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Glutathione-dependent extracellular ferric reductase activities in dimorphic zoopathogenic fungi

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Abstract

In this study, extracellular glutathione-dependent ferric reductase (GSH-FeR) activities in different dimorphic zoopathogenic fungal species were characterized. Supernatants from *Blastomyces dermatitidis*, *Histoplasma capsulatum*, *Paracoccidioides brasiliensis* and *Sporothrix schenckii* strains grown in their yeast form were able to reduce iron enzymically with glutathione as a cofactor. Some variations in the level of reduction were noted amongst the strains. This activity was stable in acidic, neutral and slightly alkaline environments and was inhibited when trivalent aluminium and gallium ions were present. Using zymography, single bands of GSH-FeRs with apparent molecular masses varying from 430 to 460 kDa were identified in all strains. The same molecular mass range was determined by size exclusion chromatography. These data demonstrate that dimorphic zoopathogenic fungi produce and secrete a family of similar GSH-FeRs that may be involved in the acquisition and utilization of iron. Siderophore production by these and other fungi has sometimes been considered to provide a full explanation of iron acquisition in these organisms. Our work reveals an additional common mechanism that may be biologically and pathogenically important. Furthermore, while some characteristics of these enzymes such as extracellular location, cofactor utilization and large size are not individually unique, when considered together and shared across a range of fungi, they represent an important novel physiological feature.

Introduction

Iron is an essential nutrient for the metabolism and growth of nearly all living organisms. Although iron is the second most abundant metal on earth, it is generally present in forms that are not readily bioavailable. Under aerobic conditions iron is extremely stable as ferric ions (Fe^{3+}). This form is essentially insoluble in water at neutral pH as aqueous environments bring about the formation of ferric hydroxide species polymerizing into an amorphous gel. This occurs due to a loss of protons from $\text{Fe}^{3+}(\text{H}_2\text{O})_n$ complexes, because ferric iron is a strong Lewis acid. In contrast, ferrous iron (Fe^{2+}) is a weak Lewis acid that is not able to ionize readily. Iron in this reduced form is relatively soluble at neutral pH, but also spontaneously auto-oxidizes under aerobic conditions. In general, Fe^{3+} is the major form of iron found under the aerobic atmosphere of our planet (Lancashire, 2002; Kosman, 2003).

Limitation of iron availability is utilized by many animal species as an antimicrobial defence strategy. Host mechanisms, e.g. binding of iron to proteins such as transferrin or lactoferrin, permit host cells to maintain access to the metal while preventing the invading fungal pathogens from acquiring iron and, consequently, from successful parasitism (Weinberg, 1999). Since iron is generally critical for viability, successful pathogens have developed mechanisms for iron acquisition and utilization in the face of its environmental or host-mediated scarcity (Howard, 1999). Almost all iron uptake by fungi involves reduction from the ferric to the ferrous form via two general mechanisms: (i) uptake before reduction or (ii) reduction before uptake (de Luca & Wood, 2000). The first strategy is often based on secretion of low-molecular-mass iron chelators called siderophores and is regulated by iron-activated repressors. In some cases siderophores can also be used for iron storage. The second mechanism starts with iron reduction that can be catalysed by specific membrane-associated or secreted enzymic proteins (iron reductases) or secreted external low-molecular-mass reductants. Reduction of Fe^{3+} to Fe^{2+} followed by transport of the latter form into the fungal cell may provide an effective way to acquire iron from inorganic or organic ferric salts, from Fe^{3+} -loaded siderophores, or from host Fe^{3+} -binding proteins (de Luca & Wood, 2000).

Among pathogenic fungi, *Cryptococcus neoformans* reduces iron using secreted ferric reductants (Nyhus *et al.*, 1997), whereas *Candida albicans* expresses cell-associated ferric reduction activity (Morrissey *et al.*, 1996) and a high-affinity iron permease (Ramanan & Wang, 2000). At least three strategies have been demonstrated for this process in the dimorphic pathogenic fungus *Histoplasma capsulatum* (Woods, 2003). One mechanism is the secretion of low-molecular-mass Fe^{3+} -chelating hydroxamate siderophores and utilization of xenosiderophores produced by other microbes (Howard *et al.*, 2000). A second possible route to acquire iron is its gradual release from transferrin at acidic pH (Newman *et al.*, 1994). A final iron acquisition strategy relies on secretion of enzymic and non-enzymic ferric reducing activity (Timmerman & Woods, 1999, 2001). In *H. capsulatum*, three iron-reducing activities are expressed: (i) glutathione-dependent ferric reductase (GSH-FeR), (ii) low-molecular-mass non-enzymic reductants, and (iii) cell-surface reducing activity (Timmerman & Woods, 1999). GSH-FeR could utilize siderophores and mammalian compounds such as transferrin as enzymic substrates, which is consistent with the utility of this process in the soil or in the host (Timmerman & Woods, 2001). Moreover, GSH-FeR is secreted during both mould and yeast growth and the expression of all of three iron-reducing activities is increased when growing under iron-limiting conditions (Timmerman & Woods, 2001). These findings are consistent with a role of ferric reductase activities in iron acquisition.

In this study, we provide evidence of the ability of four dimorphic zoopathogenic fungal species, *H. capsulatum*, *Blastomyces dermatitidis*, *Paracoccidioides brasiliensis* and *Sporothrix schenckii*, to express a highly similar extracellular iron reduction system based on GSH-FeRs. The data presented here also indicate an alternative for iron acquisition in these fungi distinct from siderophore production that is frequently accepted as the mechanism by which this process occurs.

Methods

Fungal strains and growth conditions

The strains used in this study are listed in Table 1. Cells were routinely grown in liquid *Histoplasma*-macrophage medium (HMM) (Woods *et al.*, 1998) at 37 °C in 5% CO₂/95% air atmosphere either for 4 days in 1.5 l batch cultures or for 2, 4 and 6 days in 20 ml cultures in time-course experiments, respectively. These time points roughly correspond to early exponential, exponential and stationary growth phases of strain G217B.

Preparation of supernatants

Cells were removed by centrifugation (3000 g, 5 min, 25 °C). Supernatants were sterilized through 0.2 µm filters and partly concentrated by ultrafiltration using an Amicon 8400 unit equipped with 10 kDa nominal molecular mass cut-off limit YM10 regenerated cellulose membranes (Millipore). A final concentration of high-molecular-mass fractions was achieved using Centrifree-15 Biomax centrifugal filter devices (Millipore) with 5 kDa cut-off polysulfone membranes, and the supernatants were diafiltered against PBS, pH 7.2. This procedure concentrates the ferric reductase enzymic activity we reported for strain G217B and removes any low-molecular-mass reductants (Timmerman & Woods, 1999).

Protein determination

The protein concentration was determined using the BCA Protein Assay Kit (Pierce), with bovine serum albumin as a standard.

Assays of GSH-FeR activities in supernatants

Ferric chloride and ferric nitrate were used as substrates at a concentration of 50 µM. Formation of Fe²⁺ was quantified with the chromogenic chelator ferrozine (Sigma) in a microtitre plate-based assay (Timmerman & Woods, 1999). The reaction was carried out in PBS (except where noted) at 37 °C for 6 h and then the absorbance was measured at 562 nm. The negative control contained all compounds (including GSH) except supernatants and no non-enzymic iron reduction was observed under the conditions described above. No differences in enzymic activity for substrates used in both free and chelated forms were observed in this and other studies reported elsewhere (Mazoy *et al.*, 1999; Timmerman & Woods, 2001). To determine the effect of pH upon GSH-FeR activity, the following buffers were used at 150 mM concentration: acetate (pH 4.0 and 5.0), MES (pH 6.0), phosphate (pH 7.0) and HEPES (pH 8.0). Al³⁺ and Ga³⁺ ions (as nitrates) at concentrations ranging from 0.05 to 350 µM were used in GSH-FeR inhibition studies. EC₅₀ values were determined by a non-linear sigmoidal curve fit using OriginPro v. 7.0383 (OriginLab).

Detection of GSH-FeR activity in native gels

Non-denaturing discontinuous one-dimensional PAGE was performed without SDS using 7.5% polyacrylamide in the separating gel and 3.5% in the stacking gel (Laemmli, 1970). Concentrated supernatant samples containing 20–120 µg protein were adjusted with 50 mM Tris/HCl (pH 8.0) to 100 µl and 50 µl loading dye (10 mM Tris/HCl, pH 7.5, 0.01% bromophenol blue, 30% glycerol) was added. Electrophoresis was carried out at a constant

current of 20 mA at room temperature overnight using an SE 410 Vertical Electrophoresis Unit (Hoefer). To detect ferric reductase activity, the gel was washed twice with distilled water and incubated in PBS (pH 7.2) for 1 h with shaking. Subsequently, the buffer was removed and the gel was immersed in 250 ml PBS containing 2 mM ferrozine, 0.1 mM FeCl_3 and 1.6 mM glutathione, and incubated until red bands became visible. The molecular masses were calculated on the basis of comparison with the following high-molecular-mass native standards: thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa) and albumin (66 kDa) (Amersham Pharmacia). A part of the gel containing standards was stained with GelCode Blue Stain Reagent Kit (Pierce).

Subunit composition of GSH-FeR

Concentrated supernatant from the strain G217B culture was separated in a non-denaturing discontinuous one-dimensional polyacrylamide gel as described above and subsequently stained for the presence of GSH-FeR activity. The visible red band was cut out of the gel and placed in Spectra/Pro Membrane dialysis tubing (3.5 kDa cut-off; Spectrum Laboratories), which was presoaked in electroeluting buffer (25 mM Tris, 192 mM glycine, pH 8.3). Electroelution was carried out at 50 V for 4 h and then at 100 V for 5 min with reverse polarity. Fluid from the dialysis bag was transferred to a new one and dialysed overnight against 2 mM Tris/HCl, pH 7.4, supplemented with 30 mM NaCl. Next, the sample was concentrated using Nanosep 10K Omega centrifugal device (Pall Life Sciences), tested for the GSH-FeR activity and separated in a standard 10% denaturing discontinuous SDS-polyacrylamide gel. To renature proteins after electrophoresis, the gel was washed with 2.5% Triton X-100 for either 1 or 12 h. Next, an in-gel GSH-FeR activity detection assay was performed as described above.

Size exclusion chromatography

The AKTA purifier system (Amersham) equipped with a HiPrep 16/60 Sephacryl S200 HR column was used. The column was equilibrated either with 375 mM Tris/HCl, pH 8.8, or with PBS, pH 7.2. Maximum 1.0 ml samples of concentrated supernatants from G217B or UCLA 531S cultures were loaded and proteins were eluted at a flow rate of 0.25 ml min⁻¹. Then, 1.5 ml fractions were collected and tested for GSH-dependent iron-reducing activity. For determination of molecular masses, the same column was calibrated with a gel filtration calibration kit, including blue dextran (~2000 kDa), α -amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa) and cytochrome *c* (12.4 kDa) (Sigma).

Results

Detection of extracellular GSH-FeR activity in dimorphic pathogenic fungi

Concentrated supernatants from several strains of dimorphic zoopathogenic fungi, belonging to four genera, were assayed for the presence of GSH-FeR activity. All species tested were able to reduce Fe^{3+} ions, but showed some variation in the level of activity (Table 2). The highest GSH-dependent iron reduction activity levels were observed in 6-day-old supernatants from *B. dermatitidis* as well as from *H. capsulatum* var. *capsulatum* strains belonging to RFLP class III (G184AS, G184AR, G184AE and G186AS). The ability for

extracellular GSH-dependent ferric iron reduction by these fungi increased during growth, whereas activity in the remaining strains was more uniform over time. Similarly, the highest culture iron reduction capacity was detected in *H. capsulatum* var. *capsulatum* G184AS, G184AR and G186AS strains. The expression activity patterns in other strains were relatively similar with the exception of *P. brasiliensis* cultures, which showed about tenfold lower total extracellular iron reduction activity.

Stability of GSH-FeR activities at different pHs

Activity of extracellular GSH-FeRs of dimorphic fungi was tested in acidic, neutral and slightly alkaline environments (Fig. 1). The activities of enzymic iron reduction by the *H. capsulatum* var. *capsulatum* Downs, UCLA 531S and G217B strains as well as by *S. schenckii* were similar over a broad pH range, whereas strains belonging to *H. capsulatum* var. *capsulatum* RFLP class III (G184AS, G184AE, G184AR and G186AS) and *B. dermatitidis* expressed the highest reduction activity under acidic conditions in which ferric iron is most soluble. Strain G222B as well as *H. capsulatum* var. *duboisii* RV26821 and *P. brasiliensis* most efficiently reduced iron in non-acidic environments, but this activity was also observed at low pH. The range of variation for all strains at all pHs was no greater than about fourfold.

Inhibition of GSH-FeRs by trivalent aluminium and gallium ions

The inhibitory actions of aluminium and gallium ions were tested against GSH-FeRs of dimorphic zoopathogenic fungi. Aluminium and gallium are, like iron, group IIIA metals. Due to their physical/chemical properties they can substitute for Fe^{3+} in many molecular processes and disrupt them by competitive inhibition (Olakanmi *et al.*, 2000). Al^{3+} and Ga^{3+} , unlike Fe^{3+} , are unable to undergo redox cycling and form Al^{2+} and Ga^{2+} ions, respectively (Chitambar *et al.*, 1988). The concentrations of Al^{3+} and Ga^{3+} ions required for 50% inhibition of ferric reductase activities (EC_{50}) are shown in Table 3. Ga^{3+} and Al^{3+} were not equivalent in their inhibitory efficacy. In general, the EC_{50} for Al^{3+} was approximately 30–45 times lower than those of Ga^{3+} . For *B. dermatitidis* and *H. capsulatum* var. *capsulatum* G184AS, EC_{50} values for Ga^{3+} were only 18 times higher than those for Al^{3+} .

GSH-FeR size determination by native PAGE and gel permeation chromatography

Assays for detection of GSH-FeR activities in non-denaturing polyacrylamide gels were performed with concentrated supernatants. Iron reductase activity could be visualized as a purple band on a pinkish background, which began to appear within 30 min and reached maximal intensity after 4–6 h incubation. Only one band per strain was observed. The bands observed in native gels had apparent molecular masses varying from 430 to 460 kDa (Fig. 2). The same protein size range was determined using size exclusion chromatography (Fig. 3). For this experiment, concentrated supernatants from two *H. capsulatum* var. *capsulatum* strains were used. Only one peak of GSH-FeR activity per strain was detected in fractions collected after protein separation (Fig. 3). In both cases active fractions had the same mobility, indicating the similar size of secreted GSH-FeRs in strains G217B and UCLA 531S.

We electroeluted the visualized band from strain G217B from the native gel and found it retained enzyme activity (data not shown). This material was electrophoresed under denaturing conditions, revealing several major and several minor bands over a wide range of sizes on a stained gel (data not shown). These bands could represent components of an enzymic protein complex and/or proteins comigrating with the ferric reductase in the native gel. After two different renaturation protocols, no enzyme activity band could be visualized in the gel (data not shown). This result indicates that the original enzyme requires the interaction of two or more polypeptides which were separated in the denaturing gel, or alternatively that any enzymically active polypeptide, a single large protein or a constituent part of a multimeric complex, was not adequately renatured to restore ferric reductase activity.

Discussion

All pathogenic fungi share the requirement to survive within an infected host. For this reason many strategies have been evolved either to adapt to or to modify this hostile environment. During infection, the level of available iron is significantly limited and therefore microbial mechanisms to acquire iron are highly adaptive and important for successful virulence (Weinberg, 1999). Siderophore production in *H. capsulatum* and many other fungi has been recognized for decades. Demonstration of this trait in a microbe is sometimes considered a sole or adequate explanation for the essential process of iron acquisition. For *H. capsulatum*, our previous work with iron reduction (Timmerman & Woods, 1999, 2001) and the work of others on pH-dependent release of iron from transferrin (Newman *et al.*, 1994) and surface haemin binding (Foster, 2002) have expanded consideration of the range of approaches this fungus may use for this critical biological and pathogenic feature. We previously characterized ferric reductase in *H. capsulatum* G217B as a proteinase K-susceptible, heat-labile protein present in the high-molecular-mass fraction of supernatant (Timmerman & Woods, 1999). This enzyme utilized GSH as an electron donor and could use Fe³⁺-loaded siderophores and Fe³⁺-binding proteins (haemin and transferrin) as substrates, indicating its potential importance in iron utilization (Timmerman & Woods, 2001). In this study, we have demonstrated GSH-FeR activities in three other dimorphic fungal pathogens that cause respiratory and systemic disease, *B. dermatitidis*, *P. brasiliensis* and *S. schenckii*, as well as other *H. capsulatum* strains.

The highest GSH-FeR activities were observed in older cultures of *H. capsulatum* var. *capsulatum* strains belonging to RFLP class III, followed by RFLP class II and the lowest in RFLP class I and other genera tested. There was also an evident trend towards accumulation of this activity over time of culture growth. In all these fungi, GSH-FeRs were found to be functionally active in a broad range of pH, which is in good agreement with other studies demonstrating the ability of many secreted enzymic proteins to function under diverse conditions, e.g. proteases from *S. schenckii* (Tsuboi *et al.*, 1987) or phospholipase B, lysophospholipase and acyltransferase produced by *C. neoformans* (Chen *et al.*, 1997). This activity would also be predicted to be effective in acidic intracellular compartments after host macrophage infection by *H. capsulatum*. All of the fungal strains we examined shared a similarly sized band of ferric reductase activity on native electrophoretic gels and showed stability of ferric reductase activity across a broad pH range in fluid-phase enzyme activity

assays of culture supernatants. There was some variation in pH optima using the latter technique, but no more than about a fourfold range of activity at different pHs. We do not know if this activity variation reflects inherent differences in the enzymes or an effect of some other supernatant component(s). Comparison of purified enzymes would address this issue and we are attempting such a purification.

Further experiments demonstrated the GSH-FeR activity to be modulated by trivalent aluminium and gallium ions, and these inhibition patterns were similar in all the strains examined. Al^{3+} was a strong inhibitor of GSH-FeR, whereas Ga^{3+} was less active. These differences in inhibition might result from different ion sizes in aqueous solutions. The Shannon-Prewitt radii of non-hydrated trivalent ions for iron is 78 pm, 67 pm for aluminium and 76 pm for gallium (Wulfsberg, 2000). Assuming their hydrated radii show similar relative sizes and Al^{3+} is significantly smaller than Ga^{3+} , this metal could more easily displace Fe^{3+} in the GSH-FeR catalytic site.

GSH-FeRs could be observed in native polyacrylamide gels and after chromatographic separation. In all cases, this activity was detected as a single band in the gel (Fig. 2), or as a single peak on the chromatogram (Fig. 3). This finding is in a good agreement with other studies of microbial ferric reductase systems in which solely single bands were detected (Adams *et al.*, 1990; Poch & Johnson, 1993; Mazoy *et al.*, 1999). The molecular mass range determined for GSH-FeRs in all tested dimorphic fungal species was similar and varied from 430 to 460 kDa. This observation is unlike other previously published reports on prokaryotic, archaeal and eukaryotic ferric reductases that showed this heterogeneous group to be smaller; for example, the molecular masses of ferric reductases from *Legionella pneumophila* were approximately 38 and 25 kDa (Poch & Johnson, 1993), from *Paracoccus denitrificans* 55 and 19 kDa (Mazoch *et al.*, 2004), from *Archaeoglobus fulgidus* 18 kDa (Vadas *et al.*, 1999) and from *Mycobacterium paratuberculosis* 17 kDa (Homuth *et al.*, 1998). Only *Geobacter sulfurreducens* produced NADPH-dependent iron reductase that had a very large native molecular mass, 320 kDa, and consisted of 87 and 78 kDa subunits (Kaufmann & Lovley, 2001). Our finding demonstrates extracellular GSH-FeRs from dimorphic fungi to be the largest among described microbial iron reductases. We have not determined whether the activity represents a single polypeptide or a multimeric protein.

The role of the GSH-FeRs in iron uptake and utilization by dimorphic fungal pathogens and specifically its importance during infection remain unknown. Work by Cowart (2002) demonstrated microbial reductases to be mechanistically and kinetically suited to participate in the initial mobilization of iron. Extracellular ferric reductases may provide a physiologically relevant pathway for iron acquisition that would provide Fe^{2+} required for cell viability and growth. They may also compete for free iron from/in host phagocytes that generate toxic free radicals, which are an important and effective weapon against the pathogenic invaders (Homuth *et al.*, 1998; Schröder *et al.*, 2003). We are attempting to purify the *H. capsulatum* GSH-FeR. In addition to allowing analysis of the isolated enzyme, this approach should allow cloning of the encoding gene followed by gene expression and mutagenesis studies that will provide information on its function and role.

In conclusion, we have shown the extracellular GSH-FeR enzymes from dimorphic fungi exhibited similar characteristics (accumulation during culture growth, similar molecular masses on substrate gel, stability in a broad pH range and comparable patterns of inhibition by trivalent aluminium and gallium ions), consistent with a family of similar iron reductases. This class of proteins has been found to be common to at least four dimorphic zoopathogenic fungal species and represents a mechanism for extracellular reductive iron acquisition. Its novelty relies on an eclectic combination of two already well recognized strategies. The first is represented by specific membrane-associated enzymes, such as those reported for several eukaryotic microbes, including *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Candida albicans* and *Cryptococcus neoformans* (de Luca & Wood, 2000). Contrary to the GSH-FeRs described here, these enzymes require NADPH or FAD for their activity. The other tactic involves secreted low-molecular-mass ferric reductants, like 3-hydroxyanthranilic acid from *C. neoformans*, or 2,5-dimethylhydroquinone from *Serpula lacrymans* and *Gloeophyllum trabeum* (de Luca & Wood, 2000). Although the extracellular reductive utilization of iron has also been reported for prokaryotic cells, e.g. FeRs have been reported for *Mycobacterium paratuberculosis* (Homuth *et al.*, 1998) and *Listeria monocytogenes* (Deneer & Boychuk, 1993), the mechanism we report for exploiting enzymic proteins (first strategy) that are secreted into culture medium (second strategy) is novel for pathogenic fungi. The large size of the enzymes and use of GSH as a cofactor are also distinctive, albeit not unique.

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- Wulfsberg, G. Inorganic Chemistry. Sausalito: University Science Books; 2000. Table C. inside back cover

Abbreviation**GSH-FeR**

glutathione-dependent ferric reductase

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Fig. 1. Effect of pH upon extracellular GSH-FeR activity from dimorphic fungi. Activities were compared to that of G217B in PBS (pH 7.2), which was set at 100%.

Fig. 2.

Native discontinuous polyacrylamide gel electrophoretogram of extracellular GSH-FeR with ferric chloride as iron source. Lanes: 1, *H. capsulatum* var. *capsulatum* G184AS; 2, *H. capsulatum* var. *capsulatum* G184AR; 3, *H. capsulatum* var. *capsulatum* UCLA 531S; 4, *H. capsulatum* var. *capsulatum* G184AE; 5, *H. capsulatum* var. *duboisii* RV26821; 6, *H. capsulatum* var. *capsulatum* G217B; 7, *H. capsulatum* var. *capsulatum* G186AS.

Fig. 3.

Determination of GSH-FeR molecular mass in *H. capsulatum* strains UCLA 531S and G217B. Proteins were separated on a HiPrep 16/60 Sephacryl S200 HR column equilibrated with PBS (pH 7.2). (a) Calibration of the column used with a set of protein standards (A, blue dextran; B, α -amylase; C, alcohol dehydrogenase; D, bovine serum albumin; E, carbonic anhydrase; F, cytochrome C). (b, c) Chromatograms of extracellular proteins from strains G217B and UCLA 531S, respectively (solid lines). Long dashed lines represent GSH-FeR activity in collected fractions.

Table 1

Strains of dimorphic fungi used in this study

Species	Strain designation *
<i>Blastomyces dermatitidis</i> Gilchrist et Stokes	ATCC 26199
<i>Histoplasma capsulatum</i> var. <i>capsulatum</i> Darling	Downs ^I (ATCC 38904)
	UCLA 531S ^I
	G217B ^{II} (ATCC 26032)
	G222B ^{II} (ATCC 26034)
	G184AS ^{III}
	G184AR ^{III} (ATCC 26027)
	G184AE ^{III}
	G186AS ^{III} (ATCC 26029)
<i>Histoplasma capsulatum</i> var. <i>duboisii</i> Vanbreuseghem	RV26821 (ATCC 32281)
<i>Paracoccidioides brasiliensis</i> (Splendore) Almeida	ATCC 32071
<i>Sporothrix schenckii</i> Hektoen et Parkins	ATCC 10212

* Roman numbers, I, II and III, indicate classification of particular strains according to RFLP analysis (Eissenberg *et al.*, 1991).

Table 2

Extracellular GSH-FeR activity in zoopathogenic fungi

The results are means of two separate experiments with triplicate repetitions per sample. Standard error did not exceed 5%. GSH-FeR activities are expressed as nmol Fe²⁺ formed (μg protein)⁻¹ h⁻¹. Culture iron reduction capability values are expressed as nmol Fe²⁺ formed (total proteins in culture)⁻¹ h⁻¹. *S. schenckii* ATCC 10212 was not tested.

Strain	GSH-FeR activity			Culture iron reduction capability		
	2 days	4 days	6 days	2 days	4 days	6 days
<i>B. dermatitidis</i> ATCC 26199	0.08	0.12	0.17	22.7	21.4	35.8
<i>H. capsulatum</i> var. <i>capsulatum</i>						
Downs	0.05	0.06	0.06	10.5	21.5	27.6
UCLA 531S	0.08	0.09	0.07	6.7	18.4	27.7
G217B	0.07	0.07	0.08	17.4	39.1	49.3
G222B	0.06	0.08	0.07	20.3	37.2	50.5
G184AS	0.07	0.09	0.20	13.4	64.6	149.0
G184AR	0.05	0.06	0.19	9.3	66.9	145.5
G184AE	0.05	0.09	0.14	13.0	33.3	56.8
G186AS	0.07	0.10	0.17	19.0	45.5	132.6
<i>H. capsulatum</i> var. <i>duboisii</i> RV26821	0.05	0.06	0.05	5.7	16.2	24.9
<i>P. brasiliensis</i> ATCC 32071	0.07	0.06	0.08	2.1	2.6	2.6

Table 3

Inhibition of GSH-FeR activity by gallium and aluminium ions

The results reflect concentrations (μM) required for 50% inhibition of ferric reductase activity and are means from two independent experiments. Standard error did not exceed 5%. NT, Not tested.

Strain	EC ₅₀	
	Ga ³⁺	Al ³⁺
<i>B. dermatitidis</i> ATCC 26199	17.6	1.0
<i>H. capsulatum</i> var. <i>capsulatum</i>		
Downs	16.4	0.4
UCLA 531S	14.7	0.4
G217B	15.2	0.4
G222B	18.0	0.4
G184AE	14.9	0.5
G184AR	10.8	0.4
G184AS	15.7	0.9
G186AS	16.5	0.4
<i>H. capsulatum</i> var. <i>duboisii</i> RV26821	17.6	0.5
<i>P. brasiliensis</i> ATCC 32071	11.1	NT
<i>S. schenckii</i>	21.9	0.5