***Candida albicans* has a cell-associated ferric-reductase activity which is regulated in response to levels of iron and copper**

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For survival, pathogenic organisms such as *Candida albicans* must possess an efficient mechanism for acquiring iron in the iron-restricted environment of the human body. *C. albicans* can use iron from a variety of sources found within the host. However, it is not clear how biologically active ferrous iron is obtained from these sources. One strategy adopted by some organisms is to reduce iron extracellularly and then specifically transport the ferrous iron into the cell. We have shown that clinical isolates of *C. albicans* do have a cell-associated ferric-reductase activity. The determination of ferric-reductase activity of cells growing exponentially in either low- or high-iron media over a period of time indicated that *C. albicans* reductase activity is induced when in low-iron conditions. Moreover, we have demonstrated that *C. albicans* reductase activity is also regulated in response to the growth phase of the culture, with induction occurring upon exit from stationary phase and maximal levels being reached in early exponential stage irrespective of the iron content of the medium. These results suggest that *C. albicans* reductase activity is regulated in a very similar manner to the *Saccharomyces cerevisiae* ferric-reductase. Iron reduction and uptake in *S. cerevisiae* are closely connected to copper reduction, and possibly copper uptake. In this report we show that iron and copper reduction also appear to be linked in *C. albicans*. The ferric-reductase activity is negatively regulated by copper. Moreover, quantitative cupric-reductase assays indicated that *C. albicans* is capable of reducing copper and that this cupric-reductase activity is negatively regulated by both iron and copper. This is the first report that *C. albicans* has an iron- and copper-mediated ferric-reductase activity.

Keywords: *Candida albicans*, iron uptake, ferric-reductase, cupric-reductase

INTRODUCTION

Most living organisms require iron as an essential cofactor for the catalytic activity of many biologically important proteins. However, in the human body iron is not readily available to invading micro-organisms since it is complexed with high-affinity iron-binding proteins such as ferritin and transferrin. The consequent low level of available iron in the body fluids and tissues acts as a generalized defence against invading pathogens. Thus, for an organism to be successful as a pathogen it has to have a mechanism to compete with the host for this limited amount of iron. Another problem faced by all

microbial organisms is that most available sources of iron are in the insoluble ferric form. One strategy used to overcome these problems is the secretion of siderophores, high affinity iron-specific chelators that bind to insoluble ferric iron to form a soluble complex. These complexes can be taken up into the cell by specific transporters as is the case for many bacterial systems (Neilands *et al.*, 1987). An additional or alternative strategy to obtain biologically active ferrous iron involves the extracellular reduction of ferric iron followed by specific uptake of the ferrous ions; this is a mechanism used by a variety of organisms, including fungi such as *Saccharomyces cerevisiae* (Lesuisse & Labbe, 1989) and *Schizosaccharomyces pombe* (Roman *et al.*, 1993) and bacteria such as *Legionella pneumophila* (Johnson *et al.*, 1991) and *Streptococcus mutans* (Evans *et al.*, 1986). Such extracellular reductases could potentially produce

Abbreviation: BPS, bathophenanthroline disulphonate.

ferrous ions from a range of ferric iron sources including ferric-siderophore complexes. The acquisition of iron and its relationship to bacterial virulence has been extensively studied (reviewed by Wooldridge & Williams, 1993). However, little is known about the role or importance of iron uptake in relation to fungal pathogens, such as *Candida albicans*.

C. albicans is a dimorphic fungus which can be found in either yeast or hyphal form. This fungus is a common commensal of the gut, mouth and vagina, but under certain circumstances it can cause both superficial infections, and in the case of immunocompromised patients, serious systemic infections (Odds, 1987). It is thought that both the yeast and hyphal forms of *C. albicans* can be pathogenic (Kerridge, 1993; Ryley & Ryley, 1990). Investigations concerning *C. albicans* iron acquisition and uptake have to date been limited. It has been observed that *C. albicans* can secrete siderophores. Ismail *et al.* (1985) reported the secretion of both hydroxamate and phenolate siderophores, whereas other reports only confirm the production of the hydroxamate type (Sweet & Douglas, 1991a). These differences may be due to strain variations. However, although evidence has been presented which indicates that the hydroxamate siderophores are capable of stimulating *C. albicans* growth (Minnick *et al.*, 1991), it has not yet been established how *C. albicans* obtains biologically active ferrous iron from these ferric-siderophore complexes or indeed from any other sources.

The well-characterized yeast *S. cerevisiae* does not secrete its own siderophores but it does possess both a specific ferric-siderophore uptake mechanism and a reductive ferric iron uptake system (Lesuisse & Labbe, 1989). Since genetic analysis of *Candida* is difficult, as it is diploid with no sexual cycle, *S. cerevisiae* is successfully being used both as a model and as a tool to investigate a diverse range of *C. albicans* physiological functions, and it is now clear that the two yeasts are phylogenetically closely related and that genes with homologous functions are highly conserved (reviewed by Sudbery, 1994). Therefore it is likely that such an approach will also be fruitful in the investigation of iron metabolism.

S. cerevisiae has a ferric-reductase which is a multimolecular complex consisting of several proteins (Lesuisse *et al.*, 1990). To date, two genes have been identified, *FRE1* and *FRE2*, which are believed to encode proteins required for ferric-reductase activity (Dancis *et al.*, 1990; Georgatsou & Alexandraki, 1994). Biochemical assays have demonstrated that during exponential growth ferric-reductase activity is induced by iron starvation from the low basal level seen when the organism is iron-replete (Lesuisse *et al.*, 1987; Dancis *et al.*, 1990; Eide *et al.*, 1992; Georgatsou & Alexandraki, 1994). However, this iron regulation is overcome by growth-phase-dependent regulation, such that reductase activity is repressed upon entry into stationary phase and induced on entry into exponential growth, regardless of the iron content of the medium (Eide *et al.*, 1992).

Copper, like iron, is an important nutrient which is also able to undergo reversible redox reactions. Consequently,

copper is also an essential cofactor for a variety of enzymes. It has been demonstrated that the *S. cerevisiae* ferric-reductase can also reduce cupric copper to cuprous copper (Jungmann *et al.*, 1993; Hassett & Kosman, 1995; Georgatsou *et al.*, 1995). This activity has been shown to be regulated by the levels of both copper and iron in the medium (Hassett & Kosman, 1995). The *FRE1*- and *FRE2*-encoded components of the reductase can reduce copper. However only *FRE1* transcription and reductase activity are induced by the absence of copper (Georgatsou *et al.*, 1995). It is possible that extracellular copper reduction is necessary to produce cuprous ions which can then be transported into the cell. Hence there may be close parallels and links between copper and iron metabolism in *S. cerevisiae*.

We report the beginning of an analysis of iron uptake in clinical isolates of *C. albicans*. We demonstrate that *C. albicans* has ferric and cupric-reductase activities which are both regulated in a similar manner to the *S. cerevisiae* reductase. The *C. albicans* ferric-reductase activity may be important for the organisms' acquisition of ferrous iron from ferric-siderophore complexes and other ferric iron sources.

METHODS

Strains and growth conditions. The *C. albicans* isolates 85/031 and 81/40 were obtained from A. Abbott, Department of Microbiology, Leicester University, and were originally obtained from superficial clinical specimens. Isolate S/01 was obtained from R. Matthews, Department of Microbiology, University of Manchester, UK, and originally came from a systemic infection.

S. cerevisiae strain S150-2B (*MATa leu2-3, 112 his3-Δ trp1-289 ura3-52*) was obtained from J. Hicks, Cold Spring Harbor Laboratory, NY, USA. The defective ferric-reductase isolates *fre3.1* and *fre4.1* were obtained by UV mutagenesis of S150-2B (unpublished data).

Yeast cultures of both species were grown at 30 °C in either YEPD (1 %, w/v, yeast extract; 2 %, w/v, Bactopeptone; 2 %, w/v, glucose) or minimum defined medium (MD). MD medium was based on Wickerham's nitrogen base recipe (Wickerham, 1951) except that 20 mM sodium citrate, pH 4.2, was also added (Eide *et al.*, 1992). Amino acid supplements were added to the MD medium at final concentrations as indicated by Sherman *et al.* (1986) and glucose was added at 2 %. FeCl_3 and/or CuSO_4 were omitted when the media was to be rendered iron- and/or copper-restricted. Growth in liquid cultures was monitored by observing cells by phase-contrast microscopy and counting cell numbers using a haemocytometer. For growth on solid media, 2 % (w/v) agar was added to YEPD or MD.

MD medium was rendered iron-restricted by the addition of 300 μM $\alpha\alpha'$ -dipyridyl (Sigma). This MD-dipyridyl medium was unable to support the growth of all isolates used in this investigation unless extra FeCl_3 was added. The threshold level of iron which would support normal growth of *C. albicans* S/01 and yet still render the cells iron deprived was determined by obtaining growth curves from a series of cultures grown in media to which different amounts of FeCl_3 were added. Thus, it was determined that a final concentration of 100 μM FeCl_3 was the lowest amount of added iron required to support growth at a mean doubling time of 77.5 ± 3.5 min. All iron concentrations

below 100 μM only supported growth for a maximum of two doublings. All iron concentrations above 100 μM to a maximum of 2 mM had similar mean doubling times of 77.5 ± 3.5 min.

$\alpha\alpha'$ -Dipyridyl was not added to the MD medium used during the cupric-reductase activity experiments (Fig. 4). In this case, the medium was rendered both iron- and copper-restricted by the addition of 50 μM bathophenanthroline disulphonate (BPS) as described by Hassett & Kosman (1995). This medium supported growth without additions. In the experiments described in Fig. 4, either 40 μM FeCl_3 (Cu^+Fe^+) or 40 μM CuCl_2 (Cu^+Fe^-) was added to the MD-BPS medium to give the high-iron or high-copper conditions.

Qualitative ferric-reductase assay. A modified version of a qualitative, solid-phase reductase assay (Dancis *et al.*, 1990) was used to identify extracellular ferric-reductase activity. Cells were streaked onto solid YEPD medium, grown overnight at 30 °C, then transferred to nylon filters and incubated for 24 h on the surface of solid MD-dipyridyl medium, to which either 100 μM (low iron/MD100) or 2 mM FeCl_3 (high iron/MD2000) was added. After overnight incubation the nylon filters were removed from the plates and incubated for 5 min on Whatman 3MM paper soaked in assay buffer (50 mM sodium citrate, pH 6.5, 5% glucose). The filters were then transferred onto fresh 3MM paper soaked in 50 mM sodium citrate, 5% glucose, 1 mM FeCl_3 and 1 mM BPS, and incubated for a further 5 min. Colonies with ferric-reductase activity stained the filter red due to the formation of $\text{BPS}[\text{Fe}^{2+}]$ complex.

Quantitative liquid-phase ferric- and cupric-reductase assays. Liquid-phase reductase assays were used to give a quantitative measure of either ferric- or cupric-reductase activities. Samples containing 1×10^7 cells for the ferric-reductase assay and 5×10^7 cells for the cupric-reductase assay were taken from liquid cultures. Cells were harvested by centrifugation, washed in distilled water, repelleted, and resuspended in 1 ml assay buffer (50 mM sodium citrate, pH 6.5, 5% glucose) containing 1 mM FeCl_3 and 1 mM BPS for the ferric-reductase assay; or 1 mM CuCl_2 and 1 mM bathocuproine sulphonate for the cupric-reductase assay. The samples were incubated at 30 °C for 10 min, after which the cells were removed by centrifugation and the A_{520} of the supernatant was measured using a CE272 spectrophotometer (Cecil Instruments). The levels of ferrous or cuprous ions produced were estimated from calibration curves constructed from solutions of known ion concentrations. From these data the ferric or cupric-reductase activity was calculated as nmol Fe(II) or Cu(I) that would be expected to be produced by 1×10^7 cells in 1 h.

RESULTS

C. albicans has a cell-associated ferric-reductase activity

We have used a modified version of a qualitative, solid-phase assay to determine whether *C. albicans* has an extracellular ferric-reductase activity. *C. albicans* isolates obtained from various superficial and systemic clinical infections were streaked onto solid YEPD medium in duplicate and grown overnight at 30 °C. The *S. cerevisiae* strain S150-2B (wild-type) and the S150-2B derivatives *fre3.1* and *fre4.1* were also grown on YEPD plates as positive and negative controls. The isolate *fre3.1* is defective in ferric-reductase activity, whereas *fre4.1* is constitutive for ferric-reductase activity at all iron concentrations (unpublished data). These YEPD-grown cells

were then transferred to nylon filters, preincubated on low- or high-iron media and the filters assayed qualitatively for ferric-reductase activity as described in Methods. Examples of filters are shown in Fig. 1. It must be noted that the *C. albicans* and the *S. cerevisiae* strains demonstrated different levels of growth on the YEPD medium and therefore more *C. albicans* cells were transferred to the assay filters. Consequently this assay cannot be used to make quantitative comparisons of the activity demonstrated by the two species. However, it does demonstrate presence or absence of reductase activity and enables a comparison to be made after preincubation of a particular species on low- or high-iron media.

Wild-type *S. cerevisiae* cells preincubated on low-iron medium have an induced ferric-reductase activity, which was demonstrated by the red staining of the nylon filter. This red colouration is due to the extracellular reduction of the ferric iron, present in the assay buffer, to ferrous iron, which then forms a red-coloured complex with BPS ($\text{BPS}[\text{Fe}^{2+}]$). The basal level of ferric-reductase activity which occurs in high-iron conditions was demonstrated by the slight red colour associated with wild-type *S. cerevisiae* cells preincubated on the high-iron medium, which indicates that only a small amount of ferrous iron had been produced. The isolate *fre3.1* is known to be defective in reductase activity (J. Morrissey, unpublished observation) and, as expected, there was no colour change associated with *fre3.1* cells incubated on either high- or low-iron medium. The isolate *fre4.1* is known to have reductase activity which is constitutive and, again as expected, high levels of $\text{BPS}[\text{Fe}^{2+}]$ complex were produced irrespective of iron concentration.

C. albicans cells incubated on the same low-iron medium produced a high level of the red $\text{BPS}[\text{Fe}^{2+}]$ complex. This indicates that *C. albicans* cells can reduce extracellular ferric iron to ferrous iron. This reductase activity was lowered when cells were incubated on high-iron medium. These results suggest that *C. albicans* has an extracellular ferric-reductase activity which appears to be regulated by the iron content of the medium.

To ascertain whether this reductase activity is associated with the cell surface or is due to a secreted or exported reductant, cells were separated from their liquid growth media by centrifugation. The cell-free supernatant would be expected to contain any proteins that had been previously secreted or exported by the cells. The reductase activities of the cells and the resultant cell-free growth media were then compared using a quantitative form of the reductase assay.

Cell samples from *C. albicans* S/01 cultures grown in low- or high-iron MD-dipyridyl media were resuspended in ferric-reductase assay buffer. Ferric-reductase activity of both cell-containing and cell-free fractions was then determined. There was a significant difference in ferric-reductase activity between the cell-containing fraction (2820 nmol per 10^7 cells h^{-1}) and the cell-free growth media (720 nmol per 10^7 cells h^{-1}) (Table 1). This suggests that the majority of the ferric-reductase activity is not

Fig. 1. Different clinical isolates of *C. albicans* show an extracellular ferric-reductase activity. Cells grown on duplicate YEPD plates were replica-plated onto duplicate nylon filters placed on the surface of MD-dipyridyl plates with either (a) low or (b) high concentrations of iron. The plates were incubated at 30 °C for 24 h. The filters were then removed and ferric-reductase activity was identified as described in Methods. The isolates used were: (a) *C. albicans* S/01, *C. albicans* 85/031 and *C. albicans* 81/40; (b) *S. cerevisiae* S150-2B, *S. cerevisiae* *fre3.1* and *S. cerevisiae* *fre4.1*.

Table 1. *C. albicans* reductase activity is cell-associated

Samples of 5×10^7 cells were taken from cultures growing exponentially in either MD-dipyridyl medium with 100 μM FeCl_3 or MD-dipyridyl medium with 2 mM FeCl_3 . The cells were washed in distilled water and resuspended in ferric-reductase assay buffer at a cell density of 5×10^7 cells ml^{-1} . Every 30 min a sample containing 1×10^7 cells was taken. The cell suspension was pelleted, the supernatant was transferred to a fresh Eppendorf tube and FeCl_3 and BPS were added, both to a final concentration of 1 mM. The pellet was resuspended in prewarmed ferric-reductase assay buffer, and again FeCl_3 and BPS were added to final concentrations of 1 mM. Both samples were incubated at 30 °C for 10 min, and the reductase activity was then determined as described in Methods. The figures given are means of at least three experiments and standard deviations are indicated in brackets. Reductase activity is expressed as nmol per 10^7 cells h^{-1} .

Time (min)	100 μM FeCl_3		2 mM FeCl_3	
	Cells present	Cells absent	Cells present	Cells absent
0	2820 (888)	720 (150)	1386 (294)	396 (16.8)
30	2148 (288)	498 (102)	1278 (252)	378 (16.8)
60	2046 (396)	540 (< 1)	1140 (120)	408 (34.2)
90	2076 (432)	558 (198)	1110 (126)	528 (66)
120	1686 (240)	570 (48)	1104 (228)	450 (60)

secreted/exported but is associated with the intact cells. Moreover, this cell-associated activity is regulated by iron, since the maximum rate of reduction by cells grown

previously in the low-iron medium was 2820 nmol per 10^7 cells h^{-1} , whereas cells grown in high-iron medium had a maximum rate of only 1386 nmol per 10^7 cells h^{-1} .

Fig. 2. *C. albicans* extracellular reductase activity is negatively regulated by iron. Cells growing exponentially in MD medium (without dipyrindyl) were washed in distilled water, then were transferred into duplicate fresh prewarmed MD media at a cell titre of 1×10^6 cells ml^{-1} . The MD-dipyrindyl media contained either 100 μM FeCl_3 (MD 100; $\circ$), or 2 mM FeCl_3 (MD 2000; $\blacktriangle$) medium. These cultures were then incubated with shaking at 30 °C. At various time points, the cell number was determined using a haemocytometer. Samples containing 1×10^7 cells were then obtained, and the ferric-reductase activity of these cells was determined as described in Methods. The results presented are a mean of at least three separate experiments, and the standard deviations are shown.

Some ferric-reductase activity was present in the cell-free growth media; this activity represents on average 20 % of the total ferric-reductase activity seen under these conditions. However, this level of reductase activity is not dependent on concentration of iron in the medium. It is possible that this activity is due to the incomplete removal of cells from the media after culture. Alternatively, in addition to the cell-associated iron-dependent reductase, *C. albicans* may also secrete or export an iron-independent reductant. Indeed some evidence has been presented that this is the case for *S. cerevisiae* (Lesuisse *et al.*, 1987, 1992; Georgatsou & Alexandraki, 1994). However, irrespective of the source of the small amount of reductase activity in the medium it is clear that there is a cell-associated ferric-reductase, which appears to be regulated by levels of available iron.

C. albicans cell-associated ferric-reductase activity is regulated by iron

The experiments described above indicate that the *C. albicans* cell-associated ferric-reductase activity is dependent on iron concentration. To confirm this, the difference in reductase activity between cells growing exponentially in low- or high-iron media was determined. Exponentially

growing cells of *C. albicans* S/01 were used to inoculate low- and high-iron media. Samples were taken periodically from these cultures and assayed for ferric-reductase activity. Growth was monitored throughout the experiment. After an initial lag period due to the cells recovering from the washing and inoculating procedures, the growth rate was seen to be the same in both high- and low-iron cultures, although the doubling time (77.5 ± 3.5 min) varied among experiments.

The data presented in Fig. 2 clearly show that the *C. albicans* ferric-reductase activity is regulated by extracellular iron concentration. Cells grown in MD 100 had a mean maximum ferric-reductase activity of 2810 nmol per 10^7 cells h^{-1} , whereas cells grown in MD 2000 had a mean maximum ferric-reductase activity of only 1230 nmol per 10^7 cells h^{-1} . This maximal ferric-reductase activity was reached after 150 min of growth in the low-iron medium and after 100 min in the high-iron medium. At this stage the cells were in an early stage of exponential growth. In both cases, ferric-reductase activity then steadily decreased throughout the time-course of the experiment. This iron-dependent regulation of *C. albicans* ferric-reductase activity was kinetically very similar to the regulation of *S. cerevisiae* reductase activity (Dancis *et al.*, 1990; Georgatsou & Alexandraki, 1994). *S. cerevisiae* reductase activity is also maximal when cells are growing exponentially in low-iron medium. *S. cerevisiae* cells only reach this maximum after 240 min of growth (Dancis *et al.*, 1990), whereas *C. albicans* cells reached a maximum activity in half this time. Moreover, *S. cerevisiae* maintains this maximal activity for approximately 120 min, whilst *C. albicans* reductase activity started to decrease after only 50 min. These differences probably reflect a difference in growth rate, since the *S. cerevisiae* cultures in these experiments had a doubling time of 150 min (Dancis *et al.*, 1990), whereas the *C. albicans* cultures in our experiments had a doubling time of only 77.5 ± 3.5 min.

S. cerevisiae reductase activity decreases as cells enter stationary phase, regardless of iron availability (Eide *et al.*, 1992). Thus, it is possible that the observed decrease in *C. albicans* reductase activity may also be due to a similar growth-dependent response.

The effect of growth phase on *C. albicans* reductase activity

Ferric-reductase activity was determined throughout the growth of a culture which progressed from stationary phase through exponential phase and back into stationary phase.

Stationary phase cells of *C. albicans* S/01, previously grown overnight in iron-rich YEPD medium, were used to inoculate fresh YEPD medium. YEPD medium was used throughout these experiments to ensure that cells were iron-replete. This limited the induction caused by iron deprivation, so that any change observed would be due to the growth phase of the cells. Samples were taken periodically throughout the growth of the culture and were assayed for ferric-reductase activity as described in

Fig. 3. *C. albicans* reductase activity is dependent on the growth phase of the cell. Stationary phase cells were taken and inoculated into fresh prewarmed YEPD media at a concentration of 1×10^6 cells ml^{-1} . Cell number was determined periodically ($\blacktriangle$) and samples containing 1×10^7 cells were obtained and assayed for ferric-reductase activity ($\circ$). The graphs represent the mean of three experiments, and the standard deviations are shown.

Methods. Fig. 3 clearly shows that ferric-reductase activity increased as cells left stationary phase, reaching a maximum of 1686 nmol per 10^7 cells h^{-1} 200 min after being inoculated into the fresh medium. This peak in ferric-reductase activity corresponds to an early-exponential stage of growth. This growth-induced response was also observed with *S. cerevisiae*. Under similar growth conditions *S. cerevisiae* reached a maximal reductase activity after 140 min, which corresponds to a very early stage of exponential growth. Both *C. albicans* and *S. cerevisiae* reductase activities decreased by half by mid-exponential stage, and as the cells entered late-exponential/stationary phase the activity decreased to a basal level. The *C. albicans* basal level of 400 nmol per 10^7 cells h^{-1} was maintained as long as the cells were in stationary phase. Consequently, these results confirm that in addition to iron-dependent regulation there is also a growth-phase-dependent regulation of the *C. albicans* ferric-reductase activity.

C. albicans has a cupric-reductase activity

There is a clear link between iron and copper reduction and uptake in *S. cerevisiae* (Hassett & Kosman, 1995). The ferric-reductase can also reduce cupric ions and both activities are regulated by iron and copper. We used quantitative iron and copper reductase assays to determine whether there is a similar link in *C. albicans*.

C. albicans cells taken from an exponentially growing MD culture were transferred into three aliquots of fresh MD medium which was restricted for iron and copper but still

Fig. 4. *C. albicans* has a copper(II) reductase activity which is regulated by both iron and copper. *C. albicans* cells growing exponentially in MD medium (without dipyrldyl) were washed in distilled water, then transferred into three aliquots of fresh prewarmed MD media at a cell titre of 1×10^6 cells ml^{-1} . The MD media contained BPS, and no added iron or copper ($-\text{Fe} -\text{Cu}$, $\blacksquare$), FeCl_3 ($+\text{Fe} -\text{Cu}$, $\square$) or CuCl_2 ($+\text{Cu} -\text{Fe}$, $\blacksquare$). These cultures were then incubated with shaking at 30°C . Once cultures were growing and in exponential phase, samples containing 1×10^7 cells were taken and the ferric-reductase activity was determined. Samples containing 5×10^7 cells were taken at the same time and the cupric-reductase activity was also measured as described in Methods. The graphs represent the mean of three experiments, and the standard deviations are shown.

supported growth (Methods). These aliquots were either unsupplemented with iron or copper or supplemented by the addition of $40 \mu\text{M}$ FeCl_3 or by the addition of $40 \mu\text{M}$ CuCl_2 . Once cells were growing exponentially in the fresh media, samples were taken and tested for both ferric- and cupric-reductase activities as outlined in Methods. The data in Fig. 4 demonstrate that *C. albicans* does have a cupric-reductase activity, which is repressed by the addition of both iron and copper. Moreover, the ferric-reductase activity is also repressed by the addition of copper in the medium, although, under these conditions, copper only repressed activity by 53% whereas iron repressed ferric-reductase activity by 71% (where 100%

is the level of induced reductase activity seen in $-Fe - Cu$ medium). These results do suggest that there is a close relationship between iron and copper metabolism in *C. albicans*. However, it is not yet clear whether the same *C. albicans* enzymes are responsible for both ferric- and cupric-reductase activities.

DISCUSSION

This is the first report of a cell-associated ferric-reductase activity in *C. albicans*. This ferric-reductase activity is clearly induced by iron starvation and is maximal during exponential growth. Studies of the *S. cerevisiae* cell-associated ferric-reductase (Lesuisse *et al.*, 1987; Dancis *et al.*, 1990; Eide *et al.*, 1992; Georgatsou & Alexandraki, 1994) indicate that in this respect regulation of the two reductases is comparable.

Our results also demonstrate that iron and copper metabolism appear to be very closely connected in *C. albicans*. We have demonstrated that the *C. albicans* ferric-reductase activity is regulated in response to levels of copper in the medium. Moreover, *C. albicans* cells can reduce copper and this activity is also regulated by levels of iron and copper. In *S. cerevisiae*, the enzyme components encoded by *FRE1* and *FRE2* are responsible for both iron and copper reductase activities (Hassett & Kosman, 1995; Georgatsou *et al.*, 1995). However, it is not yet clear whether this is the case for *C. albicans*.

Following reduction of ferric iron, the high-affinity transport of ferrous iron into *S. cerevisiae* cells is dependent on the presence of copper (Askwith *et al.*, 1994; Dancis *et al.*, 1994). It has been proposed that copper is essential for the catalytic activity of a protein, encoded by the *FET3* gene, which is required to oxidize ferrous iron in order to release it from the transporter. Therefore this mechanism of iron uptake is dependent on copper and this may explain the closely linked regulation of iron and copper reductase activities in *S. cerevisiae*. The same situation may occur in *C. albicans*, although to date there is no evidence of a ferrous-specific transporter.

Our results were obtained from clinical isolates of *C. albicans* growing in flask cultures at 30 °C. It has not been determined whether this is indicative of *in vivo* growth. However, the experiments were repeated at 37 °C (data not presented) and it was found that the regulation and magnitude of *C. albicans* reductase activity was unchanged at the higher temperature. In all the experiments reported here, *C. albicans* was present in the yeast morphological form. Therefore it will be interesting to investigate the ferric-reductase activity of the hyphal form of *C. albicans*, and to determine the kinetics of any changes that may occur during the dimorphic switch.

We are now in the position to use molecular genetics to investigate the components of the reductase, how the system is regulated and to establish the importance of the reductive iron-uptake mechanism. Since there are many similarities between *C. albicans* and *S. cerevisiae* with respect to the regulation of ferric- and cupric-reductase activities, it is likely that *S. cerevisiae* can be used to clone *C. albicans*

genes involved in both iron and copper metabolism, either by complementation of *S. cerevisiae* mutants with *C. albicans* genomic DNA or by homology to known *S. cerevisiae* genes. These techniques have already been used to successfully clone many *C. albicans* genes involved in a variety of cell structures and functions (reviewed by Sudbery, 1994).

To date, the role and implication of iron acquisition and iron metabolism for virulence of *C. albicans* has not been established. It is known that human serum inhibits the growth of *C. albicans* cells in culture due primarily to the presence of the high-affinity iron-binding protein transferrin, which sequesters serum iron. The related proteins lactoferrin and ovotransferrin have also been shown to have antifungal activity (Valenti *et al.*, 1986). These observations suggest that competition for iron is an important virulence factor for *C. albicans*. Moreover the expression of two virulence characteristics, adherence to epithelial cells and the ability to form germ tubes, has been shown to be dependent on iron, and there are quantitative differences between the protein profiles of cells grown in iron-deficient and iron-replete conditions (Sweet & Douglas, 1991b).

It is probable that *C. albicans* can acquire iron from a variety of sources. These include siderophores, complement-coated red blood cells and haemoglobin, since addition of exogenous haemoglobin or haem can reverse serum inhibition (Moors *et al.*, 1992). However, it is still not clear how biologically active ferrous iron is obtained from these or any other sources. The action of the cell-associated ferric-reductase, either solely or in conjunction with other uptake mechanisms, may be important for iron acquisition.

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