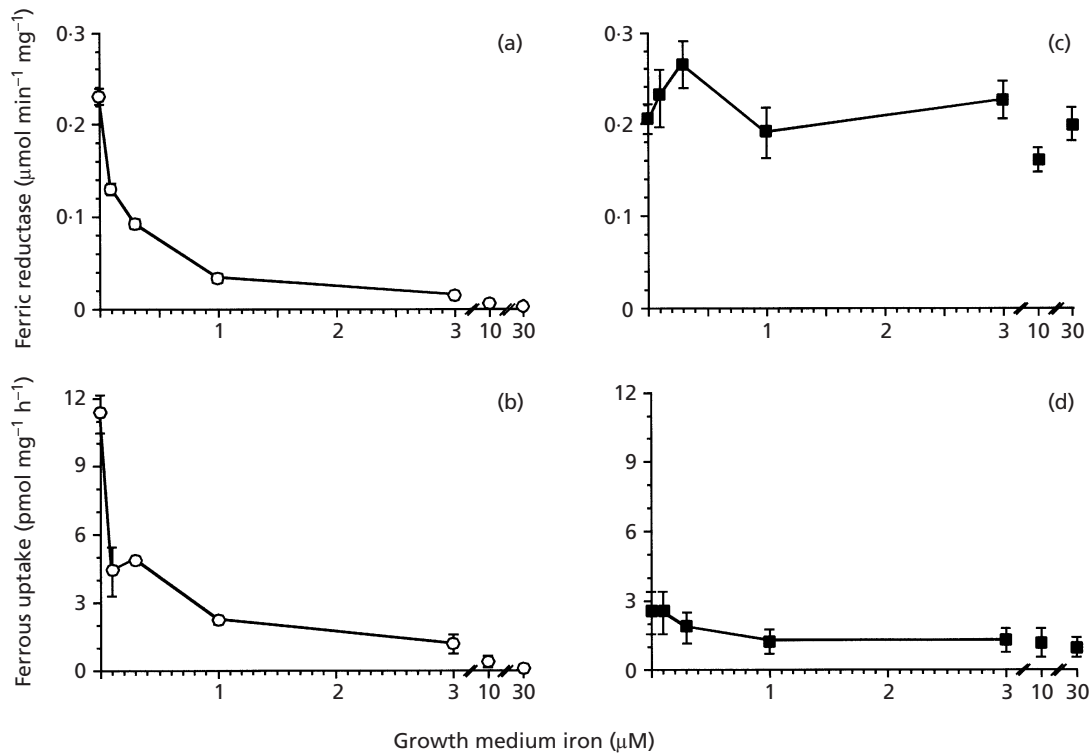


Fig. 6. *TUP1* is required for iron-dependent regulation of cell-surface ferric reductase activity and ferrous iron uptake in *C. albicans*. Wild-type *C. albicans* strain SC5314 (a and b) and the congenic $\Delta tup1/\Delta tup1$ mutant (BCa02-10) (c and d) were grown overnight in low-iron defined medium containing 1 μ M copper sulfate and iron (ferric ammonium sulfate) as indicated. The cells were diluted into fresh medium and grown for 4 h. Cells were washed and assayed for cell-surface ferric reductase activity (a and c) or high-affinity ferrous uptake (b and d). Each point shows the mean value \pm SD of six assays.

quence similarities were detected by pairwise BLAST at default settings (www.ncbi.nlm.nih.gov/blast/). We amplified the *CaFTR1* ORF from genomic DNA by PCR and used it for further studies.

The $\Delta ftr1$ strain of *S. cerevisiae* exhibited negligible high-affinity iron uptake (Fig. 5b). Expression of *CaFTR1* from the PGK promoter in this strain restored high-affinity iron uptake to 77% of the *S. cerevisiae* wild-type level. Expression of *CaFTR2* in a parallel construct increased high-affinity iron uptake to 42% of the wild-type level (Fig. 5b). Both *CaFTR1* and *CaFTR2* were able to mediate ferrous uptake into cells, although *CaFTR1* exhibited more efficient complementing activity than *CaFTR2* (Fig. 5b). The function of Ftr1p in *S. cerevisiae* requires assembly with the multicopper oxidase partner protein Fet3p, and thus *CaFTR1* and *CaFTR2* very likely encode proteins capable of assembly and functional interaction with the heterologous Fet3p. Complementation was incomplete in contrast to the reductase complementation, perhaps because of the added constraint for Fet3p interaction.

CaFET99, a candidate multicopper oxidase gene

A mutant of *S. cerevisiae* deleted for *FET3*, which like the *FTR1* mutant grows slowly on iron-chelated medium, was transformed with the *C. albicans* library.

However, no complementing clones were identified after screening 50000 transformants or the equivalent of multiple genome coverage. The genome sequence database for *C. albicans* contains five genes with significant homology to *FET3*, the multicopper oxidase of *S. cerevisiae* implicated in iron uptake. *CaFet99p* bears the highest homology to *S. cerevisiae* Fet3p (60% identity, 74% similarity), retaining all of the copper-binding sites, a putative iron-binding site (Bonaccorsi di Patti *et al.*, 1999), as well as 10 of 14 potential *N*-glycosylation sites. Therefore, we amplified this gene directly from genomic DNA and cloned the ORF for further studies. The *CaFET99* ORF was unable to complement the *FET3* deletion strain of *S. cerevisiae*. We then noticed that two CUG codons were present in the *CaFET99* ORF. These would be decoded as leucine in *S. cerevisiae* and as serine in *C. albicans*, because of non-classical codon usage in the latter organism. Therefore, the codons in question were changed by site-directed mutagenesis (CUG changed to AGC and TCT, respectively) and the modified clone was retested for complementing activity. Complementing activity again was not conferred. Co-expression with *CaFTR1* or *CaFTR2* likewise did not confer complementation. The reason for the inability of *CaFET99* to complement the $\Delta fet3$ mutant of *S. cerevisiae* remains unexplained. Nonetheless, *C. albicans* possesses PPD oxidase activity and five

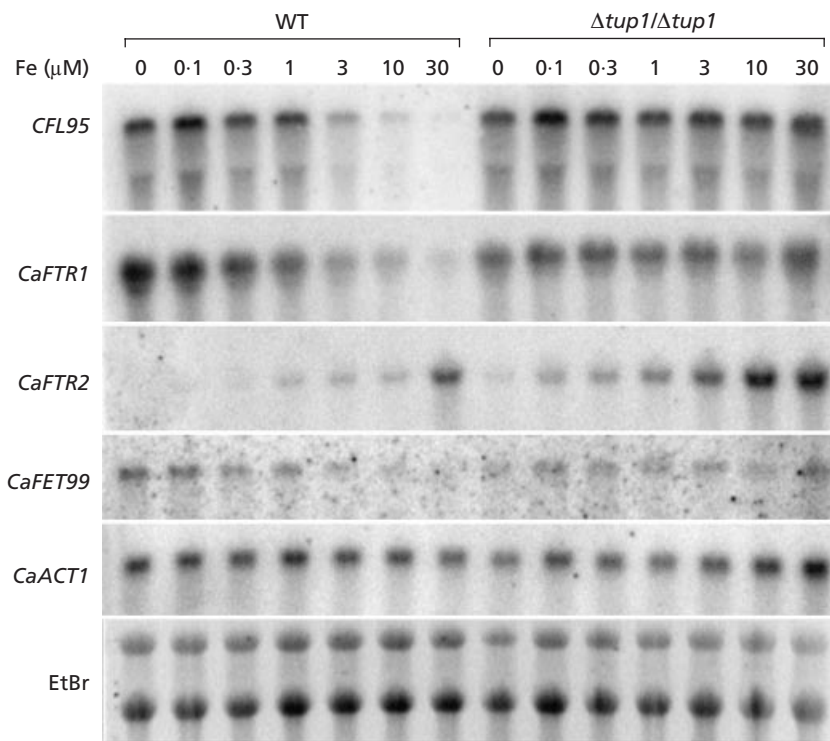


Fig. 7. Expression of genes involved in iron acquisition is misregulated in the $\Delta tup1/\Delta tup1$ mutant. Wild-type *C. albicans* strain SC5314 and the congenic $\Delta tup1/\Delta tup1$ mutant (BCa02-10) were grown in defined medium with varying amounts of iron (as described in the legend to Fig. 6). Total RNA (30 μ g per lane) was isolated and analysed by Northern blotting with probes for the indicated genes.

homologous genes belonging to the multicopper oxidase family.

TUP1 required for iron regulation of ferric reductase and ferrous transport activities

TUP1 of *C. albicans* functions as a repressor of gene expression. *TUP1* mutants grow as filamentous pseudohyphae, indicating a role in repressing genes involved in morphologic change. A role in iron metabolism was suggested by the identification of *CFL95*, the ferric reductase gene (also called *RBT2*), as a target of *TUP1*-mediated repression. We therefore decided to compare wild-type and homozygous $\Delta tup1/\Delta tup1$ deletion strains for cell-surface ferric reductase activity and high-affinity ferrous iron uptake.

The $\Delta tup1/\Delta tup1$ mutant of *C. albicans* BCa02-10 grew entirely in filamentous form as reported by Braun & Johnson (2000) and this form was unaltered by manipulations of medium iron availability. Microscopic examination revealed elongated branching forms consisting of discrete septated cells (pseudohyphae), presenting difficulties in expressing ferric reductase and ferrous iron uptake activities in terms of cell number. Instead, for both wild-type and the $\Delta tup1/\Delta tup1$ mutant, assays performed on whole cells were expressed on the basis of cellular protein (see Methods). Surprisingly, effects of the $\Delta tup1/\Delta tup1$ mutation on ferric reductase and ferrous iron uptake were quite different. In wild-type cells, ferric reductase activity was progressively down-regulated by addition of 0.1 μ M iron to the growth medium (Fig. 6a). Addition of 0.1 μ M iron decreased activity to

almost 50% and 30 μ M iron decreased activity to 1% compared to cells grown in the absence of iron (Fig. 6a). In contrast, ferric reductase activity of $\Delta tup1/\Delta tup1$ cells was constitutively elevated and unaffected by iron exposure (Fig. 6c). Ferrous iron uptake in the wild-type strain, like ferric reductase, was progressively repressed by iron exposure during growth (Fig. 6b). However, ferrous iron uptake in the $\Delta tup1/\Delta tup1$ mutant, while no longer responsive to iron exposures, was neither maximally induced nor repressed (Fig. 6d).

TUP1 required for iron regulation of key transcript levels

The pathway by which iron availability in the medium is transduced into changes in cellular iron uptake might involve regulated changes in mRNA, proteins or activities of critical elements of the iron uptake system. As a first step to studying iron regulation, transcript levels for candidate genes involved in iron uptake were examined. Transcripts for the ferric reductase gene *CFL95* were examined in wild-type *C. albicans* by Northern blotting. In these cells *CFL95* mRNA levels declined as iron concentrations in the growth medium increased (Fig. 7). For the most part, *CFL95* mRNA abundance and ferric reductase activity declined in parallel with increasing iron exposure of the cells. An exception was noticed in cells exposed to very low levels of iron in the medium (0.1, 0.3 and 1 μ M, Fig. 6a). Under these conditions, ferric reductase activity was repressed, whereas *CFL95* mRNA levels were not substantially altered (Fig. 7). Iron-dependent repression of *CFL95* mRNA translation, iron-dependent protein turnover,

(a)

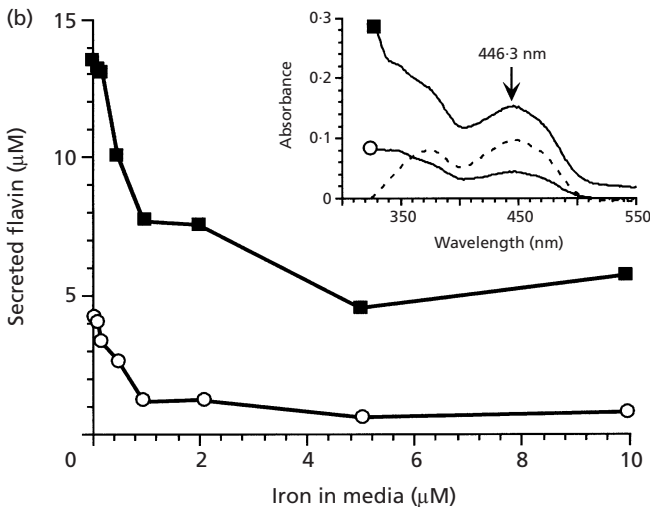
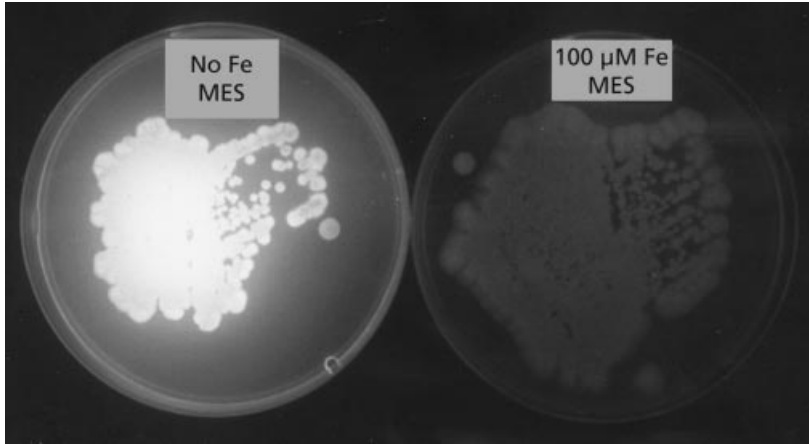


Fig. 8. Flavin secretion by *C. albicans*: regulation by iron and repression by *TUP1*. (a) Wild-type *C. albicans* strain SC5314 was grown on solid agar defined medium containing 50 mM MES, pH 6.2, 1 μM copper sulfate and 1 mM ferrozine with no added iron (left) or with 100 μM ferric ammonium sulfate (right). After incubation for 3 d at 30 °C the plates were photographed under a UV lamp. The iron-chelated culture (left) emitted a bright yellow colour. (b) Wild-type *C. albicans* strain SC5314 (○) and $\Delta tup1/\Delta tup1$ (■) were grown in defined medium containing 1 μM copper sulfate and with iron additions as indicated. After 40 h at 30 °C the cell cultures were centrifuged to pellet the cells (1500 g, 10 min) and supernatants were assayed for flavin concentration. The insert shows an absorbance scan of conditioned medium from wild-type (■) and $\Delta tup1/\Delta tup1$ (○) cells grown in low iron medium. A scan for 10 μM riboflavin is shown as a dashed line and the 446.3 nm maximum is indicated.

repression of enzymic activity or effects on protein partners could account for these post-transcriptional effects. *CFL1*, another *C. albicans* gene able to confer reductase activity to a $\Delta fre1\Delta fre2$ mutant of *S. cerevisiae* (Hammacott *et al.*, 2000), was not detected by Northern blotting under iron-starved or iron-replete conditions (data not shown) and so conclusions could not be drawn regarding its regulation.

CaFTR1 mRNA was also down-regulated by iron exposure during growth (Fig. 7). The decline was progressive with increasing iron addition and correlated well with progressive decreases in ferrous uptake activity (Fig. 6b). By contrast, *CaFTR2* levels increased with increasing concentrations of iron in the growth medium. A large increase in mRNA abundance was seen between 10 and 30 μM iron. These results are consistent with previous observations regarding the bidirectional regulation of the two *C. albicans* *FTR1* orthologues.

In *C. albicans*, five multicopper oxidase homologues are present in the genome. *CaFET99* was investigated

because it showed the highest level of sequence identity to *S. cerevisiae* Fet3p (60% amino acid identity over the entire protein). Similar to *CaFTR1* regulation, the levels of *CaFET99* mRNA progressively decreased with addition of iron to the growth medium (Fig. 7). *CaFET99* transcripts required a 6 d exposure for visualization by Northern blotting versus 1 d for the *CaFTR1* and *CaFTR2* transcripts. The decreased signal could represent decreased mRNA abundance for this gene or decreased reactivity of the probe. The probe was directed to a small region 3' of the ORF, and the precise margins of the 3' untranslated region have not yet been mapped. Another multicopper oxidase homologue, *CaFET3*, with 56% amino acid identity to Fet3p of *S. cerevisiae*, was not detected by Northern blotting with a probe directed to the 3' untranslated region of that gene (data not shown and Eck *et al.*, 1999).

To assess *TUP1* effects on iron regulation, total RNA was isolated from the $\Delta tup1/\Delta tup1$ strain grown under different iron conditions and probed to analyse ex-

pression of various genes implicated in iron uptake (Fig. 7). Surprisingly, although in each case iron-dependent regulation was blunted or abolished, the effects were not the same for each gene. *CFL95* mRNA levels were maximally induced and unresponsive to iron exposure. These results correlated with the constitutively high ferric reductase activities of the $\Delta tup1/\Delta tup1$ strain (Fig. 6c). By contrast, *CaFTR1* mRNA in the $\Delta tup1/\Delta tup1$ strain was expressed at a medium level, much less than the maximally induced level observed in wild-type cells grown in the absence of iron. Iron-dependent changes were abrogated. These results correlated well with the ferrous uptake data, which also showed mid-level unregulated activities (Fig. 6d). *CaFET99* transcripts in the $\Delta tup1/\Delta tup1$ strain were present at moderately induced levels (see 1 μM level for comparison) and iron-dependent changes were absent.

The iron response profile of the *CaFTR2* transcripts in the $\Delta tup1/\Delta tup1$ mutant was unique (Fig. 7). *CaFTR2* expression was induced by iron exposure in the wild-type, with a large step increase at 30 μM medium iron. In the $\Delta tup1/\Delta tup1$ strain, the induction threshold was shifted to a much lower iron level (compare *CaFTR2* mRNA level at 30 μM in the wild-type with 3 μM in the mutant). Furthermore, the large step increase of the wild-type was not seen, but rather a graded increase from 0 to 30 μM medium iron. The effects of the $\Delta tup1/\Delta tup1$ mutation were to shift downward the threshold for iron-dependent induction of expression of *CaFTR2* and to generally derepress expression. However, iron-dependent expression of the transcripts was preserved over a wide range of medium iron exposures.

CaACT1 transcript levels and ethidium bromide staining of 25S and 18S rRNAs served as loading controls for these experiments.

Regulation of flavin secretion: role of iron and *TUP1*

During the course of these experiments, we observed an intense yellow-green colour of the culture medium from stationary-phase iron-limited cultures of *C. albicans*. The medium (not shown) and colonies (Fig. 8a) also exhibited bright yellow fluorescence in response to UV excitation. The visible absorbance spectrum of the secreted pigment exhibited a peak at 446.3 nm (Fig. 8b insert), suggesting that a flavin was responsible. The concentrations of flavin in the medium decreased with increasing medium iron concentrations up to 1 μM (Fig. 8b). Flavin production by the $\Delta tup1/\Delta tup1$ mutant was markedly elevated at all growth medium iron concentrations, although some level of iron response was maintained (Fig. 8b).

DISCUSSION

C. albicans possesses more than one type of iron acquisition system (Eck *et al.*, 1999; Hammacott *et al.*, 2000; Lesuisse *et al.*, 1998; Manns *et al.*, 1994; Ramanan & Wang, 2000), perhaps because each system is specifically adapted for an environmental niche or because redundancy is desired for this crucial function. As shown

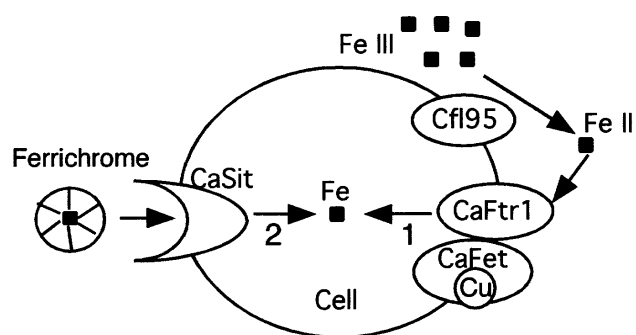


Fig. 9. Model of the iron acquisition systems of *C. albicans*. 1. The copper-dependent reductive system. A ferric reductase (*Cfl95*) reduces iron to the ferrous form which is taken into the cell by a complex consisting of the ferrous permease (*CaFtr1*) and a multicopper oxidase (*CaFet*). 2. The copper-independent siderophore permease (*CaSit*) system for ferrichrome uptake.

here, at least two systems can be distinguished: the reductive copper-dependent iron uptake of elemental iron and a copper-independent siderophore transport system (Fig. 9). In the former, iron uptake from ferric chelates involves a surface reductase encoded by *CFL95* and a ferrous permease encoded by *CaFTR1*. The ferrous transport component is copper-dependent, as is the PPD oxidase activity found in an isolated membrane fraction. Therefore, a multicopper oxidase is likely to be involved, analogous to the situation in *S. cerevisiae* (Askwith *et al.*, 1994; Stearman *et al.*, 1996). The gene or genes responsible for the oxidase activity mediating iron uptake have been difficult to ascertain, because five homologous multicopper oxidase genes exist in the *Candida* genome and the most homologous, *CaFET99*, was unable to complement the orthologous mutant of *S. cerevisiae*. The copper dependence of elemental iron uptake contrasts with the copper independence of iron uptake from the siderophore ferrichrome. A single siderophore permease homologue is present in the genome of *C. albicans* that may mediate ferrichrome uptake (Lesuisse *et al.*, 2001; Ardon *et al.*, 2001), *C. albicans* may even synthesize its own siderophores (Ismail *et al.*, 1985), providing even greater versatility in scavenging iron.

The reductive iron uptake system of *C. albicans* resembles that of *S. cerevisiae*, but is distinguished by greater regulatory complexity and the existence of multiple genes for each component. Of special interest are the two forms of the permease, *CaFTR1* and *CaFTR2* (Ramanan & Wang, 2000). These are highly homologous within their coding regions but dissimilar in their untranslated and flanking regions, consistent with their very different patterns of expression. *CaFTR1* is induced by iron deprivation while *CaFTR2* is induced by iron exposure. A conundrum is raised by the complementation data presented here. Both genes are able to complement the orthologous $\Delta ftr1$ mutant of *S. cerevisiae* by conferring high-affinity iron uptake activity upon this mutant. In *C. albicans*, however, under

conditions in which *CaFTR2* is induced (> 30 μ M medium iron concentration), high-affinity iron transport into the cell does not occur. The implication is that another level of control exists. Perhaps in this setting, the CaFtr2p functions elsewhere in the cell or with an alternative oxidase (Fet3p) homologue that confers iron sequestering or iron detoxifying activity. Such an activity might be needed under special iron stress conditions.

Regulation of genes involved in iron uptake responds to iron availability, either by down-regulation (*CFL95*, *CaFTR1*, *CaFET99*) or by up-regulation (*CaFTR2*). The sensor-regulator that transduces changes in available iron into effects on transcription has not been identified for *C. albicans*. A homologue of *AFT1* (Yamaguchi-Iwai *et al.*, 1996), the sensor-regulator in *S. cerevisiae*, exists (<http://www-sequence.stanford.edu/group/candida>) and might provide such a function, although no direct information exists regarding the function of this gene. Post-transcriptional iron regulatory effects might also occur as shown by the marked repression of *CFL95* that occurs at low iron levels without changes in transcript levels. Mechanisms for these regulatory effects also remain to be defined.

Iron-dependent gene regulation is linked to changes in the morphological growth form of *C. albicans* by the role of the *TUP1* repressor in both processes. In rich laboratory medium, the organism grows as a budding yeast, but in some settings it changes to a filamentous or hyphal growth form (Brown & Gow, 1999; Ernst, 2000). The ability to effect this change is correlated with virulence (Lo *et al.*, 1997; Mitchell, 1998), and *TUP1* maintains the organism in the yeast growth form through repressive effects on numerous target genes (Braun *et al.*, 2000). Expression of *CFL95*, the ferric reductase gene, was previously shown to be repressed by *TUP1* (Braun *et al.*, 2000). Here we show that *TUP1* is required for iron-dependent repression of the *CFL95* transcript and more generally for correct iron sensing and iron-dependent gene regulation. Extensive characterization of the *S. cerevisiae* *TUP1* homologue shows that the protein does not interact directly with DNA. Instead it is brought to the promoter regions of specific target genes by DNA-interacting regulatory proteins where it acts to shut off transcription (Smith & Johnson, 2000). *TUP1* of *S. cerevisiae* has been implicated in diverse processes such as mating type silencing, response to nitrogen starvation (Smith & Johnson, 2000) and regulation of siderophore iron uptake (Lesuisse *et al.*, 2001). In *C. albicans*, an iron sensor-regulator protein might interact with a specific DNA binding site in the *CFL95* promoter region. Interaction of this sensor protein with *TUP1* protein in turn might mediate iron-dependent repression of gene expression. How specific nutritional signals (such as iron availability) are transduced by a general and global repressor protein remains a mysterious and central problem in understanding *TUP1* function. Iron-dependent regulation of other genes was also *TUP1*-dependent, although different effects were discerned. For *CaFTR1* and *CaFET99*, *TUP1* was required for iron regulation, although the

Atup1/Atup1 mutant showed mid-level expression for these transcripts, suggesting a requirement of *TUP1* for both maximally induced and maximally repressed transcript levels. For *CaFTR2*, iron-dependent regulation was still observed in the absence of *TUP1*, although the threshold for induction was shifted downward and the expression level was increased.

During growth of *C. albicans* in low iron conditions, the medium became yellow-green due to accumulation of flavins. The amount of flavin was inversely related to iron availability in the medium and was markedly increased in the absence of *TUP1*. Flavins are versatile molecules that can catalyse two-electron dehydrogenations and participate in single-electron reductions (Massey, 2000). The synthesis and excretion of flavins in response to iron deficiency has been observed in other yeasts as well as plants (Fedorovich *et al.*, 1999; Susin *et al.*, 1993). The switch to flavin production might be advantageous by making available an alternative to iron- and cytochrome-dependent electron transport for various metabolic pathways. Alternatively, reduced flavins might facilitate release of iron from ferric chelates or ferrisiderophores, thereby facilitating cellular iron acquisition (Coves & Fontecave, 1993).

Previous work has shown that inactivation of *CaFTR1* leads to decreased killing of mice subjected to intravenous injections of this mutant of *C. albicans* (Ramanan & Wang, 2000). Therefore, the copper-dependent pathway of iron acquisition involving ferric reductase, multicopper oxidase and ferrous permease must be involved in a critical step in pathogenesis of infections following intravenous dissemination of the organism. Macrophages are mainstays of the circulating immune defence, able to phagocytose *Candida* in the circulation (Vazquez-Torres & Balish, 1997). Exposure of *C. albicans* to the environment of the phagosome apparently causes deprivation for some nutrients, thereby inducing genes of the glyoxylate cycle (Lorenz & Fink, 2001). Changes in iron availability might occur within the phagosome (Kuhn *et al.*, 1999) and survival of *C. albicans* might require specific iron-regulated responses. The progression of *C. albicans* infections can be viewed in terms of complex host-pathogen interactions specific to various niches (De Bernardis *et al.*, 1998). Each niche has peculiar features regarding the form and availability of iron. The regulation and function of iron uptake genes within host niches still needs to be defined and the use of reductive versus siderophore pathways for iron acquisition will require further study. Exploration of these factors may better define the role of iron in virulence and suggest therapeutic approaches.

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