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# Utilization of Ferroproteins by *Candida albicans* during Candidastasis by Apotransferrin

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Many reports have stated that some of the pathogenic bacteria can obtain iron from ferroproteins, such as cytochrome C, ferritin, hemin, hemoglobin, and myoglobin. These reports prompted us to determine if an opportunistic pathogenic fungus, *Candida albicans*, can utilize ferroproteins to circumvent the iron-regulatory effect of transferrin. The following assays were carried out to measure *in vitro* growth stimulation by the ferroproteins: as an initial step, *C. albicans* was cultured in iron-free (pretreated with apotransferrin for 24 h) culture medium. Once *Candida albicans* yeast cell growth reached stasis from iron starvation, individual ferroproteins were added to the culture media. Results showed that hemin, hemoglobin, and myoglobin supported a partial growth recovery. Additional studies with haptoglobin, a serum protein that interacts with the globin moiety of certain ferroproteins, established that *C. albicans* could obtain iron from the haptoglobin-ferroprotein complexes. These data indicate that the heme part of the ferroproteins is the source of iron. This implies that heme oxygenase, CaHMX1 might be involved in bringing about dissociation of heme-containing protein for iron-acquisition. In addition, anticandidal activity of transferrin takes place not only by the process of iron regulation, but also by direct interaction with the yeast cells.

**Key words:** *Candida albicans*, Transferrin, Ferroproteins, Haptoglobin, Heme

## INTRODUCTION

*Candida albicans*, like most pathogenic microorganisms requires iron, a mineral that is abundant in nature to perform its own metabolic activities (Young *et al.*, 1967; Weinberg, 1971; Howard, 1999). However, due to the extreme ($10^{-38}$ M) insolubility of ferric ions ($Fe^{+3}$) at neutral or alkaline pH, only limited amounts of free iron are available for microbial metabolism. Furthermore, as in vertebrate tissues iron is tightly bound to particular iron-chelating homologous glycoproteins, called transferrins, this element is available only in minute quantities within the host. One of the major functions of transferrin is iron-chelation, which is an important component of mammalian host defense against infection (Finkelstein *et al.*, 1983; Griffiths, 1983). Three proteins typify the transferrin family: serum transferrin which is found in circulating plasma and lymph, transports iron from the intestine to the bone marrow for incorporation into hemoglobin, and also transfers iron to iron storage depots maintained in the liver and spleen (Fletcher and Huehns, 1986); secretory lactoferrin found in milk (Masson and Heremans, 1971), other external secretion (Masson, *et al.*, 1966), as well as in leukocytes (Green *et al.*, 1971); and ovotrasferrin (conalbumin) present in egg white.

In normal human plasma, the concentration of transferrin is about 30 μM (2 mg/mL), whereas iron is approximately 18-21 μM (Young *et al.*, 1967; Weinberg, 1971; Howard, 1999). As the reported plasma protein concentration has the capacity to bind to 60 μM iron, in healthy human beings, transferrin is 30 to 35% saturated with iron. This situation results in a net free serum iron concentration of about $10^{-18}$ M (Bullen *et al.*, 1978), which is well below the concentration required for microbial growth (Weinberg 1978; Bullen, 1981; Cowart and Foster, 1985; Simonson *et al.*, 1982). Nevertheless, under appropriate conditions certain pathogenic bacteria can obtain sufficient iron *in vivo* from extracellular ferroproteins such as heme or hemoglobin for successful multiplication under iron-depletion conditions by transferrin (Perry and Brubaker, 1979; Gentry *et al.*, 1986; Pidock *et al.*, 1998). It is not

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clearly determined whether fungi can utilize ferroproteins for their metabolic activities. The growth of *C. albicans* is retarded by transferrin (Roth and Goldstein, 1961; Caroline *et al.*, 1964; Esterly *et al.*, 1967). According to a recent report by Santos (Santos *et al.*, 2003), *C. albicans* heme oxygenase gene (CaHMX1) was required for iron assimilation. A more recent report (Pendrak *et al.*, 2004) showed that induction of CaHMX1 occurred by exposure to hemin was further activated by deficiency of iron. In these studies, the expression of CaHMX1 gene upon iron-regulation was mostly examined at the molecular level, but growth-recovery pattern of *C. albicans* by CaHMX1 expression remains unclear when the fungus confronts a condition of the iron-starvation in a culture medium.

In this present study, we have demonstrated that *C. albicans* yeast cells facilitate the utilization of ferroproteins to circumvent candidastasis produced by iron-free (apo) transferrin *in vitro*. Additionally, we also determined the growth-recovery pattern of the fungus under a condition of candidastasis by apotransferrin.

## MATERIALS AND METHODS

### Reagents

Iron-free (apo) transferrin (apoconalbumin, chicken egg white), hemin (bovine), and hemoglobin (human) were purchased from U.S. Biochemical Co. (Cleveland, OH). Haptoglobin (humin), myoglobin (horse skeleton muscle), ferrous ammonium sulfate [$Fe(NH_4)_2(SO_4)_26H_2O$], ethyleneglycol-bis-(β-amino-ethyl-ether) *N,N'*-tetra acetic acid (EGTA) were obtained from Sigma (St. Louis, MO). Hematoporphyrin IX (deferrated hemin) was purchased from Aldrich Chemical Company (Milwaukee, WI). Dialysis bag [6~8000 daltons (Da) mol. wt. cut-off] was obtained from Spectrum Medical Industries, Inc., (Los Angeles, CA).

### Organism and culture media

*C. albicans* 3153A and 8011 strains were obtained from the Mycological Reference Laboratories, Public Health Laboratory Service and previously characterized CA-1 strain(Han *et al.*, 2000, 2001; Han and Lee, 2005a; Han, 2005b) were also used. The strains were maintained on Sabouraud dextrose agar (SDA; Difco Laboratories, Detroit, MI) slants and cloned on this medium every month to ensure homogeneity in culture. Inocula were prepared from overnight cultures (usually 15-16 h incubation) in a Sabouraud dextrose broth at 37°C. Experiments with the yeast cells were carried out in a modified Winge (Garcia-Mendoza and Novales-Lindieu, 1968) liquid medium (pH 7.5; 0.3% yeast extract, 0.2% glucose) supplemented with 15 mM $NaHCO_3$. Prior to use, the modified Winge medium was sterilized by filtration with a disposable filter apparatus (pore size = 0.45 μM; Nalge Company, Rochester, NY). Sodium bicarbonate was added to enhance antifungal activity of apotransferrin (Phelps and Antonini, 1975; Graham and Bates, 1976).

### Ferroprotein utilization assay

A volume of 2 mL of apotransferrin (20 mg/mL) dissolved in the modified Winge medium and filter sterilized was added into 18 mL of the same medium (total volume = 20 mL) in a 125 mL Erlenmeyer plastic flask (Nalge Company). The final concentration of apotransferrin was 2 mg/mL. Culture media were pre-incubated at 37°C for 24 h before inoculation with *C. albicans* yeast cells to allow the apotransferrin to bind to endogenous free iron in the culture media, which were inoculated with $1\times10^5$ yeast cells/mL from an exponentially growing (15-16 h) culture. At various time periods, the cell number was enumerated with a hemocytometer. The employed test ferroproteins were hemoglobin, myoglobin, hemin (ferriprotoporphyrin, oxidized heme), and hematoporphyrin IX (deferrated hemin). Hemin and hematoporphyrin IX are actually not ferroproteins, but were considered as such in this study. Once yeast cell growth reached stasis, ferroproteins were each added to the culture media at an equimolar iron concentration (10 μM). As a control, ferrous ammonium sulfate was used. Hemin and hematoporphyrin IX were prepared in triethanolamine (0.01%). Preliminary data showed that triethanolamine was ineffective against yeast cell growth. The remaining ferroproteins and the inorganic iron were prepared in the Winge liquid media and filter-sterilized as described above. After the addition of the ferroproteins, the culture flasks were incubated at 37°C for 5 days. In some of the experiments, EGTA (300 nM as a final concentration) was incorporated with apotransferrin to ensure the absence of free iron.

### Utilization of haptoglobin-treated ferroprotein assay

Filter-sterilized haptoglobin-ferroprotein mixture was prepared as previously described (Eaton *et al.*, 1982) and added to the culture medium at time 48 h, and recovery of growth was measured. Haptoglobin may act bacteriostatically by binding to free hemoglobin at a 1:1 molar ratio, thus making heme iron unavailable for microbial growth (Eaton *et al.*, 1982; Pendrak *et al.*, 2004). Concentration of the ferroproteins was chosen at an equimolar iron concentration of 200 nM. Prior to these experiments, a maximal iron concentration for maximal yeast growth under iron regulation by apotransferrin was determined by adding decreasing concentrations of a ferroprotein at time 48 h as described above in the ferroprotein utilization assay. The range of ferroprotein concentration was from 4 to 1,024 nM equimolar iron.

### Dialysis bag assay

The modified Winge liquid medium was distributed into a dialysis bag (2 mL) and outside a dialysis bag (18 mL) in a 125 mL flask. Each portion of the culture medium contained 2 mg/mL of apotransferrin. After pre-incubation of the apotransferrin with the culture medium for 24 h, *C. albicans* (100 μL at $1\times10^6$ yeast cells/mL) was inoculated into the medium both inside and outside the dialysis bag. When growth of yeast cells reached the stasis stage (48 h) from iron deprivation by apotransferrin, the ferroproteins (hemin, hemoglobin, and myoglobin) in 300 nM EGTA were added separately into the dialysis bags of individual flasks. Ferroproteins were at equimolar concentrations to 10 μM of iron. Controls contained the culture medium and a) lacked treatment with apo-protein, or b) apotransferrin (inside and outside the dialysis bag) but lacked the ferroprotein addition. The enumeration of cells was carried out for yeast cells present in the medium outside the dialysis membrane.

### Statistical analysis

The results are expressed as mean ± standard error. The significance of difference among the groups was determined by Student *t*-test. *P* values less than 0.05 were considered to be statistically significant.

## RESULTS

### *C. albicans* yeast cells utilize ferroproteins during candidastasis by apotransferrin

To determine if *C. albicans* yeast cells utilize ferroproteins, ferroproteins were added to the culture media under iron-deficient condition by apotransferrin. Results showed that all of the test ferroproteins prompted an immediate growth recovery (Fig. 1). The increase in cell number was due to release of the yeast cells from the inhibitory static growth during the regulation of transferrin. Hemoglobin, myoglobin, and hemin almost equally supported the growth of yeast cells (approximately 50 fold increase) when compared to the apotransferrin-regulated control. Among the three kinds of the ferroproteins, growth stimulation by hemoglobin was slightly greater than the other two ferroproteins. The magnitude of the growth recovery was not equal to the growth as observed for the untreated controls. Difference between ferroprotein-treated and untreated cultures was significant ($P < 0.05$). Hematoporphyrin IX, a negative control, was ineffective in stimulating the growth of yeast cells. Growth stimulation was not observed even by the addition of 10 μM inorganic iron (ferrous ammonium sulfate, data not shown). The same experiments were performed with two other strains of *C. albicans*, CA-1 and 8011. The CA-1 (Fig. 2) and strain 8011 (data not shown) produced results very similar to those of strain 3153A.

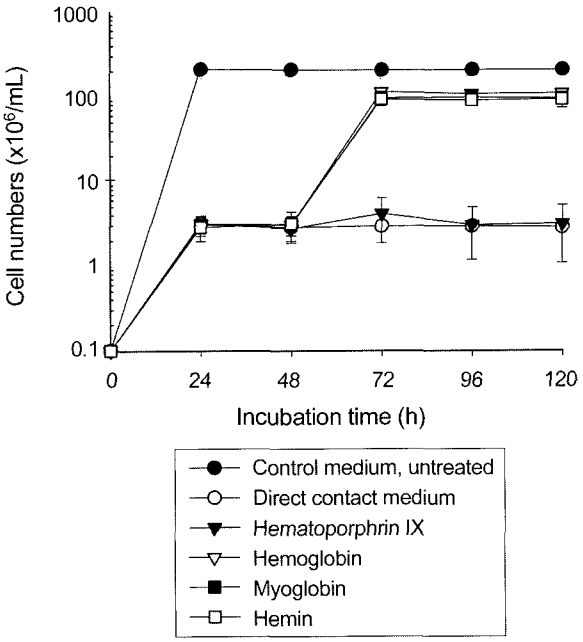


**Fig. 1.** Effect of ferroproteins on growth of *C. albicans* yeast cells (strain 3153A) while under fungistatic regulation by apotransferrin. *C. albicans* yeast cells were cultured in apotransferrin-pretreated Winge broth medium. After 48 h, hemoglobin, myoglobin, or hemin at equimolar levels to iron (10 μM) was added into the medium. For a control, 10 μM of hematoporphyrin IX was added into the culture medium instead of a ferroprotein. Cell counts were made by microscopy. Results showed that all test ferroproteins stimulated growth-recovery when compared with control medium containing no ferroprotein. The difference was significant ($P < 0.05$). Values were expressed as mean ± S.E.

Unless specified, all other remaining experiments were carried out on strain, 3153A.

In other experiments, when EGTA (300 nM) was incorporated into the experimental design, the patterns of growth stimulation recovering from transferrin regulation in the presence of EGTA were very similar to those observed in the presence of EGTA (Fig. 3).

### *C. albicans* yeast cells can still utilize the haptoglobin-treated ferroproteins

Results from the addition of decreasing concentration of ferroprotein experiments showed that concentrations of three ferroproteins (hemin, hemoglobin, and myoglobin) as low as 4 nM permitted some recovery from apotransferrin growth regulation (data not shown). Maximal growth was attained at an iron concentration of 128 nM for those ferroproteins (data not shown). Based on these data, when a haptoglobin (300 nM)-treated ferroprotein (200 nM) mixture (1.5:1 molar ratio) was added into the culture media as a source of iron, yeast cell growth was still recovered (Fig. 4A). Growth stimulation was similar in the presence (Fig. 4A) or absence (Fig. 4B) of haptoglobin,

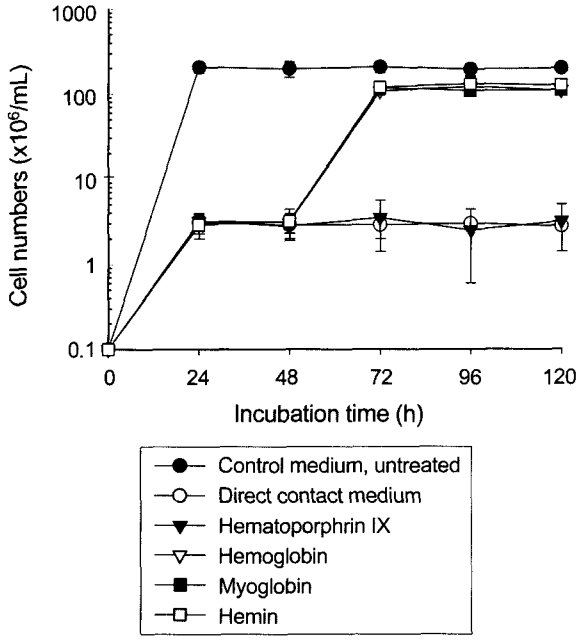


**Fig. 2.** Effect of ferroproteins on growth of *C. albicans* (strain CA-1) while under fungistatic regulation by apotransferrin. The same experiments were performed with another strain of *C. albicans*, CA-1. The CA-1 strain produced results very similar to those of strain 3153A. Difference in growth between the ferroprotein received and control media was significant ($P < 0.05$). Values were expressed as mean ± S.E.

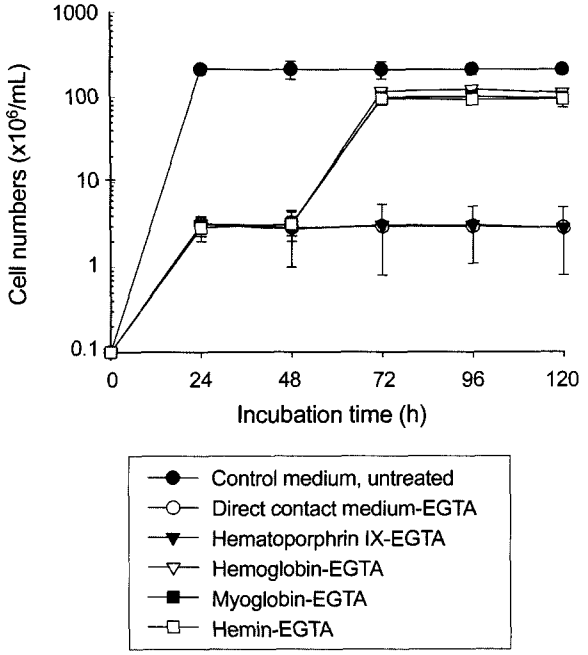


**Fig. 3.** Effect of ferroproteins on growth of *C. albicans* yeast cells while under fungistatic regulation by apotransferrin plus EGTA. In these experiments, EGTA was incorporated into the culture medium to ensure the condition of iron-deprivation. Data showed that EGTA addition resulted in no influence on the growth-recovery by ferroproteins as shown in Figs. 1 and 2. Values were expressed as mean ± S.E.

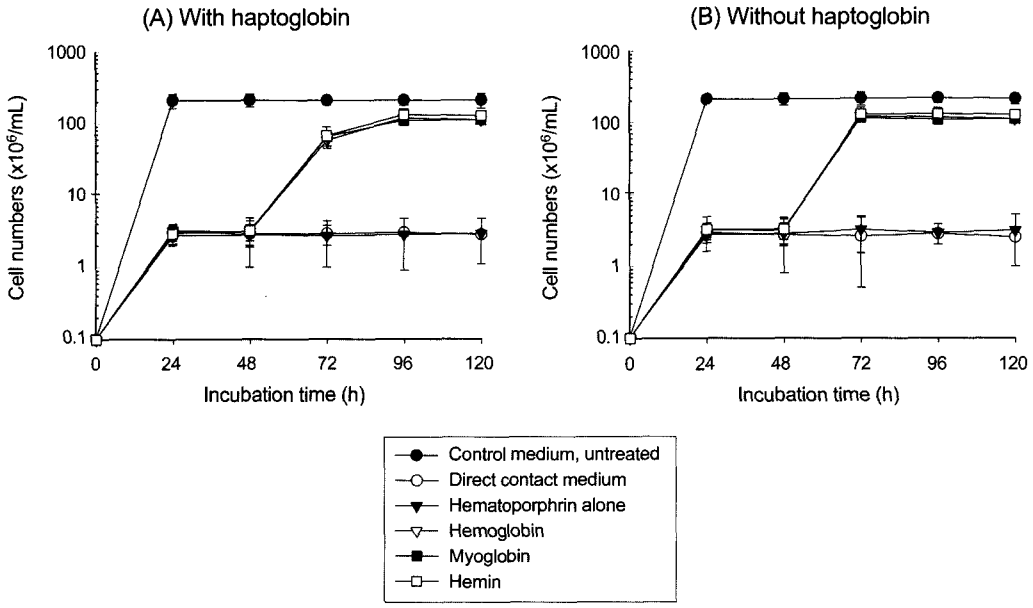


**Fig. 4.** Effect of haptoglobin on the growth stimulation by the ferroproteins under fungistatic regulation by apotransferrin. *C. albicans* yeast cells were cultured in apotransferrin-pretreated Winge broth medium. At 48 h, hemoglobin, myoglobin, or hemin pretreated (A) or untreated with haptoglobin were added into the culture medium. Cell counts were made by microscopy. The haptoglobin treatment of ferroproteins had no interference on iron utilization from the ferroproteins ($P < 0.05$). Values were expressed as mean ± S.E.

thus stating that this protein neither interfered with nor facilitated the iron utilization from the test ferroproteins. Although growth during the first 24 h after the addition of the mixture appeared to be slightly delayed, there was the

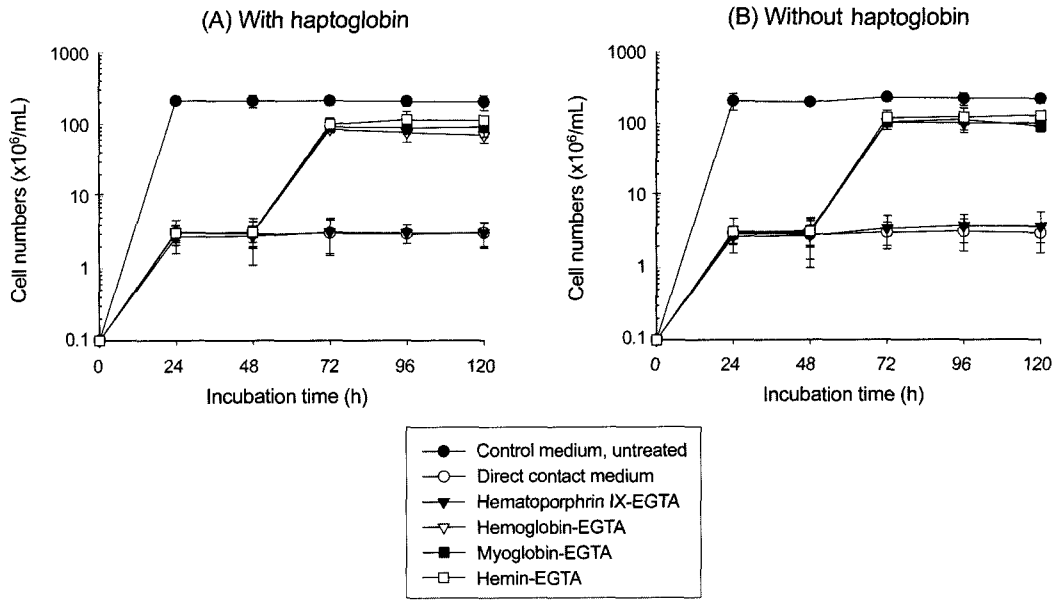


**Fig. 5.** Effect of haptoglobin on the growth stimulation by the ferroprotein under fungistatic regulation by apotransferrin plus EGTA. For confirmation of the results from Fig. 4, respective ferroprotein pretreated (A) or untreated (B) with haptoglobin was added in combination with EGTA into the culture media. The incorporation of EGTA resulted in similar pattern of growth as shown in Fig. 5. Values were expressed as mean ± S.E.

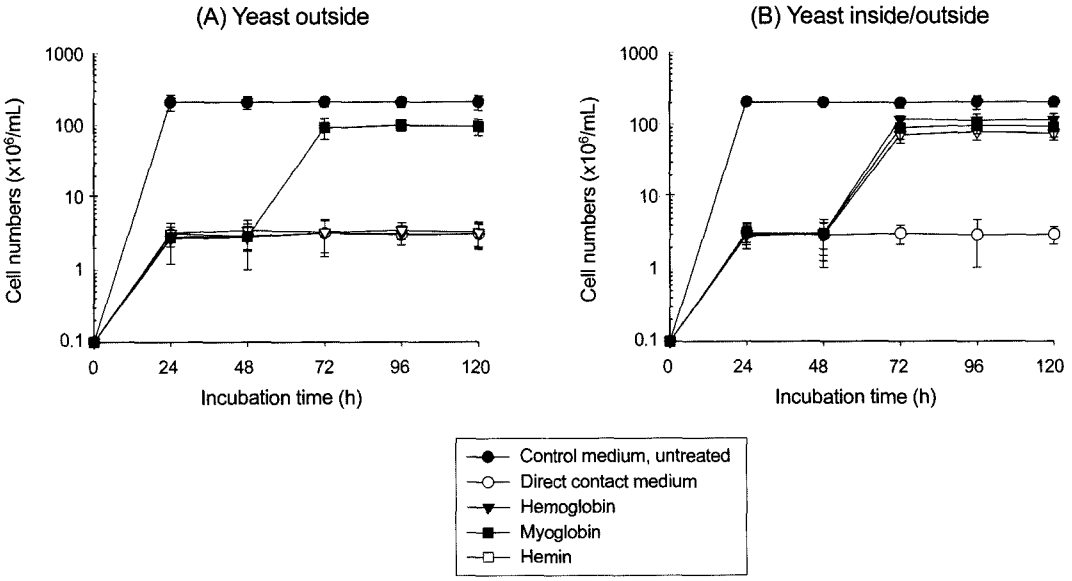


**Fig. 6.** *C. albicans* yeast cell bioassay for detection of ferroprotein degradation and release of iron-containing nutrients. Apotransferrin-pretreated with medium was dispensed into a dialysis bag and into a flask. To one set of flask, yeast cells were added to the outside of the bag. To the second set, yeast cells were added to both outside and inside the bag. After 48 h, ferroproteins in combination with EGTA were added into the dialysis bag lacking the yeast (A) or containing the yeast (B). Hemin supported the growth-recovery only when the yeast was present outside bag, whereas hemoglobin and myoglobin gave no response. Values were expressed as mean ± S.E.

same degree of growth recovery even after the lapse of initial 24 h (Fig. 4). Similar experiments were repeated with the incorporation of EGTA (300 nM) into the culture media to ensure the absence of free iron. The obtained data showed that the presence of EGTA had no effect on the growth recovery stimulated by haptoglobin-complexed ferroproteins (Fig. 5A & B).

## Iron acquisition from ferroproteins by *C. albicans* yeast cells

When *C. albicans* yeast cells were inoculated into the medium outside the dialysis bag (Fig. 6A), the sole ferroprotein to stimulate growth recovery was only hemin, whereas when yeast cells were inoculated into the medium both inside and outside the dialysis bag (Fig. 6B),

all of the ferroproteins, hemin, hemoglobin, and myoglobin stimulated growth recovery. Control cultures resulted in maximal growth in the untreated medium and minimal growth in the apotransferrin-containing medium in both sets of cultures.

## DISCUSSION

In essence, the concentration of free ionic iron in body fluids is far too low to support normal microbial growth. To successfully multiply under these conditions, pathogenic microbes must possess a mechanism(s) for scavenging the iron associated with host iron-binding protein, such as transferrin. Some pathogenic bacteria can utilize iron-containing proteins, namely ferroproteins, as sources of iron (Otto *et al.*, 1990; Stull, 1997; Zakaria-Meehan *et al.*, 1998). In case of *C. albicans*, there is a general agreement that this fungus requires iron for growth and the candidastatic activity of human serum is attributed to the unavailability of free iron (Cutler and Han, 1996). In such a harsh environment in the host, how the fungus obtains iron for its metabolism is an interesting question to probe.

In the present work, we determined the utilization of ferroproteins by *C. albicans* as one alternative means to bypass the iron regulatory effects of apotransferrin. When apotransferrin was incorporated into the culture medium, there was an initial growth phase followed by stasis, which was presumably due to exhaustion of the intracellular iron storage pool. The iron concentration of 10 μM was chosen to provide a sufficient amount of iron for maximal growth, but was insufficient to fully saturate the dissolved apotransferrin, if iron bound to ferroprotein was released into the surrounding milieu. Any free iron should have been removed *via* chelation and thereby must have not affected cell growth in the culture medium containing 2 mg/mL (or 23 μM) of apotransferrin. Because transferrin chelates iron in an iron to transferrin molar ratio of 2:1, theoretically it would require 46 μM iron to fully saturate the apotransferrin. For the purpose of calculation, the endogenous iron content of the medium was considered inconsequential. Thus, the possibility that iron was first released from the ferroproteins and utilized by *C. albicans* was invalidated because growth recovery ensured, although the culture medium contained apotransferrin and EGTA. This suggests that larger protein component carries the iron into the yeast cells. In this part of the study, this question was investigated by using haptoglobin that binds to the globin moiety and not the heme group (Hamaguchi *et al.*, 1971; Eaton, *et al.*, 1982). Results showed that *C. albicans* yeast cells were able to obtain iron from ferroprotein-haptoglobin complexes, indicating that the heme group was the iron source from the ferroprotein. Experiments with ferroprotein within or outside a dialysis bag, and with or without yeast cells defined more definitively size of moiety providing iron for yeast cells during apotransferrin regulation. In addition, this assay method was performed to determine if yeast cells could degrade the ferroproteins to release iron into the surrounding milieu. Theoretically, hemoglobin (64.5 KDa) and myoglobin (16.9 KDa) are unable to pass through the dialysis bag unless and until they are degraded. Hemin (652 Da), being of small size, is able to pass through the dialysis membrane unimpeded. Our results showed that only hemin stimulated the growth-recovery, which supports that degradation by *C. albicans* was unnecessary.

Throughout the studies, it was noteworthy that growth recovery of *C. albicans* yeast cell by utilizing ferroproteins never achieved the maximal growth as observed with untreated control cells. For this remark, three possible explanations can be given. First, the choice of 10 μM of ferroproteins was insufficient to promote maximal growth. However, maximal growth was attained at a concentration of 128 nM for the test ferroproteins. Therefore, more than sufficient ferroprotein was added in the initial studies to support maximal growth of yeast cells. A second explanation would be that the addition of ferroprotein resulted in a disruption of the surface interaction of yeast cell to apotransferrin, therefore permitting growth. Growth-recovery did not reach control values because the apotransferrin within the medium chelated the necessary iron. Such a possibility eliminates any utilization of the ferroprotein as source of iron. This explanation seems unlikely because hematoporphyrin IX failed to support any recovery of growth. Finally, maximal growth could not be attained due to some unknown regulatory influence exerted by apotransferrin while in contact with the cells. Regarding this aspect, it has been suggested that apotransferrin possess an anticandidal activity by mediating direct interaction with *C. albicans* yeast cells as another possible mechanism, besides the chelation of iron (Valenti, 1986). Currently, in our lab, the possibility of the first mechanism is under investigation. Collectively, these data indicate that *C. albicans* possess the ability to dissociate hemoglobin and myoglobin and utilize the heme group as a source of iron. However, the manner in which *C. albicans* dissociates hemoglobin and myoglobin was not determined in this study.

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