The iron- and cAMP-regulated gene *SIT1* influences ferrioxamine B utilization, melanization and cell wall structure in *Cryptococcus neoformans*

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The mechanisms by which pathogens sense and transport iron are important during infection, because of the low availability of free iron in the mammalian host. Iron is a key nutritional cue for the pathogen *Cryptococcus neoformans*, because it influences expression of the polysaccharide capsule that is the major virulence factor of the fungus. In this study, *C. neoformans* mutants were constructed with a defect in the iron-regulated gene *SIT1* that encodes a putative siderophore iron transporter. Analysis of mutants in serotype A and D strains demonstrated that *SIT1* is required for the use of siderophore-bound iron, and for growth in a low-iron environment. The *sit1* mutants also showed changes in melanin formation and cell wall density, and it was found that mutants defective in protein kinase A, which is known to influence melanization and capsule formation, showed elevated *SIT1* transcripts in both the serotype A and the serotype D backgrounds. Finally, the mutants were tested for virulence in a murine model of cryptococcosis, and it was found that *SIT1* was not required for virulence. Overall, these studies establish links between iron acquisition, melanin formation and cAMP signalling in *C. neoformans*.

INTRODUCTION

Cryptococcus neoformans is the leading cause of fungal meningitis in immunocompromised individuals (Casadevall & Perfect, 1998). Five serotypes (A, B, C, D and AD) are recognized, based on the antigenicity of the polysaccharide capsule, and three varieties have been described: *neoformans* (D), *grubii* (A) and *gattii* (B and C). Several virulence factors have been identified for the fungus, including the polysaccharide capsule, production of melanin by the enzyme laccase, the ability to grow at 37 °C, and survival within macrophages (Casadevall & Perfect, 1998). The polysaccharide capsule is antiphagocytic and suppresses the immune response, while the expression of laccase and melanin formation are necessary for survival within alveolar macrophages, resistance to oxidative stress, and extrapulmonary dissemination to the brain (Bose *et al.*, 2003; Casadevall & Perfect, 1998; Gomez &

Nosanchuk, 2003; Janbon, 2004; Liu *et al.*, 1999; Noverr *et al.*, 2004; Perfect, 2005; Williamson, 1997). Capsule and melanin production are regulated by several factors. For example, capsule size is influenced by iron and CO_2 levels, serum, and the location of the fungus in host tissue (Bose *et al.*, 2003; Janbon, 2004; Vartivarian *et al.*, 1993; Zaragoza *et al.*, 2003). Melanin synthesis is regulated by iron and copper, and by low glucose levels (Alspaugh *et al.*, 1997; Jacobson & Compton, 1996; Polacheck *et al.*, 1982; Salas *et al.*, 1996; Zhu *et al.*, 2001; Zhu & Williamson, 2004). The cAMP pathway is known to regulate both capsule and melanin, and the *PKC1*/MAP kinase pathway has also been implicated in melanin production, because loss of the C1 domain of *PKC1* leads to reduced laccase activity (Alspaugh *et al.*, 1997; D'Souza *et al.*, 2001; Heung *et al.*, 2005; Hicks *et al.*, 2004).

We are interested in the mechanisms of iron regulation and uptake in *C. neoformans*. Physiological and genetic studies indicate that iron acquisition is mediated by both high- and low-affinity uptake systems, with a role for cell surface ferric reductase activity, secreted reductants such as 3-hydroxyanthranilic acid, and melanin (Jacobson *et al.*, 1998; Lian *et al.*, 2005; Nyhus *et al.*, 1997; Nyhus & Jacobson, 1999). Based on similarities with *Saccharomyces cerevisiae* (Stearman *et al.*, 1996), the high-affinity uptake system is

believed to be composed of an iron permease, Ftr1, and a multicopper oxidase, Fet3, and iron regulation of these components has been confirmed by transcriptome analysis (Lian *et al.*, 2005). Iron uptake is also potentially mediated by siderophores that are exported from cells to bind ferric iron, with subsequent import. This strategy is common for both fungi and bacteria, and siderophores may allow pathogens to steal iron from host proteins such as transferrin or ferritin, or from nutritional competitors in their environment (Barasch & Mori, 2004; Haas, 2003). However, Jacobson & Petro (1987) were unable to identify extracellular iron chelators and hydroxamate-type siderophores in *C. neoformans*, although they did demonstrate that the fungus can use the siderophore deferoxamine in the presence of $FeCl_3$.

Specific transporters are required for non-reductive uptake of ferric–siderophore complexes. Interestingly, some fungi, such as the pathogen *Candida albicans* and *S. cerevisiae*, have homologues for siderophore transporters, even though they apparently do not possess the enzymes for the synthesis of siderophores (reviewed by Haas, 2003). It is presumed that the transporters allow these fungi to utilize siderophores from competitors. In *S. cerevisiae*, there are four siderophore transporters: *ARN1*, *ARN2/TAF1*, *ARN3/SIT1* and *ARN4* (Lesuisse *et al.*, 2001; Haas, 2003; Yun *et al.*, 2000a, b). The specificity of some of these transporters has been examined and, although issues of redundancy exist, Arn3p/Sit1p has high affinity for the hydroxamate siderophore ferrioxamine B (FOB; feroxamine), and lesser affinity for ferrichromes and ferricrocin (Kosman, 2003; Lesuisse *et al.*, 2001; Yun *et al.*, 2000a, b). *C. albicans* contains a similar set of transporters to those found in *S. cerevisiae*, and one of these, CaArn1/CaSit1, is required for epithelial cell invasion but not for systemic infection (Ardon *et al.*, 2001; Haas, 2003; Heymann *et al.*, 2002; Hu *et al.*, 2002; Kosman, 2003). *Aspergillus fumigatus* is also thought to have siderophore transporters, and the loss of ability to synthesize

siderophores compromises virulence in this fungus (Haas, 2003; Hissen *et al.*, 2005; Schrettl *et al.*, 2004). Recently, we have identified the transcript for a putative siderophore transporter (*SIT1*) by serial analysis of gene expression (SAGE) in *C. neoformans*, and have found that the message is elevated in cells grown in low-iron medium (LIM) (Lian *et al.*, 2005). This discovery suggests that *C. neoformans* may be able to utilize siderophores via a specific transporter or receptor, as predicted by Jacobson & Petro (1987).

In this study, we constructed null mutants for *SIT1* in the serotype D strain B3501A and the serotype A strain H99 of *C. neoformans*, and established that the gene is required by both strains for siderophore (FOB) utilization and growth in a low-iron environment. We also found that *SIT1* influences melanization and cell wall structure, and that this role is more pronounced in the serotype D strain B3501A. As has been observed in other studies (Barchiesi *et al.*, 2005; Hicks *et al.*, 2004), our analysis revealed differences in *sit1* phenotypes for the strains of the A and D serotypes. We also extended our studies to show that the cAMP pathway influences *SIT1* transcript levels. Finally, we found that *SIT1* is not required for virulence in a mouse model of cryptococcosis.

METHODS

Strains and growth conditions. The strains used in this study are listed in Table 1. Strain B3501A was provided by Dr J. Kwon-Chung (National Institutes of Health, Bethesda. MD). Strains JEC21, JEC43 and H99, and all of the mutants with defects in cAMP signalling components, were provided by Dr J. Heitman (Duke University, Durham, NC). The *ura5* mutant of B3501A was isolated by selection on 5-fluoroorotic acid plates. LIM was prepared with 20 mM HEPES and 20 mM $NaHCO_3$, as described by Vartivarian *et al.* (1993). The water for LIM was treated with Chelex-100 resin (Bio-Rad) to chelate iron. LIM + Fe was prepared by addition of 100 μM Fe^{3+}/EDTA (Sigma catalogue ref. EDFS). LIM + bathophenanthroline disulfonic acid (BPDA) + deferoxamine was prepared by addition of 550 μM

Table 1. *C. neoformans* strains used in this study

Strain	Genotype	Serotype	Strain background	Source
B3501A	MATα wt	D	NA	J. Kwon-Chung
b-u1	MATα *ura5* (FOA^R)	D	B3501A	This study
b-s10	MATα Δ*sit1*::*URA5 ura5*	D	B3501A	This study
b-s42	MATα Δ*sit1*::*URA5 ura5*	D	B3501A	This study
b-s42R1	MATα Δ*sit1*::*URA5 ura5*+*SIT1*::*NAT*	D	B3501A	This study
b-s42R2	MATα Δ*sit1*::*URA5 ura5*+*SIT1*	D	B3501A	This study
H99	MATα wt	A	NA	J. Heitman
h-s1	MATα Δ*sit1*::*NEO*	A	H99	This study
h-s2	MATα Δ*sit1*::*NEO*	A	H99	This study
h-s1R1	MATα Δ*sit1*::*NEO*+*SIT1*::*NAT*	A	H99	This study
cdc 40	MATα *pka1*::*ADE2 ade2*	D	JEC21	J. Heitman
cdc 99	MATα *pka2*::*URA5 ura5*	D	JEC21	J. Heitman
cdc 2	MATα *pka1*::*ADE2 ade2*	A	H99	J. Heitman
JH3	MATα *pka2*::*URA5 ura5*	A	H99	J. Heitman

BPDA (Sigma) and 100 μM deferoxamine (deferrioxamine; Sigma). To measure growth in liquid media, 5 ml yeast extract peptone dextrose (YPD) medium was inoculated with a single colony, and grown overnight at 30 °C in a gyratory shaker at 250 r.p.m. One hundred microlitres of culture was transferred to 5 ml yeast nitrogen base medium, and grown overnight at 30 °C. Cells were washed four times with sterile water, and transferred to flasks containing 50 ml LIM, LIM + Fe or LIM + BPDA + deferoxamine at a concentration of 1×10^6 cells ml$^-$. The cultures were shaken at 250 r.p.m. at 30 °C, and growth was monitored by measuring OD$_{600}$ on a Beckman DU530 UV/VIS spectrophotometer with a 1 cm path length.

Identification of *SIT1* and siderophore transporter orthologues. *SIT1* and additional candidate siderophore transporter orthologues were identified by using the sequences of the characterized *S. cerevisiae* siderophore transporters (Lesuisse *et al.*, 2001; Yun *et al.*, 2000a, b) to search the *C. neoformans* genome sequence databases for strains JEC21 (http://www.tigr.org/tdb/e2k1/cna1/), B3501A (http://www-sequence.stanford.edu/group/C.neoformans/index.html) and H99 (http://cneo.genetics.duke.edu). The gene identifications assigned to *SIT1* in JEC21 and H99 are CNA07920 and GLEAN05672, respectively. Transmembrane regions for the Sit1 sequence were predicted using TMHMM (http://www.cbs.dtu.dk/services/TMHMM).

RNA isolation and analysis. Cells were grown in LIM and LIM + Fe, as described above, harvested by centrifugation, flash-frozen in a dry ice/ethanol bath, and lyophilized overnight at −20 °C. Cell pellets were pulverized with glass beads for 10 min, and the cell powder was resuspended in 15 ml Trizol extraction buffer (Invitrogen). RNA was isolated according to the manufacturer's recommendations, with the additional step of LiCl precipitation at 4 °C after ethanol precipitation. Northern blot preparation and hybridization were performed as described by Sambrook *et al.* (1989), and all hybridization experiments were performed with two independent preparations of RNA from cells grown in LIM or LIM + Fe. The hybridization probe was a PCR-amplified 409 bp DNA fragment from exon 6 of the *SIT1* gene, using primers SITEXON6F and SITEXON6R (all primer sequences are listed in Supplementary Table S1). The probe was labelled with ^{32}P using an oligolabelling kit (Amersham Pharmacia Biotech).

Primers for real-time RT-PCR analysis were designed for each serotype using Primer Express software 3.0 (Applied Biosystems) (Supplementary Table S1). Cell cultures were prepared as described below for the laccase assay. Total RNA was purified with the RNeasy kit (Qiagen), treated with DNase (Qiagen), and cDNA was generated using the SuperScript First-Strand Synthesis system (Invitrogen). PCR reactions contained 750 nM of each primer, 1 × Power SYBR Green PCR Master Mix (Applied Biosystems) and 100 ng cDNA in a final volume of 20 μl. PCR reactions were performed on the Applied Biosystems 7500 Fast Real-Time PCR system, with 50 °C incubation for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Relative gene expression was quantified using the SDS software 1.3.1 (Applied Biosystems), based on the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001), and *ACT1* was used as an endogenous control for normalization.

Construction of *sit1*::*URA5* (serotype D) and *sit1*::*NEO* (serotype A) alleles. A PCR overlap strategy was used to prepare a deletion allele for strain B3501A (Davidson *et al.*, 2002). DNA fragments containing the 5′ (742 bp) and 3′ (853 bp) regions flanking the *SIT1* gene were amplified from genomic DNA from strain JEC21. Note that JEC21 and B3501A are closely related strains (Loftus *et al.*, 2005). The primers adjacent to the 5′ and 3′ regions of the gene were SIT1A and SIT1C, and SIT1D and SIT1F, respectively. The *URA5* marker was amplified from plasmid pJHM973 using hybrid primers SIT1B and SIT1E for *SIT1* and *URA5*, respectively.

The three fragments were joined by overlapping PCR (Davidson *et al.*, 2002) using primers SIT1A and SIT1F. For strain H99, a modified overlap strategy was employed that increased the yield of the final construct (Yu *et al.*, 2004). DNA fragments containing the 5′ (1151 bp) and 3′ (1162 bp) regions flanking the *SIT1* gene were amplified from genomic DNA of H99. The primers adjacent to the 5′ and 3′ regions of the gene were SITA1 and SITA3, and SITA4 and SITA6, respectively. The neomycin cassette was amplified from pJAF1 using hybrid primers SITA2 and SITA5 for *SIT1* and *NEO*, respectively. Fragments were used in second- and third-round PCR reactions, as described by Yu *et al.*, 2004. The nested primers used in the third round of PCRs were SITANF and SITANR. The DNA constructs were introduced into the appropriate parent strains by biolistic transformation (Toffaletti *et al.*, 1993). Transformants were screened by colony PCR using primers SIT1UP and 6328 for strain B3501A, and SITAUP and NEOPS for strain H99. Transformants in which the wild-type (wt) allele was replaced were confirmed by Southern analysis using the 5′ fragment (generated with primers SIT1A and SIT1C) as a probe; the absence of *SIT1* transcripts in the mutants was confirmed by quantitative real-time PCR. Two mutants for each strain were retained for analysis. To complement the mutations, the wt *SIT1* gene was reintroduced by biolistic transformation into one mutant of each strain background, on the vector pCH233 that confers resistance to nourseothricin (NAT).

Plate assays. Siderophore utilization assays were performed as described by Heymann *et al.* (2002). Plates of ferrous-iron-free medium (IFM) were prepared in the same manner as for LIM, with the addition of 20 g Bacto-agar l^{-1} and 550 μM BPDA chelator. Two hundred microlitres of cell suspension containing 10^7 c.f.u. ml^{-1} were spread on IFM, and a disk of Whatman paper saturated with 10 μl 100 μM deferoxamine or ferrichrome (Sigma) was placed in the centre of the plate. Plates were incubated for 2 days at 30 °C, and zones of growth were recorded by digital photography. Melanin assays were performed on 3,4-dihydroxyphenylalanine (DOPA; Sigma) medium that was prepared with 20 g Bacto agar l^{-1} in 900 ml distilled water. Separately, 1 g L-asparagine (Sigma), 1 g glucose, 3 g KH$_2$PO$_4$, 0.25 g MgSO$_4$.7H$_2$O and 200 mg DOPA were dissolved in 100 ml distilled H$_2$O and the pH was adjusted to 5.6. One milligram of thiamine/HCl (Sigma) and 5 μg biotin (Sigma) were added, the mixture was filter-sterilized, and then added to the autoclaved agar after cooling to 50 °C. The following modifications were made to DOPA medium prior to filter-sterilization to test various parameters: 4× DOPA contained 4 mM instead of 1 mM DOPA (Sigma), and 4 × DOPA + glucose contained 4 mM DOPA and 1.0 % glucose (instead of 0.1 %). To test for melanin production, 10 μl 10^7 c.f.u. ml^{-1} cell suspension was spotted on the plates and pigment was assessed after 2 days incubation at 30 °C. Temperature sensitivity assays were performed on low-glucose asparagine (LGA) medium and on DOPA medium. Tenfold serial dilutions of cells (5 μl) were spotted on either LGA or DOPA plates, with an initial inoculum of 10^7 c.f.u. ml^{-1}. Plates were incubated for 2 days at 30 or 37 °C. Cell wall integrity assays were performed as above, except that 0.015 % SDS, 300 μg Congo red ml^{-1} or 30 μg calcofluor white ml^{-1} was added to the medium. All experiments were repeated at least three times.

Laccase assays. Laccase assays were performed by inoculating 50 ml LGA (asparagine medium with 0.1 % glucose) with a single colony and growing the cells for 2 days at 30 °C. Cells were washed three times with 50 ml sterile deionized water, resuspended in 50 ml asparagine medium with no glucose, and grown for 4 h at 30 °C. In experiments to examine the effect of metal availability on laccase activity or *LAC1* transcript levels, 100 μM CuSO$_4$ or 100 μM Fe^{3+}/EDTA was included during the 4 h incubation. Cells were pelleted and washed three times with 50 ml 0.05 M NaPO$_4$ and resuspended at 10^8 c.f.u. ml^{-1}. To measure activity, 10^8 (1 ml) cells were incubated

with 1 mM DOPA for 30 min at 30 °C, the cells were pelleted, and A_{475} of the supernatant was recorded (A_{475} 0.001 corresponds to 1 U laccase). The laccase assays were repeated three times.

Microscopy. Transmission electron microscopy (TEM) was performed on a Hitachi H7600 microscope. The cells were grown, as described for the DOPA plate assays, on DOPA or LGA medium for 4 days at 30 °C. The full 10 μl spot of cell growth from DOPA or LGA medium was resuspended in 1 ml water, vortexed for 10 min, and then centrifuged for 5 min at 13 200 r.p.m. Cells were fixed in 2.5 % glutaraldehyde in 0.05 M cacodylate buffer at 28 °C under vacuum. Microwave (Ted Pella Microwave) processing was employed at 100 W, with 2 min on and 2 min off (repeated twice). Samples were washed twice with cacodylate buffer at 28 °C for 40 s, using power level 2. Cells were fixed in osmium tetroxide at 28 °C under vacuum, by microwave processing at 100 W, 2 min on and 2 min off (repeated twice). Samples were dehydrated in increasing concentrations of 50, 70, 90 and 100 % ethanol (the final step was repeated three times). Samples were infiltrated with Spurrs resin in increasing ratios of Spurrs : acetone, 1 : 3, 1 : 1 and 3 : 1, at vacuum power level 3 for 3 min. Resin was polymerized by baking the sample overnight at 60 °C. Samples were then cut into 70 nm sections on a Leica Ultracut T Ultramicrotome, and stained with 2 % uranyl acetate for 14 min and lead citrate for 7 min.

Virulence assays. Cells were prepared by inoculating 5 ml YPD with a single isolated colony, followed by overnight growth on a gyratory shaker (250 r.p.m.) at 30 °C. Cells were washed three times with sterile PBS and resuspended in 5 ml PBS. Cells were counted with a haemocytometer, and counts were confirmed by plating serial dilutions of strains on YPD agar, with subsequent growth for 2 days at 30 °C to determine c.f.u. The serotype D strain B3501A and the corresponding *sit1* mutant were tested initially using DBA1 mice (16–20 g; Jackson Laboratories) with a defect in C3 complement. Five mice were used for wt B3501A, and four mice were inoculated with the *sit1* mutant. The mice were inoculated by intranasal inhalation of 5×10^{6} cells in 50 μl PBS per mouse. For serotype A, A/Jcr mice (16–20 g; NIH Program) were infected intranasally with an inoculum of 5×10^{4} fungal cells in 50 μl PBS per mouse. Ten mice were used for each of the wt, *sit1* and *sit1* + *SIT1* strains. An additional experiment was performed using a 10-fold smaller inoculum of 5×10^{3} cells in 50 μl PBS, and five mice per strain. Animals that appeared moribund or in pain were euthanized.

RESULTS

Characterization of the *SIT1* gene

The *SIT1* gene was originally identified during the analysis of SAGE data for the serotype D strain B3501A, and was found to have a 3.3-fold higher transcript level in cells grown in LIM compared with LIM + Fe (Lian *et al.*, 2005). Examination of the gene indicated that it contained five introns and six exons, and encoded a predicted polypeptide of 604 aa with 13 putative transmembrane regions. We compared Sit1 to related sequences in other fungi and found that the closest orthologue, Arn3/Sit1 of *S. cerevisiae*, showed 39 % identity and 55 % similarity, resulting in an e value of 10^{-112} using the BLASTP algorithm (Altschul *et al.*, 1990). *SIT1* was also the best match with two of the three other siderophore transporter genes (*ARN1* and *ARN2*) of *S. cerevisiae*. This was true in searches of all three *C. neoformans* genomes (strains JEC21, B3501A and H99), although we did identify three other candidates for siderophore transporter

genes in each genome. Alignments of the Sit1 amino acid sequence with Arn3/Sit1 and orthologues from other fungi are presented in Supplementary Fig. S1. To begin a functional analysis of *SIT1*, we used RNA blot analysis to confirm that the transcript was elevated in cells from LIM in strains B3501A and H99 (serotype A) (Fig. 1).

SIT1 is required for iron acquisition under iron-limited conditions

To investigate the role of the *SIT1* gene in iron acquisition, null mutants were constructed in strains B3501A and H99. The entire coding region was deleted with transformation cassettes constructed by overlap PCR with a *URA5* marker for strain B3501A and a neomycin marker for H99 (Fig. 2) (Davidson *et al.*, 2002; Varma *et al.*, 1992; Yu *et al.*, 2004; Methods). Two independent transformants were obtained and characterized for both B3501A and H99. Replacement of the wt allele by the appropriate marker in the mutants was confirmed by colony PCR and Southern analysis (Fig. 2). The *sit1* mutants were reconstituted by transformation with the wt *SIT1* gene from strain JEC21 cloned into a vector containing an NAT-resistance gene.

Growth assays were initially performed on iron-free solid medium supplemented with deferoxamine to examine siderophore utilization in the *sit1* mutants. This medium contains the ferrous iron chelator BPDA (Cowart *et al.*, 1993) to establish conditions in which the cells must use ferric iron sequestered from the medium by deferoxamine. Under these conditions, zones of growth were apparent for all wt and reconstituted strains, but not for any of the *sit1* mutants at 2 or 7 days (Fig. 3). These assays indicate that the *sit1* mutants were unable to use FOB as a sole source of iron. In *S. cerevisiae*, Arn3/Sit1 can mediate the uptake of FOB or ferrichrome (Lesuisse *et al.*, 1998; Yun *et al.*, 2000a, b). We therefore tested our strains in the same plate assays with ferrichrome in place of deferoxamine, and we observed growth for all of the strains (data not shown). This result
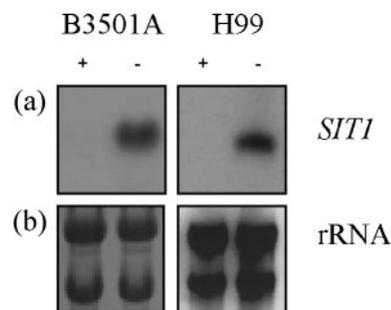

Fig. 1. Iron regulation of *SIT1* transcript levels. A Northern blot was prepared with total RNA isolated from cells grown in LIM (−) or iron-replete medium (+) at 37 °C for 6 h. Blots were hybridized with a DNA fragment from exon 6 of the *SIT1* gene of strain JEC21. (a) *SIT1* transcript levels; (b) rRNA (18S and 28S) bands as a loading control.
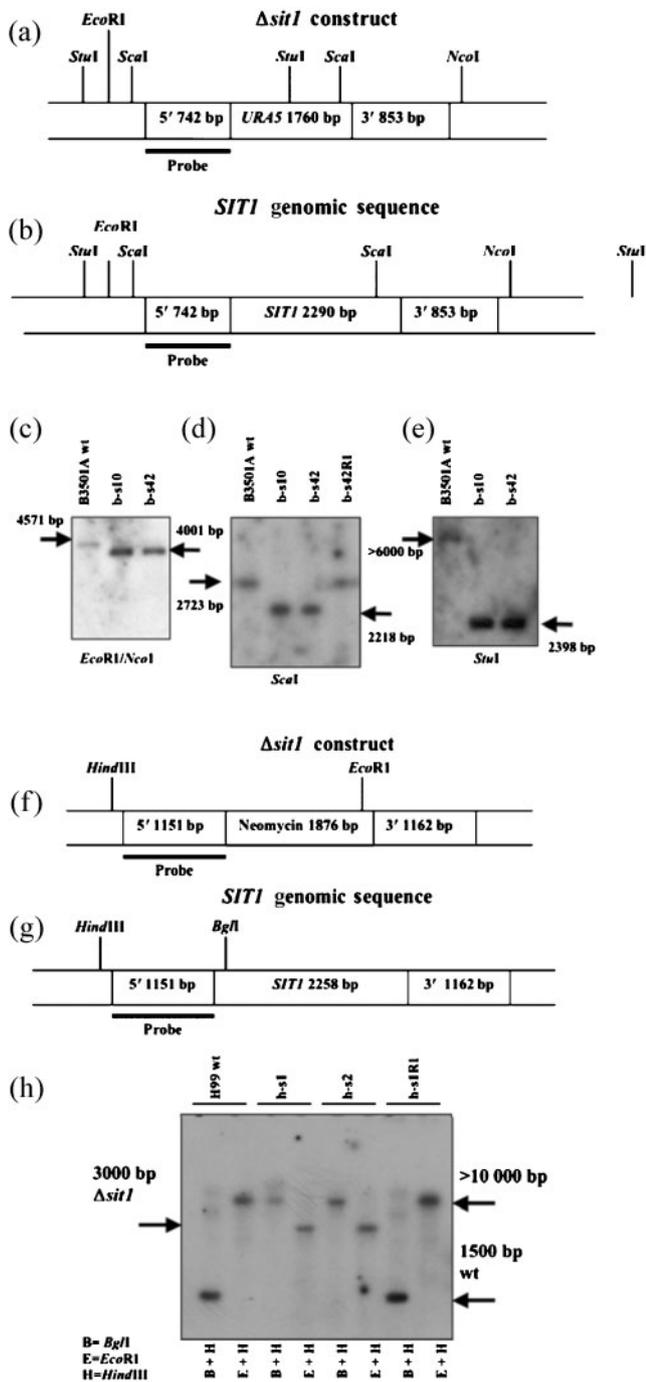
Fig. 2. Structure of *SIT1* deletion constructs and confirmation of homologous replacements in strains B3501A and H99. (a) Restriction enzyme sites in the Δ*sit1* construct. (b) The corresponding sites in the *SIT1* gene from strain B3501A. (c–e) Southern blots of genomic DNA digested with *Eco*RI/*Nco*I, *Sca*I or *Stu*I as indicated, and hybridized with a fragment of the *SIT1* gene [labelled as probe in (a) and (b)]. (f) *Bgl*I and *Hin*dIII restriction enzyme sites in the Δ*sit1* construct. (g) The corresponding sites in the *SIT1* gene from strain H99. (h) Southern blot of genomic DNA digested with *Bgl*I and *Hin*dIII or *Eco*RI and *Hin*dIII, and hybridized with the *SIT1* probe indicated in (f) and (g).
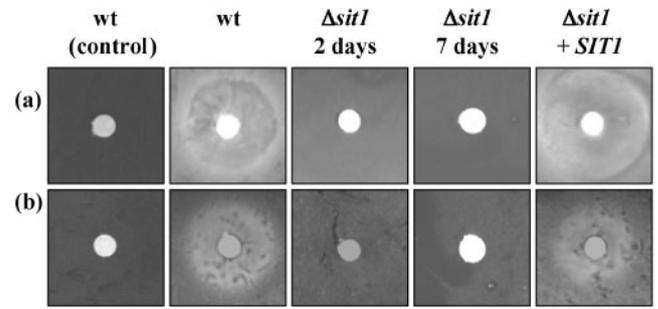

Fig. 3. *sit1* mutants are unable to acquire iron from a siderophore. Siderophore utilization was tested by spreading cells on LIM plates containing BPDA. A disk containing deferoxamine was placed in the centre of each plate, and the cultures were incubated for 2 or 7 days at 30 °C. (a) Strain B3501A; (b) strain H99. The assays were repeated several times.

indicates that *SIT1* is not required for ferrichrome utilization, although it is not possible to rule it out as a substrate from this experiment.

The analysis of growth in liquid media revealed that loss of *SIT1* greatly reduced growth of the mutants in LIM, and when siderophore was provided as the sole iron source (BPDA chelator + deferoxamine) (Fig. 4). None of the strains (wt or mutant) was able to grow in LIM with the BPDA chelator (data not shown), and all strains showed similar growth patterns in LIM + Fe, although growth was slightly delayed for *sit1* mutants (wt levels were achieved by 36 h; Fig. 4). The wt H99 strain also showed reduced growth in LIM (OD$_{600}$ ∼5 at 54 h) compared with that of strain B3501A (OD$_{600}$ ∼8 at 54 h), suggesting that this strain may be more sensitive to low-iron conditions. Overall, these results indicate that Sit1 plays a role in iron acquisition in a low-iron environment, and in the use of FOB as a sole iron source.

The *sit1* mutants exhibit altered melanin deposition and laccase activity

Previous studies have indicated a potential connection between melanin and iron in *C. neoformans* (Polacheck *et al.*, 1982; Jacobson & Compton, 1996; Jacobson, 2000). Therefore, we tested the ability of the *sit1* mutants to produce melanin, and found enhanced melanization in the B3501A mutant grown on agar medium containing the substrate DOPA (Fig. 5a). In particular, the mutant produced melanin more rapidly, and the surface of the colony appeared rougher, with a higher sheen compared to that of the wt (Fig. 5a). In addition, we noted that B3501A wt cells were able to invade the agar, whereas the *sit1* mutant could not (data not shown). No obvious change in melanization was observed for the H99 *sit1* mutants compared to that for the wt (Fig. 5a). We also tested a higher substrate concentration to determine if wt melanization would be enhanced to match the *sit1* mutant phenotype

(Fig. 5a). We found that a fourfold higher level of DOPA enhanced melanin formation in strain B3501A but not in H99, suggesting that substrate availability may have been a factor in the melanization difference between the B3501A *sit1* mutant and the wt. We also found that 1.0 % glucose could repress melanin production in all strains, indicating that the *sit1* mutation did not affect the known negative regulatory influence of glucose (Fig. 5a) (Nurudeen & Ahearn, 1979; Pukkila-Worley *et al.*, 2005).

We looked more closely at melanin production by measuring laccase activity, and found slightly elevated levels in the *sit1* mutants in both strain backgrounds (Fig. 5b). These results indicated that laccase activity was affected by the loss of *SIT1* in each strain, even though no difference in melanization was noted for the *sit1* mutant of strain H99 grown on solid medium. It is possible that the variation in laccase activity was the result of differences in metal availability to the enzyme between mutant and wt
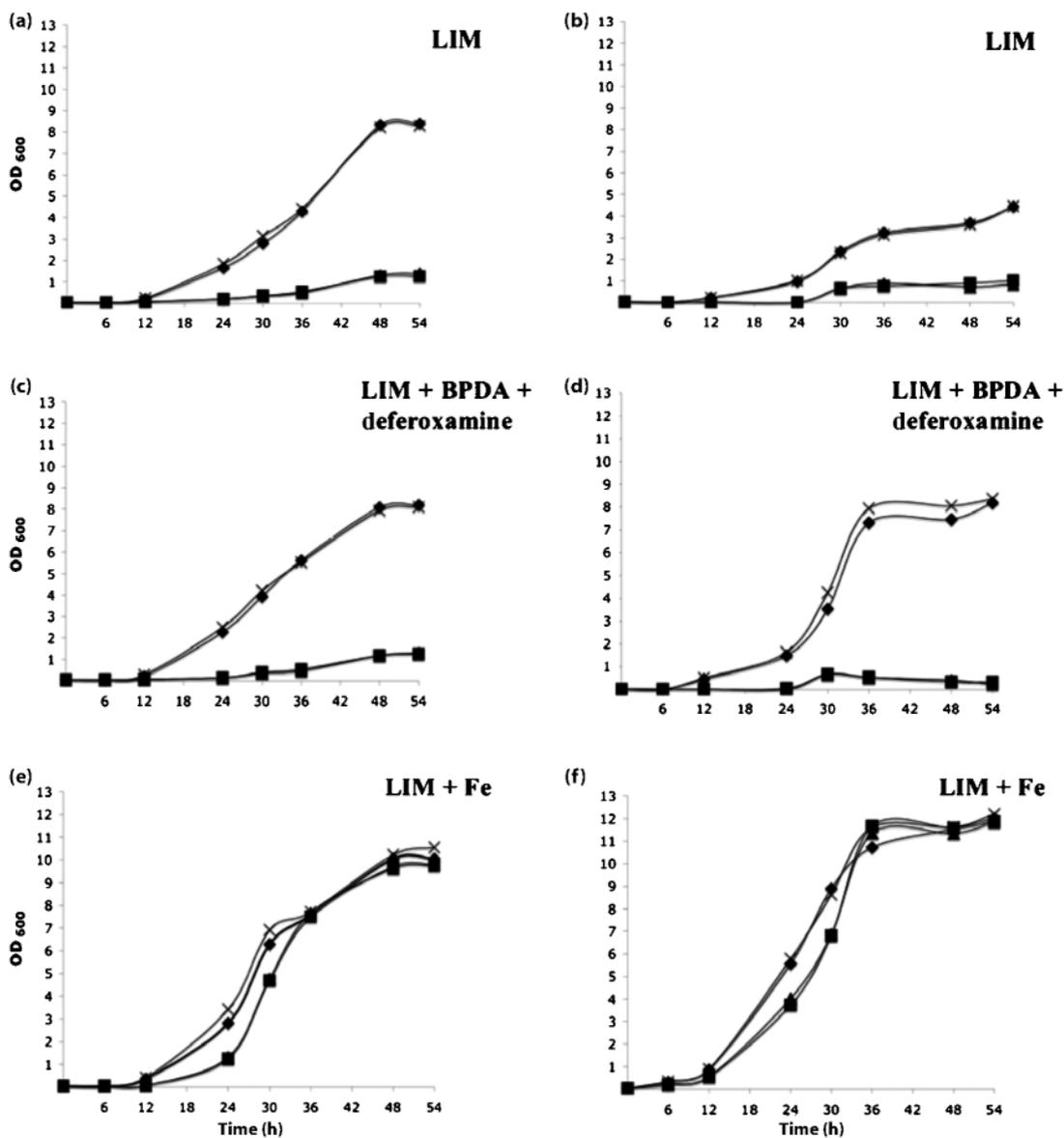

Fig. 4. Disruption of *SIT1* affects growth in LIM and LIM+BPDA+deferoxamine. Growth of cells in LIM (a), LIM+BPDA+deferoxamine (c) and LIM+Fe (e) is shown for the serotype D strain B3501A (wt; ◆), two *sit1* :: *URA5* disruption mutants of B3501A (b-s10, ■; b-s42, ▲), and two reconstituted strains of B3501A (*sit1* :: *URA5*+*SIT1*) (b-s42R1 and b-s42R2, ×). Comparison is also shown in (b, d, f) with strain H99 (wt, ◆), two *sit1* :: *NEO* disruption mutants in H99 (h-s1, ■; h-s2, ▲), and the reconstituted H99 mutant (*sit1* :: *NEO*+*SIT1*) (h-s1R1, ×) grown in LIM (b), LIM+BPDA+deferoxamine (d) and LIM+Fe (f). Graphs show the mean of three separate experiments.

cells. That is, loss of *SIT1* may have influenced metal homeostasis in the cells, resulting in a change in laccase activity due to metal loading on the copper-requiring enzyme. To test this hypothesis, we repeated the assays with cells incubated in the presence of added copper, and found that laccase activity was elevated in the wt and reconstituted strains, matching the level in the mutants (Fig. 5c). These results suggest that loss of *SIT1* influences copper loading on laccase, perhaps through altered metal homeostasis and/or changes in laccase expression or localization (normally in the cell wall) between mutant and wt strains.

We also examined whether the changes in laccase activity could have been due in part to differences in transcript levels between mutant and wt cells. Real-time PCR was used to measure laccase (*LAC1*) transcript levels in wt, mutant and reconstituted cells grown in low-glucose medium, with or without the addition of iron or copper (see Methods). As

shown in Fig. 5(d), no differences in *LAC1* transcript levels were found for the H99 strains grown under any conditions, or for the B3501A strains grown in medium without metal supplementation. However, higher transcript levels were found in the B3501A mutant grown in medium with iron or copper compared with those in the wt or reconstituted strains. These findings suggest that loss of *SIT1* influences laccase expression at the transcript level in the serotype D strain, and that metal repletion is important for this effect.

Loss of *SIT1* in the serotype D background results in temperature sensitivity and altered cell wall structure

We were intrigued by the idea that loss of *SIT1* might influence the localization of laccase, as suggested by the influence of substrate concentration and metal availability described above. Given the location of laccase in the cell
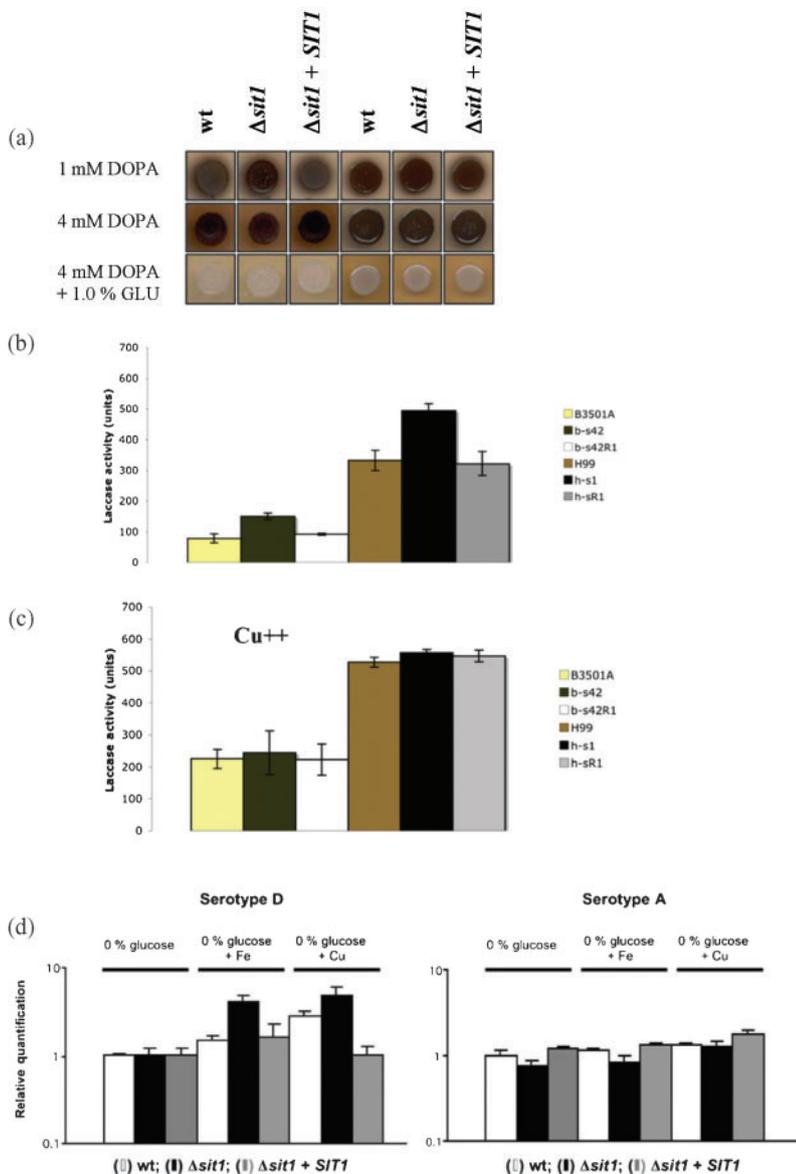

Fig. 5. Melanin production, laccase activity and *LAC1* transcript levels in *sit1* mutants. (a) To test for melanin production, 10 μl cultures of wt or mutant cells (10^7 ml^{-1}) were spotted onto 0.1 % glucose plates containing 1 mM DOPA, 4 mM DOPA or 4 mM DOPA + 1.0 % glucose. Plates were incubated for 2 days at 30 °C. (b, c) Laccase assays were performed for all strains with 10^8 cells (see Methods) and for (c), the cells were grown in the presence of 100 μM copper sulfate. (d) Relative quantification of *LAC1* transcriptional levels (*y* axis) was performed as described in Methods. Strains of B3501A (Serotype D) and H99 (Serotype A) were used for the analysis, and the *LAC1* expression levels in the wt strain of each serotype were used as a calibrator. The results are from triplicate assays, and the error bars represent standard errors of the mean expression levels.
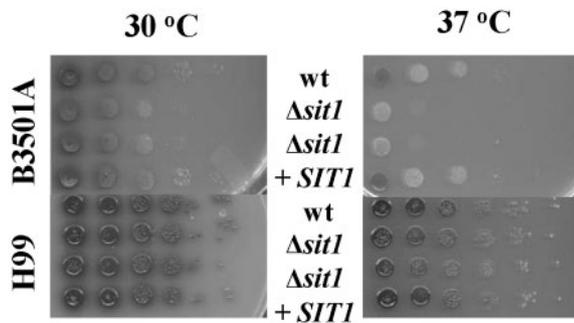
Fig. 6. Temperature sensitivity assays. To test for temperature sensitivity and cell wall integrity, serial dilutions of cells (from 10^2 to 10^7 c.f.u. ml^{-1}) were spotted onto solid 0.1% glucose + 1 mM DOPA medium, and plates were incubated for 2 days at 30 or 37 °C. The assays were repeated three times with similar results.

wall, we investigated whether the mutants showed differences in wall integrity. First, we tested growth in the presence of calcofluor white, SDS and Congo red on low-glucose or DOPA plates. Only slight sensitivity to these compounds was observed for the B3501A sit1 mutant compared with that for the wt, and no difference was noted for the H99 strains (data not shown). The cells were also tested for temperature sensitivity (a phenotype associated with cell wall integrity) by spotting serial dilutions of cells on DOPA plates (Fig. 6). The sit1 mutants showed slightly less growth than wt at 30 °C, and reduced growth at 37 °C for the

B3501A mutant (Fig. 6). The temperature sensitivity was particularly apparent in the B3501A background, and no difference was noted for the H99 strains (Fig. 6). Similar results were obtained in medium without DOPA (data not shown). The temperature sensitivity of the sit1 mutant in the B3501A background was confirmed in growth assays in liquid LGA medium (data not shown).

The changes in melanin production and the temperature sensitivity prompted a closer examination of the cell wall of the sit1 mutants. We employed TEM of melanized cells (grown for 4 days on DOPA plates) and non-melanized cells (grown for 4 days on LGA plates), and observed a difference in cell wall organization between the sit1 mutants, the reconstituted strains and the wt cells (Fig. 7). The cell walls of the wt and reconstituted cells contained distinct and dense concentric rings, and ordered polysaccharide fibrils, consistent with the observations of Eisenman et al. (2005). In contrast, the mutant walls appeared to be less dense, and they varied in thickness, with less ordered fibrils. The mutant cells in the B3501A strain were also easily distorted by the TEM processing (Fig. 7). For example, among a sample of 40 cells examined for each strain, 89% of sit1 mutant cells from strain B3501A were distorted (not spherical) versus 7.4% of the wt cells (data not shown). An independent preparation for TEM with prolonged dehydration steps reduced the distortion in the mutant cells, but the defect was still more apparent in the mutant cells (data not shown). These results suggest that loss of sit1 caused an altered organization of the cell wall in strain B3501A. Although the mutant cells in the H99 background
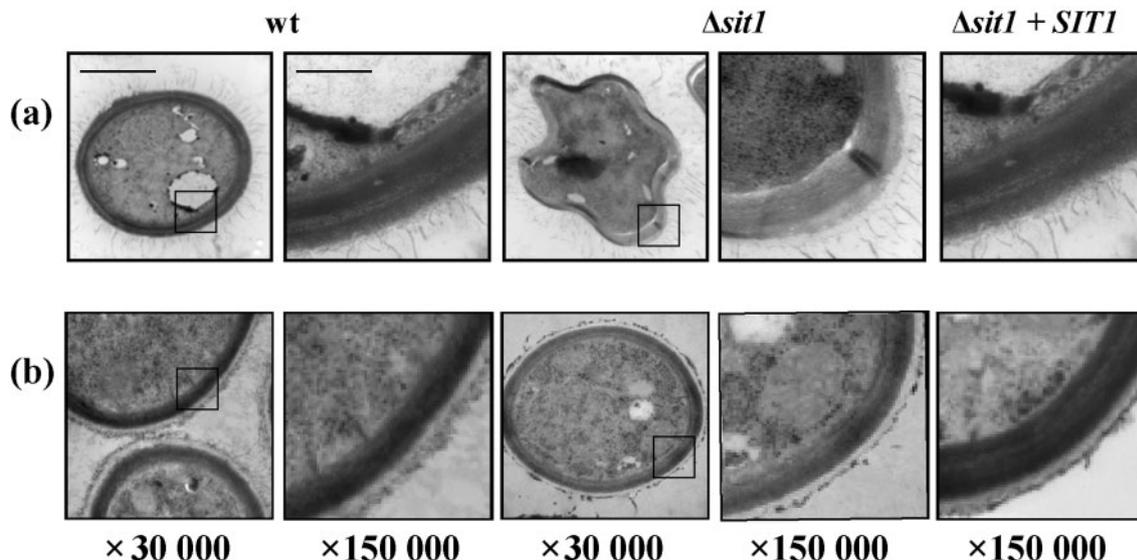

Fig. 7. TEM of melanized cells for wt and sit1 mutants. Two magnifications of the same cell are shown for the mutant and parental strains, and the reconstituted strains are shown at the higher magnification. The images show a cross section of the cell wall, cytoplasm and polysaccharide capsule for strains B3501A (a) and H99 (b). Approximately 50 cells were examined for each strain, and representative images are shown. Bars: ×30 000, 4 μm; ×150 000, 0.8 μm.

appeared to have a less dense wall, they did not show the same distortion upon processing; this is consistent with the lack of changes in melanization, and temperature and cell wall sensitivity, in the H99 mutants. Finally, the differences in the cell wall noted for the *sit1* mutants were apparent by TEM even in the non-melanized cells grown on LGA plates (data not shown). Taken as a whole, these results suggest that cell wall changes were present in the *sit1* mutants, and that these were particularly apparent in the serotype D strain B3501A.

Protein kinase A (PKA) influences *SIT1* transcript levels

In *S. cerevisiae*, the cAMP pathway and the *TPK2* gene encoding a catalytic subunit of PKA have been implicated in the negative regulation of iron-uptake genes, including *ARN3/SIT1* (Robertson *et al.*, 2000). We were therefore interested in the effect of the cAMP pathway on siderophore utilization in *C. neoformans*, especially given the influence of the pathway on melanin. We obtained mutants defective in the genes encoding the catalytic subunits of PKA in both serotype A and serotype D strains (D'Souza *et al.*, 2001; Hicks *et al.*, 2004), and used RT-PCR to measure *SIT1* transcript levels. We found that a defect in *pka1*, but not *pka2*, resulted in elevated *SIT1* transcripts in strain JEC21 (a close relative of B3501A), while both the *pka1* and the *pka2* mutants showed elevated transcripts in strain H99 (Fig. 8). These results suggest that similarities exist between *S. cerevisiae* and *C. neoformans* in the regulation of siderophore transport expression by PKA. The possible connection between siderophore utilization (perhaps involving *SIT1*) and the cAMP pathway prompted us to examine the *sit1* mutants for defects in other traits associated with cAMP signalling, including capsule formation (D'Souza *et al.*, 2001) and mating (Alspaugh *et al.*, 2002). No difference in capsule size or induction was noted between any of the wt or mutant cells grown in LIM and LIM + Fe conditions, suggesting that *SIT1* is not required for this trait (data not shown). Similarly, mating tests with serotype A or D MATa strains and the corresponding MATα *sit1* mutants indicated that no unilateral mating defect was caused by the loss of *SIT1* (data not shown).

SIT1 is not required for virulence in a murine model of cryptococcosis

We next tested the virulence of the *sit1* mutant, because of the importance of iron acquisition during infection, and the fact that the mammalian host environment is low in free iron. Initially, we used strain B3501A and its *sit1* mutants in a mouse inhalation model (DBA1 mice), but differences in virulence were not observed (data not shown). However, B3501A strains were generally only weakly virulent in our assays. Therefore, virulence was also tested for the H99 strains using the murine model. In the first trial, A/Jcr mice were infected intranasally with an inoculum of 5×10^4 cells per mouse, but no differences in survival ($\sim 20 \pm 2$ days) were noted between these strains (data not shown). Fungal cell counts from brain and lung tissue were performed for two mice for each of the wt and the mutant strains, and one mouse for the *sit1* + *SIT1* reconstituted strain. Cell counts were equivalent for the brains [$\sim 10^5$ cells (g tissue)$^{-1}$] and lungs [$\sim 10^6$ cells (g tissue)$^{-1}$] for all of the infections, further indicating that *SIT1* is not necessary for extra-pulmonary dissemination in this model. The *sit1* mutant cells isolated from the brain were retested in the siderophore utilization assay to confirm that the strain was unable to use deferoxamine (data not shown). In a second trial, a 10-fold lower inoculum of 5×10^3 cells was used with five mice per strain. Again, there was no difference in virulence between *sit1* mutants and wt strains, although the mice survived slightly longer with the lower inoculum (26–27 days; data not shown). Overall, these results indicated that *SIT1* is not required for virulence in this model.

DISCUSSION

The role of *SIT1* in iron acquisition

The acquisition of iron is essential for the proliferation of pathogens in the host environment, and siderophores are known to be important facilitators of iron uptake in several bacterial pathogens (Ratledge & Dover, 2000). Siderophore production by *C. neoformans* has not been detected (Jacobson & Petro, 1987), but the fungus may use transporters to capitalize on siderophores produced by nutritional competitors in the environment. We therefore
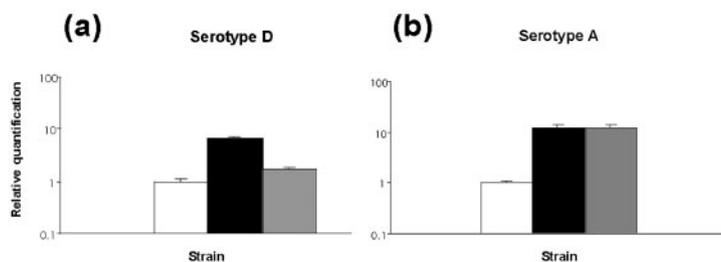

Fig. 8. Influence of cAMP signalling mutants on *SIT1* transcript levels. (a, b) Relative quantification of *SIT1* transcriptional levels (*y* axis) was accomplished as described in Methods. Strains of JEC21 (Serotype D) and H99 backgrounds (Serotype A) were used for analysis, and the *SIT1* expression levels in the wt strain of each serotype were used as a calibrator. The results are from tri-plicate assays, and the error bars represent standard errors of the mean expression levels. White bars, wt; black bars, Δ*pka1*; grey bars, Δ*pka2*.

characterized the putative siderophore transporter gene *SIT1*, and found that the gene is essential for the use of FOB, but not ferrichrome, as a sole source of iron. In addition, the lack of growth of *sit1* mutants in LIM may indicate a connection to components of the high- or low-affinity uptake systems, such as the iron permease *FTR1* or the multicopper oxidase *FET3* (Lian *et al.*, 2005). With regard to the use of FOB, our observations suggest that none of the other candidate genes for siderophore transporters is functionally redundant with *SIT1* under the conditions tested. However, the interpretation of redundancy can be complicated, as demonstrated by attempts to examine the specificity of the four siderophore transporters in *S. cerevisiae* (discussed by Kosman, 2003; Lesuisse *et al.*, 2001; Yun *et al.*, 2000a, b). In brief, it appears that there is considerable redundancy among the four transporters, although there is some specificity of Sit1 for FOB and Taf1/Arn2 for triacylfusarinine. In addition, variation is observed when the influence of transporter deletions is assessed in different strain backgrounds or laboratories. This suggests that transporter specificity may be a multigenic trait (Kosman, 2003). Given the variation that we have already observed between serotype A and D strains, the evaluation of specificity for Sit1 in *C. neoformans* and for the other candidates for siderophore transporters will likely require careful analysis of single and multiple deletion mutants in a single strain background.

Connections between Sit1, melanin, cell wall and signalling pathways

Loss of Sit1 in the B3501A background resulted in enhanced melanin formation, and increased laccase activity was seen for the *sit1* mutants in both B3501A and H99. Our results suggest that loss of *SIT1* results in increased laccase activity by multiple mechanisms, including transcription of the *LAC1* gene, access of the enzyme to substrate (perhaps due to altered localization or cell wall structure), and copper loading of the enzyme. *LAC1* transcription is known to be responsive to iron and copper (Zhu *et al.*, 2003), and our observations are consistent with a role for Sit1 in metal homeostasis. Additionally, Walton *et al.* (2005) have demonstrated the importance of copper and iron homeo-stasis for melanization, through an insertional mutagenesis approach to identify mutants with reduced melanin production. Specifically, they have identified mutations in *C. neoformans* homologues of the copper transporter Ccc2 and the copper chaperone Atx1, along with a chitin synthase gene and two transcriptional regulators. The mutants defective in *CCC2* or *ATX1* fail to grow on medium with an iron chelator, perhaps due to a role in providing copper to iron-uptake functions, such as the Fet3 multicopper oxidase. The role of the chitin synthase gene is interesting and provides a link between cell wall defects and laccase localization. Mutants with a defect in the chitin synthase gene give a 'leaky' melanin phenotype, likely due to a failure of laccase retention in the wall (Walton *et al.*, 2005).

Our TEM observations suggest a reduced density for the cell wall in the *sit1* mutants in both strain backgrounds. A recent description of the microstructure of the wall of *C. neoformans* has revealed that melanin is present in concentric layers of spherical granular particles with a diameter of 40–130 nm (Eisenman *et al.*, 2005). In light of this description, it is possible that *SIT1* contributes to laccase localization and therefore to melanin deposition in the wall. However, changes in the cell wall were noted even in the absence of the melanin substrate, and were also observed in strain H99, in which loss of *SIT1* did not noticeably affect melanin production (although laccase activity was slightly increased). Therefore, it seems that melanin localization is not the sole cause of the cell wall changes. Although further analysis is needed, it is possible that Sit1 may play a role in the exocytosis or endocytosis of components such as laccase, or factors required for laccase activity, to the cell wall, particularly in the serotype D strain. Connections between siderophore transporters, trafficking and the cell wall have been established in *S. cerevisiae*. For example, Arn1 cycles from the plasma membrane to endosomes, depending on the concentration of siderophore (Kim *et al.*, 2002). Additionally, Arn3 has been shown recently to interact with a cell wall protein, Sed1, and a role for the cell wall Fit proteins in siderophore transport has been established in yeast (Park *et al.*, 2005; Protchenko *et al.*, 2001).

Our observations on the influence of copper addition on *LAC1* transcription and laccase activity in the *sit1* mutants are similar to the results reported by Zhu *et al.* (2003). They have found that a defect in vacuolar acidification, due to deletion of the *VPH1* gene (encoding a vacuolar H^+-ATPase), leads to a defect in copper assembly into laccase. The mutants properly translate, secrete and localize laccase, but the enzyme is inactive. Copper addition restores laccase activity and results in increased transcription of the *LAC1* gene. They discuss the possibility that insertion of copper into laccase, perhaps in the outer Golgi network where the ATPase is located, might require acidification. The similarities with the influence of Sit1 on laccase support the hypothesis that Sit1 is important for metal homeostasis, perhaps through a role in trafficking or storing iron.

The influence of Sit1 on melanin production suggests possible connections with known regulators of this process in *C. neoformans*. For example, the *PKC1*/MAPK pathway has been recently implicated in melanization because the loss of the C1 domain (for diacylglycerol binding) of *PKC1* leads to loss of laccase activity and melanin production (Heung *et al.*, 2005). The C1 mutants display a similar phenotype to the serotype D *sit1* mutant, in that they have disorganized melanization (due to displacement of laccase) and reduced cell wall integrity. The cAMP pathway also controls laccase expression (D'Souza *et al.*, 2001; Pukkila-Worley *et al.*, 2005), and we found that mutations in the genes encoding the catalytic subunits of PKA influenced *SIT1* transcript levels. These results suggest that, as in *S. cerevisiae*, PKA negatively regulates some iron-uptake

functions (Robertson *et al.*, 2000). In this regard, *SIT1* will be a useful target gene for future analysis of gene expression, including transcription factor characterization, because of the co-regulation of transcript levels by the cAMP pathway and iron.

SIT1 and fungal infection

We found that *SIT1* was not required for virulence in a murine inhalation model of cryptococcosis, even though the *sit1* mutants showed poor growth in LIM. We were prompted to test the virulence of the *sit1* mutants because of observations in bacterial (Ratledge & Dover, 2000) and other fungal pathogens. Specifically, a siderophore transporter is not required for the virulence of *C. albicans* in a murine systemic infection model, but is required for epithelial cell invasion (Heymann *et al.*, 2002). In contrast, the high-affinity iron permease Ftr1 is required for systemic infection with *C. albicans* (Ramanan & Wang, 2000). Iron removal from holotransferrin may be siderophore-mediated in *A. fumigatus*, and the *SidA* gene that encodes an ornithine monooxygenase involved in siderophore synthesis is essential for virulence of *A. fumigatus* (Hissen *et al.*, 2004, 2005; Schrettl *et al.*, 2004). However, reductive iron assimilation by the high-affinity uptake system through FtrA is not required for virulence. There are a number of explanations for why *SIT1* may not be required for virulence in *C. neoformans*. First, *C. neoformans* does not appear to produce its own siderophores (Jacobson & Petro, 1987), so it is unclear whether a transporter would be useful during infection. Second, even if siderophores were available in the host, other siderophore transporters might contribute to iron acquisition in place of Sit1. That is, there are issues of specificity and redundancy to consider, and we have so far only found that Sit1 can promote use of the bacterial siderophore FOB (not expected to be present in the host). Finally, it has recently been shown in *C. albicans* that the reductive iron-uptake system is responsible for the acquisition of iron that is tightly bound to ferritin (Knight *et al.*, 2005). If the same were true for *C. neoformans*, then the functional *FTR1/FET3* system in the *sit1* mutants might effectively acquire iron from ferritin in the mouse. Generally, it is possible that sufficient iron is available *in vivo* to allow survival of the fungus by other iron-uptake mechanisms, even in the absence of Sit1. An exploration of the role of other uptake systems (e.g. Ftr1/Fet3) in virulence is currently under way.

ACKNOWLEDGEMENTS

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