The CaCTR1 gene is required for high-affinity iron uptake and is transcriptionally controlled by a copper-sensing transactivator encoded by CaMAC1

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INTRODUCTION

Iron and copper are essential nutrients to almost all known organisms. However, there are problems associated with the acquisition of both these transition metals, as they are not readily available in the environment (reviewed by Crichton & Pierre, 2001; Linder & Hazegh-Azam, 1996). Iron and copper are predominantly found in nature as insoluble complexes. Thus, in order to allow cellular uptake both ions must be separated from their environmental ligands. Once inside the cell, the concentration of iron and copper must be carefully monitored and controlled because of the potential toxic effects of free radical formation catalysed by the Fenton reaction (Halliwell & Gutteridge, 1999).

Iron and copper uptake in Saccharomyces cerevisiae has been well characterized and many of the component genes have been identified (reviewed by Eide, 1998). High-affinity iron and copper uptake is initiated by the ScFre1p/ScFre2p cell-surface-associated ferric/cupric reductase. Reduction of Fe$^{3+}$ and Cu$^{2+}$ to Fe$^{2+}$ and Cu$^{+}$ separates both metals from their environmental ligands, to allow uptake by specific cell membrane transporters. High-affinity iron uptake is achieved by a transporter complex that consists of a ferrous permease (ScFtr1p) and a multi-copper oxidase (ScFet3p). High-affinity copper uptake is achieved by two functionally redundant but distinct copper transporters, ScCtr1p and ScCtr3p. On delivery to the cytosol, Cu$^{+}$ ions are taken to the intracellular copper transporter ScCcc2p by the copper chaperone ScAtx1p. ScCcc2p is then responsible for the translocation of copper into the lumen of the Golgi, where four Cu$^{+}$ ions are incorporated into ScFet3p to confer biological activity. Due to the fact that ScFet3p function has an absolute requirement for copper, a defect in the process which leads to its incorporation into this protein causes severely defective high-affinity iron uptake. S. cerevisiae also has a low-affinity iron and copper uptake system that is supplied by the activity of Fet4p (Dix et al., 1994; Hassett et al., 2000). A second mechanism for iron uptake has also evolved in S. cerevisiae that makes use of low-molecular-mass compounds called siderophores that have a high affinity for the ion. This iron uptake system is not dependent upon copper and consists of four transporter proteins, ScArn1–4p (Heymann et al., 1999; Lesuisse et al., 1998; Yun et al., 2000b). Each transporter displays varying specificity to a range of ferri-siderophores from both the hydroxamate and catechol classes (Yun et al., 2000a). However, siderophore production has never been...
unequivocally proven in *S. cerevisiae*, so the organism may rely on those that are produced by other species.

In bacteria the acquisition of iron has long been known to be important for virulence (reviewed by Ratledge & Dover, 2000). It has now been identified that this is also the case for the fungal pathogen *Candida albicans* (Ramanan & Wang, 2000). Iron acquisition presents a particular problem to pathogenic micro-organisms due to the hostile environment of the human host. The level of free iron in the bloodstream is limited, as it is bound to proteins with a high affinity for the metal such as haemoglobin and transferrin. During an infection, systemic free-iron levels are further reduced by the ‘hypoferaemic response’ which is mediated by the reticulo-endothelial system (reviewed by Ward & Bullen, 1999). This results in iron being transported from the cardiovascular system and into the cells, where it is tightly bound due to increased synthesis of the iron storage protein ferritin. Conversion of ferritin to its insoluble degradation product, haemosiderin, further reduces the iron available to the intracellular pool and to serum transferrin. Pathogens such as *C. albicans* must therefore possess mechanisms that overcome the host physical barriers and defence mechanisms, to enable it to acquire iron and establish an infection.

Haemolytic activity has been identified in *C. albicans* and the pathogen has been proposed to obtain iron by attaching to and lysing complement-coated erythrocytes (Manns et al., 1994; Moors et al., 1992). In addition, the inhibitory growth effects of transferrin on *C. albicans* can be reversed by the addition of haem or haemoglobin (Moors et al., 1992). Although the genes that encode the haemolysins have not yet been identified, the mechanisms involved in acquiring extracellular iron have begun to be elucidated. As in *S. cerevisiae*, a reductive iron uptake system containing several component genes has been identified that is dependent upon copper. Cell-surface-associated ferric/cupric reductase activity has been observed in *C. albicans* and is regulated in response to iron and copper availability in the growth medium (Morrissey et al., 1996). Two component genes (*CaCFL1* and *CaCFL95*) have been characterized that have the ability to rescue *S. cerevisiae* mutants defective in cell-surface-associated reductase activity (Hammacott et al., 2000; Knight et al., 2002). The predicted proteins of *CaCFL1* and *CaCFL95* share significant sequence identity with ScFer1p and ScFer2p respectively. Furthermore, *CaCFL1* is transcriptionally regulated in response to both iron and copper availability (Hammacott et al., 2000). The function of a further ten ORFs whose predicted proteins share significant identity to those encoded by the *S. cerevisiae* FRE genes remains unknown. Two *C. albicans* genes which encode a high-affinity iron transporter complex have also been identified. The *CaFET3* gene encodes a multi-copper oxidase with the ability to rescue a *S. cerevisiae* fet3-null mutant (Eck et al., 1999) and *CaFTR1* encodes an iron permease that is functionally homologous to ScFtr1p (Ramanan & Wang, 2000). *C. albicans* strains that carry deletions in *CaFET3* or *CaFTR1* display defects in reductive high-affinity iron uptake, and most notably a *CaFTR1*-null mutant was unable to set up a systemic infection in mice (Eck et al., 1999; Ramanan & Wang, 2000). A siderophore uptake system has also been identified in *C. albicans* that is encoded by CaARN1 (Hu et al., 2002). However, siderophore production in this organism has not been unequivocally proven.

Reductive iron uptake in *C. albicans* has been shown to be dependent upon copper (Knight et al., 2002). This is proposed to be due to the requirement of the multi-copper oxidase component of the high-affinity iron transporter complex, *CaFet3p*. There are also four additional ORFs that may encode multi-copper oxidases present on the *C. albicans* genomic database (Knight et al., 2002). Evidence for the requirement of copper for reductive iron uptake in *C. albicans* is further strengthened by the characterization of a gene homologous to ScCCC2 (Weissman et al., 2002). A homozygous deletion of *CaCCC2* results in strains with defective high-affinity iron uptake, presumably as a result of incorrect delivery and incorporation of copper into *CaFet3p* (Weissman et al., 2002). However, although reductive iron uptake has been shown to be reliant on copper in *C. albicans*, *CaFet3* and *CaCC2*-null mutants were still able to set up a systemic infection in mice (Eck et al., 1999). Furthermore, the virulence of a Gaarn1-null mutant was also unaffected (Hu et al., 2002). These findings are in contrast to those found with a *CaFTR1*-null mutant and may suggest that additional mechanisms for iron and copper metabolism in *C. albicans* have yet to be elucidated.

We have previously described *CaCTR1*, a gene that is required for copper uptake in *C. albicans*. A *CaCTR1*-null mutant displayed phenotypes that were similar to those of a *S. cerevisiae* mutant defective in high-affinity copper uptake. Furthermore, this mutant grew predominantly in the filamentous form and also displayed aberrant morphology in response to copper-depleted growth conditions (Marvin et al., 2003). We demonstrate here that invasive growth by *C. albicans* is induced by low-copper conditions and that this is augmented in a *Catr1*-null strain. We also demonstrate that high-affinity iron uptake is not detectable in a *Catr1*-null strain. Interestingly, the phenotypes displayed by a *Catr1*-null mutant did not coincide with a drop in cell membrane *p*-phenylenediamine (PPD) oxidase activity. *CaCTR1* is transcriptionally controlled by the copper-sensing transactivator ScMac1p when it is heterologously expressed in *S. cerevisiae* (Marvin et al., 2003). Here, we show that a similar transactivator exists in *C. albicans* and is required for expression of *CaCTR1* in response to low-copper conditions.

### METHODS

**Strains.** The *C. albicans* strains used in this study are listed in Table 1. Strain BWP17macA::URA3/macA::ARHG was constructed using primers with 70 bp of homology to genomic sequences to enable the deletion of 1201 bp from the *CaMAC1* ORF.
Table 1. C. albicans strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source/reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SC5314</td>
<td>Clinical isolate from a patient with disseminated candidosis</td>
<td>Gillum et al. (1984)</td>
</tr>
<tr>
<td>BWP17</td>
<td>ura3Δ::::imm434/ura3Δ::::imm434; arg4::::hisG/arg4::::hisG; his1::::hisG/his1::::hisG</td>
<td>Wilson et al. (1999)</td>
</tr>
<tr>
<td>BWP17ctr1Δ::URA3/CTR1</td>
<td>ura3Δ::::imm434/ura3Δ::::imm434; arg4::::hisG/arg4::::hisG; his1::::hisG/his1::::hisG, ctr1A::URA3/CTR1</td>
<td>Marvin et al. (2003)</td>
</tr>
<tr>
<td>BWP17ctr1Δ::URA3/ctr1Δ::ARG4</td>
<td>ura3Δ::::imm434/ura3Δ::::imm434; arg4::::hisG/arg4::::hisG; his1::::hisG/his1::::hisG, ctr1A::URA3/arg4::::ARG4</td>
<td>Marvin et al. (2003)</td>
</tr>
<tr>
<td>BWP17ctr1Δ::/HIS1</td>
<td>ura3Δ::::imm434/ura3Δ::::imm434; arg4::::hisG/arg4::::hisG; his1::::hisG/his1::::hisG, HIS1::::HIS1, ctr1A::URA3/ctr1Δ::ARG4</td>
<td>Marvin et al. (2003)</td>
</tr>
<tr>
<td>BWP17ctr1Δ::/HIS1::CTR1</td>
<td>ura3Δ::::imm434/ura3Δ::::imm434; arg4::::hisG/arg4::::hisG; his1::::hisG/his1::::hisG, HIS1::::HIS1, ctr1A::URA3/arg4::::ARG4</td>
<td>Marvin et al. (2003)</td>
</tr>
<tr>
<td>BWP17mac1Δ::URA3/mac1Δ::ARG4</td>
<td>ura3Δ::::imm434/ura3Δ::::imm434; arg4::::hisG/arg4::::hisG; his1::::hisG/his1::::hisG, mac1A::URA3/mac1Δ::ARG4</td>
<td>This study</td>
</tr>
<tr>
<td>BWP17mac1Δ::/HIS1</td>
<td>ura3Δ::::imm434/ura3Δ::::imm434; arg4::::hisG/arg4::::hisG; his1::::hisG/his1::::hisG, HIS1::::HIS1, mac1A::URA3/mac1Δ::ARG4</td>
<td>This study</td>
</tr>
<tr>
<td>BWP17mac1Δ::/HIS1::MAC1</td>
<td>ura3Δ::::imm434/ura3Δ::::imm434; arg4::::hisG/arg4::::hisG; his1::::hisG/his1::::hisG, HIS1::::HIS1, mac1A::URA3/mac1Δ::ARG4</td>
<td>This study</td>
</tr>
<tr>
<td>DAY185</td>
<td>ura3Δ::::imm434/ura3Δ::::imm434; HIS1::::hisG/his1::::hisG, ARG4::::URA3::::arg4::::hisG/arg4::::hisG</td>
<td>Davis et al. (2000a)</td>
</tr>
</tbody>
</table>

(CaMAC1−64 and CaMAC1+1278; Table 2). These were used in PCR reactions with pGEM-URA3 and pGEM-ARG4SpeI to generate disruption cassettes carrying the wild-type CaARG4 or CaCaARG4 gene flanked by genomic targeting sequences (Wilson et al., 1999). The PCR products were then used in consecutive transformations of strain BWP17. Targeted integration at the correct genomic locus that led to two disrupted alleles of CaMAC1 was confirmed by PCR analysis using primers CaMAC1−344, 3DR and CaARG4+83 (Table 2). Strain BWP17mac1Δ::/HIS1 was constructed by transforming strain BWP17mac1Δ::URA3/mac1Δ::ARG4 with pGEM-HIS1 that had been linearized by digestion with NruI (Davis et al., 2000b; Wilson et al., 1999). In order to construct strain BWP17mac1Δ::/HIS1::MAC1, a 2362 bp fragment was amplified by PCR using primers CaMAC1−899 and CaMAC1+1443 using

Table 2. Primers used for PCR of C. albicans DNA

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′→3′)</th>
<th>Target site</th>
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</thead>
<tbody>
<tr>
<td>CaMAC1−899</td>
<td>GATCGTGCAGCATGTCATTCTGTTAAGCTAG</td>
<td>CaMAC1 ORF −899 to −880</td>
</tr>
<tr>
<td>CaMAC1+1443</td>
<td>GATCGTGCAGCATGTCATTCTGTTAAGCTAG</td>
<td>CaMAC1 ORF +1443 to +1424</td>
</tr>
<tr>
<td>CaCTR1+60</td>
<td>GGCAATGTGCGGCAATTGCGAACC</td>
<td>CaCTR1 ORF +60 to +80</td>
</tr>
<tr>
<td>CaCTR1+568</td>
<td>CTTCTAGAAATATGTTGATGCC</td>
<td>CaCTR1 ORF +568 to +549</td>
</tr>
<tr>
<td>CaMAC1−344</td>
<td>CATCTCAGCATGCAAACACAGGCC</td>
<td>CaMAC1 ORF −344 to −320</td>
</tr>
<tr>
<td>3DR</td>
<td>TTCCCATCATGACAGCTT</td>
<td>Polyn linker in pGEM-URA3, pGEM-HIS1 and pRS-ARG4SpeI</td>
</tr>
<tr>
<td>5DR</td>
<td>TGTTGAATTGTTGAGCCGATA</td>
<td>Polyn linker in pGEM-URA3, pGEM-HIS1 and pRS-ARG4SpeI</td>
</tr>
<tr>
<td>CaHIS1−287</td>
<td>GAGGTAGAATTGAGACATGAGG</td>
<td>CaHIS1 ORF −287 to −266</td>
</tr>
<tr>
<td>CaHIS1+1153</td>
<td>CCTTATGTTGCCATCTGGTGCAGG</td>
<td>CaHIS1 ORF +1153 to +1131</td>
</tr>
<tr>
<td>CaARG4+83</td>
<td>TCCATCAATGGATTGATGCGCAGGG</td>
<td>CaARG4 ORF +83 to +59 in pRS-ARG4SpeI</td>
</tr>
<tr>
<td>CaMAC1−64</td>
<td>GCTACCTTGAAAGCTCTGTAGTTCAATCGACT-</td>
<td>CaMAC1 ORF −64 to +6</td>
</tr>
<tr>
<td></td>
<td>AGATCCTCCGCAAAAAGCAAGACTGAAATAA-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GGAATGATATGATGTTGGAGGGTTGAGCGGATA</td>
<td></td>
</tr>
<tr>
<td>CaMAC1+1278</td>
<td>GCAACATGACCTCACCTGCTTGAGACACA-</td>
<td>CaMAC1 ORF +1278 to +1207</td>
</tr>
<tr>
<td></td>
<td>TCTTCTAGGTATCTGCGTGATTGACACAG-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TCTTCTAGGTATCTGCGTGATTGACACAGT</td>
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</table>
template DNA purified from the wild-type strain SC5314 (Gillum et al., 1984). The amplified fragment was then digested with SaIi and introduced into the equivalent site in pGEM-HIS1. The resulting construct (pGEM-HIS1/MAC1) contained the entire MAC1 ORF flanked by 899 bp of upstream and 147 bp of downstream sequence. Strain BWP17mac1Δ::URA3/mac1Δ::ARG4 was then transformed with pGEM-HIS1/MAC1 that had been linearized by digestion with NraI. Targeted integration of pGEM-HIS1 and pGEM-HIS1/MAC1 at the his1::hisG locus was confirmed by PCR analysis using primers CaHSI1-287, CaHSI1+1153 and 5DR (Table 2).

**Growth conditions.** *C. albicans* cultures were grown at 30°C. For growth in non-selective conditions yeast-extract/peptone medium was used, with glucose added at a final concentration of 2% (w/v) (YPD). To test the strains for the ability to grow on non-fermentable carbon sources, glucose was replaced with either 3% (w/v) glycerol (YPG) or 3% (w/v) ethanol (YPE). For yeast grown in selective conditions SD medium [0-67% (w/v) yeast nitrogen base with ammonium sulphate (B101)] was used. Minimal defined medium (MD) was used to verify the ability of strains to grow in low-copper or low-iron conditions; the medium was based on the yeast nitrogen base recipe of Wickerham (1951) with the addition of 20 mM sodium chloride pH 4.2 (Eide et al., 1992) and omitting copper or iron as necessary. Bathocuproinedisulphonic acid (BCS) or bathophosphate pH 7 (1984) with a base recipe of Wickerham (1951) with the addition of 20 mM or low-iron conditions; the medium was based on the yeast nitrogen source, glucose was replaced with either 3% (v/v) glycerol (YPD) or 3% (v/v) ethanol (YPE). For yeast grown in selective medium containing BPS or BCS, with iron chloride or copper chloride added or omitted as appropriate. The cells were then harvested by centrifugation at 4000 r.p.m. at 4°C and the supernatant was discarded. The cells were washed in sterilized distilled water, harvested and the supernatant discarded once more. They were then resuspended in breakage buffer (150 mM NaCl, 25 mM Tris/HCl pH 7-5, 5% glycerol, 1 mM DTT, 1 mM PMSF) containing 10 μl of a yeast protease inhibitor cocktail (Sigma-Aldrich). Finally, 1 g acid-washed glass beads (425–600 μm) was added to the breakage mix. The cells were then broken by agitation for 3 min in a multi-pressor at 4°C. This was repeated five times, with the cells placed on ice between agitation to prevent overheating and denaturation of the protein. The homogenate was then centrifuged at 55,000 rpm (100,000 g) for 40 min to harvest the cell membrane fraction. The supernatant was discarded and the pellet was washed with 1 ml breakage buffer. The membrane fraction was then solubilized in breakage buffer containing 1% Triton X-100 and centrifuged for 30 min at 13,000 r.p.m. (16,060 g) to yield a clarified extract. Protein content was determined by Bradford assay.

**p-Phenylenediamine (PPD) oxidase assays.** The method used was modified from that described by Spizzo et al. (1997). Liquid PPD oxidase assays were carried out using 25 μg of the cell membrane protein fraction. This was resuspended in 600 μl assay buffer (100 mM sodium acetate pH 5-7, 0-6% Triton X-100, 0-05% PPD) and incubated at 30°C for 1 h. Colour development was followed in a spectrophotometer set at 530 nm at 30 min intervals for a total of 3 h. Oxidation rates were calculated after subtracting the rate of a blank sample that contained assay buffer with no added protein.

**RESULTS**

**Low-copper conditions induce invasive growth in *C. albicans***

We have previously reported that both single- and double-disrupted *Cact1* mutants readily form hyphae on YPD medium. We have also shown that a double-disrupted *Cact1*-null mutant grows prevalently in the filamentous form in liquid MD-BCS medium, with many cells displaying aberrant morphology (Marvin et al., 2003). These findings led us to hypothesize that copper starvation also induces invasive growth in *C. albicans*. To investigate this,
MD agar plates were prepared so that a concentration gradient of copper was set up across the surface of the growth medium. This was achieved by using two layers of medium in a square Petri dish, pouring the two layers in opposition to each other at 45° to the edge of the plate. Histidine, arginine and uridine were added to the growth medium to facilitate the growth of auxotrophic strains. Addition of the cell suspensions was made in triplicate and staggered across the surface of the plate to emulate a single band of growth across the surface of the copper gradient. The cells were incubated for 10 days at 30°C. Finally, the plates were washed with copious amounts of sterilized distilled water to remove non-invasive growing cells.

To ascertain whether a Cactr1-null mutant displayed defective iron uptake, 55Fe uptake assays were carried out on strain BWP17ctr1Δ::URA3 and the wild-type strain, DAY185. Assays were performed at both 30°C and 0°C as a negative control, and 55Fe uptake was shown to be linear for at least 3 h. The data generated demonstrated that double deletion of CaCTR1 results in defective 55Fe uptake in the resulting mutant strain. Iron uptake in strain BWP17ctr1Δ::HIS1 was reduced by an average of 96% using the conditions described (Fig. 2). However, a low rate of uptake was still detectable in the Cactr1-null mutant when the assays were performed at 30°C. To ascertain whether the cells that had been incubated in assay buffer were still viable, serial dilutions of each strain were made after each experiment and plated out onto YPD. Following incubation at 30°C for 3 days, counts of the subsequent colonies typically gave a mean value of around 71% viability. The 55Fe incorporation assays were performed on four occasions and were quantitatively similar between experiments.

**Sequence analysis of a putative C. albicans CaMac1p**

We have previously shown that CaCTR1 is transcriptionally regulated in C. albicans in response to the concentration of copper in the growth medium, with maximum expression in low-copper conditions. We have also shown that when heterologously expressed in S. cerevisiae, CaCTR1 is transcriptionally controlled in a similar manner to C. albicans and this control is facilitated by the copper-sensing trans-activator ScMac1p (Marvin et al., 2003).

A sequence is present (orf6.8485) on the C. albicans genome database (http://www-sequence.stanford.edu/group/candida/search.html) which displays significant identity to ScMac1p.
Direct comparison using the Needleman & Wunsch algorithm alignment (GAP) from the GCG package showed that the putative Mac1p sequence from *C. albicans* shared 35±0% similarity and 26±2% identity with *Sc* Mac1p. In addition, further analysis showed that the putative *Ca* Mac1 protein sequence contained specific motifs that were indicative of a *Sc* Mac1p homologue (Fig. 3). Analysis using the predicted *Ca* Mac1p sequence as a query against the Conserved Domain Database (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) revealed the presence of a 'copper fist' motif at the amino terminus (Fig. 3a; Altschul *et al*., 1997). This motif is found in several copper-responsive transcription factors, including Mac1p and Ace1p of *S. cerevisiae* (Jungmann *et al*., 1993; Szczypka & Thiele, 1989), Am1t1p of *Candida glabrata* (Zhou & Thiele, 1991), Cuf1p of *Schizosaccharomyces pombe* (Labbe *et al*., 1999) and Crf1p of *Yarrowia lipolytica* (Garcia *et al*., 2002). In each corresponding organism, the motif facilitates the binding of protein to DNA in the presence of copper or silver (Furst & Hamer, 1989; Jensen *et al*., 1998; Jensen & Winge, 1998).

Further analysis of the putative *C. albicans* Mac1p sequence at the SWISS-PROT database, using the REPRO program, which searches for non-overlapping repeats within a particular protein sequence (http://ibivu.cs.vu.nl/programs/reprowww), revealed two high-scoring segments, between residues 169 and 224 and residues 246 and 301. Within these segments were cysteine-rich sequences with significant identity to the C1 and C2 (or REPI and REPII) motifs found in ScMac1p with a consensus sequence of CXC(X)4CXC(X)2C(X)2H (where X = any amino acid; Fig. 3b). These motifs facilitate the copper-mediated control of expression by preventing the binding of ScMac1p, in high-copper conditions, to copper-responsive elements (CuREs) found in the promoters of the target genes (Graden & Winge, 1997; Keller *et al*., 2000; Labbe *et al*., 1997; Yamaguchi-Iwai *et al*., 1997; Zhu *et al*., 1998). The spacing between the C1 and C2 motifs differs in each protein, being separated by 42 residues in ScMac1p and 61 residues in CaMac1p. Analysis of the CaMac1p predicted amino acid sequence using PSORT II revealed a 431-residue protein product that had a 73±9% probability of being retained in the nucleus.

**Fig. 2.** Iron uptake assays. (a) Assays were performed at 30°C and 0°C on strains DAY185 and BWP17ΔΔΔHIH1. (b) Assays were performed at 30°C on strains DAY185 (○) and BWP17mac1ΔΔΔHIH1 (●). All iron uptake rates were calculated by determining the c.p.m. of a known concentration standard of 55Fe. Cells were confirmed to be viable after incubation in assay buffer by serial dilution and plating out onto YPD. The results shown are from data taken from four repeated experiments. The uptake rates are presented as mean values; the error bars represent SD.

**Fig. 3.** Sequence comparisons. The predicted sequences of each conserved motif were aligned with PILEUP from the GCG package using the default parameters. The BOXSHADE program was used to highlight identities (black) and similarities (light grey) between the sequences. The consensus sequence of each motif is shown with identities in upper case and similarities in lower case. (a) Comparison of the putative ‘copper fist’ motif of CaMac1p with similar motifs found in the copper-responsive transcription factors of *S. cerevisiae* (Mac1p and Ace1p), *C. glabrata* (Am1t1p), *S. pombe* (Cuf1p) and *Y. lipolytica* (Crf1p). (b) Comparison between the cysteine-rich copper-binding sites found in ScMac1p and the putative-copper binding sites of CaMac1p.
Phenotypic analysis of a Camac1-null mutant

The features found during sequence analysis of the predicted protein product of orf6-8485 made this ORF a likely candidate to encode a protein functionally homologous to Mac1p of S. cerevisiae, and the gene is referred to as CaMAC1 from here onwards in the text. To investigate whether CaMAC1 was functional in C. albicans we constructed a Camac1-null mutant by disrupting the two genomic copies in strain BWP17 using PCR-directed mutagenesis (Wilson et al., 1999). We then reintegrated CaHIS1 into strain BWP17mac1Δ::URA3/mac1Δ::ARG4 and CaHIS1 along with a wild-type allele of CaMAC1 to construct strains BWP17mac1Δ/Δ/HIS1 and BWP17mac1Δ/Δ/HIS1::MAC1, respectively (see Methods).

It has been previously observed that a S. cerevisiae mac1-null mutant displays phenotypes similar to those of a strain which lacks high-affinity copper uptake. These phenotypes include respiratory deficiency and slow growth on low-copper and low-iron medium (Dancis et al., 1994a, b; Knight et al., 1996). The Camac1-null mutant (BWP17mac1Δ/Δ/HIS1) was therefore tested to see if it possessed similar mutant phenotypes (Fig. 4). The results revealed that the Camac1-null mutant displayed similar phenotypes to a S. cerevisiae strain defective in Mac1p activity, and these phenotypes were also comparable to those previously observed in a Cactr1-null mutant (Marvin et al., 2003). Like a Cactr1-null strain, the Camac1-null mutant displayed slow growth on low-copper medium (although this was not as marked as in a Cactr1-null strain), slow growth on low-iron medium, and was unable to grow on YP medium containing glycerol or ethanol as the sole carbon source. All these phenotypes could be rescued by the addition of 100 μM cupric chloride. Reintegration of a wild-type allele of CaMAC1 at the his1::hisG locus was sufficient to rescue all the mutant phenotypes. The Camac1-null mutant was also tested for the ability to form hyphae in response to the addition of bovine calf serum at 37°C. The results indicated that the production of true hyphae was unaffected by a double disruption of CaMAC1 (data not shown). We also tested the ability of a Camac1-null strain to take up iron using the 55Fe uptake assay. Interestingly, no significant difference was observed between the uptake rate of strain BWP17mac1Δ/Δ/HIS1 and the wild-type strain, DAY185 (data not shown). However, we did observe a lag period in strain BWP17mac1Δ/Δ/HIS1 lasting 60 min, during which iron uptake was induced at some point between 30 and 60 min (Fig. 2).

CaMac1p activity is required for regulated expression of CaCTR1

We have previously demonstrated that increased expression of CaCTR1 in C. albicans occurs in response to low-copper conditions in the growth medium (Marvin et al., 2003). To determine whether this is dependent on the activity of CaMac1p, transcription of CaCTR1 in both a wild-type and Camac1-null mutant background was investigated. Northern blot analysis was carried out on RNA isolated from strain BWP17 and strain BWP17mac1Δ::URA3/mac1Δ::ARG4, which had been grown in either high- or low-copper conditions. The strains were grown in either MD-BCS medium containing 100 μM cupric chloride or MD-BCS medium with no added copper and total RNA was extracted from each strain during the mid-exponential phase of growth (1 × 107 cells ml−1). Following transfer to a nylon membrane the RNA was probed with a 508 bp internal fragment of the CaCTR1 gene generated using primers CaCTR1+60 and CaCTR1+568 (Table 2). A CaCTR1 transcript was not detected in strain BWP17mac1Δ::URA3/mac1Δ::ARG4 in either low- or high-copper conditions on four separate occasions (Fig. 5). These results demonstrated that CaMac1p activity is required for expression of CaCTR1 in response to low-copper conditions.

The defective iron uptake observed in a C. albicans ctr1-null mutant does not coincide with a decrease in oxidase activity in total cell membrane extracts

It has previously been demonstrated that copper-depleted conditions result in defective PPD oxidase activity in cell membrane fractions purified from both S. cerevisiae and C. albicans (Knight et al., 2002; Yuan et al., 1995). This led us to hypothesize that PPD oxidase activity would be defective in a Cactr1-null and Camac1-null mutant due to deficient copper uptake. To ascertain PPD oxidase activity in the absence of CaCt1p and CaMac1p activity, assays were performed on total membrane fractions purified from BWP17ctr1Δ::URA3/ctr1Δ::ARG4 and BWP17mac1Δ::URA3/mac1Δ::ARG4, and from the wild-type strain, BWP17. This assay relies upon the activity of multicopper oxidases, which can also oxidize PPD as an artificial substrate. Oxidation of PPD results in a colour change that can be quantified by spectrophotometry (Spizzo et al., 1997).

Cells were grown in unsupplemented MD-BCS/BPS medium, or MD-BCS/BPS medium that had been supplemented with 100 μM cupric chloride, 100 μM ferric chloride, or both. Total cell membrane fractions were obtained from these cells and the rate of oxidase activity was determined (Fig. 6). The results obtained were corrected for background oxidation by subtracting the rate obtained from a blank sample containing no protein extract. To check that the colour change observed in these assays was due to protein activity, a boiled membrane extract was also assayed as a negative control and found to contain activity identical to a blank sample (data not shown).

Consistent with previous observations, the wild-type strain (Fig. 6a) displayed PPD oxidase activity that was dependent upon the presence of copper in the growth medium, but was not affected by iron concentration (Knight et al., 2002). The Camac1-null strain displayed similar PPD
**Fig. 4.** Growth phenotype of a Camac1-null mutant. Cells grown in unsupplemented MD-BCS medium were harvested at mid-exponential phase, washed three times and suspended in distilled water at a concentration of $1 \times 10^7$ cells ml$^{-1}$. Fivefold serial dilutions were made ($1 \times 10^7$ to $5 \times 10^4$ cells ml$^{-1}$; left to right in each panel) and 2 μl aliquots of each suspension were spotted onto the media indicated below the panels. The copper-supplemented media (+Cu) contained 100 μM cupric chloride. All growth was observed after incubation at 30°C for 3 days.
oxidase activity to the wild-type strain under the same growth conditions (Fig. 6c). The \textit{Cactr1}-null mutant also gave the same pattern of PPD oxidase activity as the wild-type, except when both iron and copper were absent from the growth medium (Fig. 6b). Following transfer to a nylon membrane the RNA was probed with an [\textit{x-32P}]CTP-labelled 508 bp internal fragment of the \textit{C. albicans} \textit{CTR1} gene. Autoradiographs were then exposed to the labelled membrane for 1 h to visualize hybridizing bands. rRNA bands were stained with ethidium bromide and visualized with UV radiation as a loading control (lower panel). The experiment was reproducible on four separate occasions.

**DISCUSSION**

We have previously reported that a \textit{Cactr1}-null mutant grows predominantly in filamentous form in low-copper conditions, with a significant number of cells displaying aberrant morphology (Marvin et al., 2003). The results presented here demonstrate that low-copper conditions induce invasive growth in \textit{C. albicans} and that this is augmented in a \textit{Cactr1}-null mutant. Therefore, the aberrant morphology that was previously observed in a \textit{Cactr1}-null mutant does not affect the ability of the cells to penetrate the growth medium. Although filamentation was induced by copper starvation, it is equally possible that it was induced by the lack of high-affinity iron transport. In this study, \textit{55}Fe uptake assays have shown that a \textit{Cactr1}-null mutant is defective in iron uptake. The source of iron in the \textit{55}Fe uptake assays is in the ferric form and so any uptake activity would require the activity of both cell-surface-associated ferric reductases and ferrous iron transport proteins. Iron uptake in the \textit{Cactr1}-null strain was reduced by an average of 96\%, which is consistent with defective high-affinity reductive iron uptake. Therefore, if copper transporters are not differentially expressed in response to stimuli other than low-copper conditions in \textit{C. albicans}, it appears that the organism does not contain two functionally distinct copper uptake systems.
redundant copper transporters like those found in *S. cerevisiae*. Furthermore, there are no sequences present on the *C. albicans* genome database that have significant identity to *ScCtr3p*.

The remaining 3.78% of iron uptake activity may be attributed to a low-affinity iron uptake system or low-affinity copper uptake system. There are sequences present on the Institut Pasteur *C. albicans* genome database with significant identity to the *S. cerevisiae* low-affinity iron/copper transporter Fet4p and also the vacuolar copper transporter Ctr2p (Hassett *et al.*, 2000; Kampfenkel *et al.*, 1995). These putative homologous genes may provide an alternative source of copper and iron to *CaCt1p*, and may account for the residual 55Fe uptake observed. In addition, a previous study in *S. cerevisiae* has proposed that *ScCc2p* may have the ability to shuttle copper between intracellular compartments and the cell membrane (Pufahl *et al.*, 1997). The other alternative source of iron uptake may be a ferri-siderophore uptake system. However, although *CaARN1* encodes a protein that facilitates the uptake of ferri-ferrichrome in *C. albicans*, the biosynthesis of siderophores in this organism has not been unequivocally proven (Hu *et al.*, 2002). Future studies using iron uptake assays should be carried out over longer time-periods and under different growth conditions to see if the ferri-siderophore uptake system is differentially regulated. The regulation of genes that facilitate iron uptake in *C. albicans* has been reported to be under the control of the *CaCt1p* transcriptional repressor (Knight *et al.*, 2002). Interestingly, cDNA micro-arrays have shown that transcriptional repression of *ScFRE2*, *ScFET3*, *ScFTR1*, *ScCCC2* and the *ScARN* genes in response to high-iron conditions in *S. cerevisiae* is under the control of the protein kinase *ScTpk2p* (Robertson *et al.*, 2000). The *ScTPK2* gene and its functional homologue in *C. albicans* are also involved in the regulation of morphogenesis via the cAMP-activated pathway (reviewed by Gancedo, 2001; Sonneborn *et al.*, 2000). This raises the possibility of copper- or iron-induced filamentation in *C. albicans* being under the regulation of the cAMP pathway, which may involve both *CaTpk2p* and *CaEfg1p* activity. Alkaline conditions are also known to induce filamentation in *C. albicans* via the Rim101p-mediated pathway (Davis *et al.*, 2000a). Therefore, *CaRim101p* is another candidate for activating filamentation in response to low-copper and low-iron conditions, as defective *CaCtr1p* activity may lead to changes in intracellular pH. Future studies on filamentation in *Catpk2*, *Catup1* and *Carim101*-null mutants in response to low copper or iron may reveal if they are involved in this process.

Identification of CuREs in the promoter of CaCTR1 raised the possibility of a similar copper-responsive transactivator to ScMac1p operating in *C. albicans* (Marvin *et al.*, 2003). We have previously shown that *CaCTR1* is transcriptionally regulated by *ScMac1p* when heterologously expressed in *S. cerevisiae*. Here we have shown that a copper-sensing transactivator similar to ScMac1p operates in *C. albicans* and is essential for transcription of *CaCTR1* in response to low-copper conditions. The predicted *Camac1p* possessed an N-terminal ‘copper fist’ motif (Alschul *et al.*, 1997) and C-terminal C1 (REP I) and C2 (REP II) motifs that facilitate copper-mediated control of expression in the ScMac1p transactivator (Graden & Winge, 1997; Jensen *et al.*, 1998; Jensen & Winge, 1998; Keller *et al.*, 2000; Labbe *et al.*, 1997; Yamaguchi-Iwai *et al.*, 1997; Zhu *et al.*, 1998). Future analysis of the CuRE sites in the promotor of *CaCTR1* will reveal whether they facilitate transcriptional control by *CaMac1p*. Similar studies on the ‘copper fist’ and the C1 and C2 motifs of *CaMac1p* will determine the importance of these motifs for metal-sensing and transactivation.

A *Camac1*-null mutant displays phenotypes that are directly comparable with those of a *Cactr1*-null mutant and are consistent with defective high-affinity copper uptake (Dancis *et al.*, 1994a, b; Knight *et al.*, 1996). Interestingly, the *Camac1*-null mutant displayed a similar rate of iron uptake to a wild-type strain, but had a lag period of approximately 1 h, with uptake being induced somewhere between 30 and 60 min. Although a *Camac1*-null mutant displayed slow growth on low-iron medium, this phenotype was not manifested so strongly as in a *Cactr1*-null mutant (Marvin *et al.*, 2003). These observations may simply reflect the effect of *CaMAC1* deletion on the growth rate of *C. albicans*. It should be noted that *CaMac1p* appears to be a copper-sensing transactivator. Therefore, a basal level of expression of *CaCTR1* may still be present in a *Camac1*-null mutant even though no transcript was detected following Northern blot analysis (Fig. 5). This may explain the anomalies in the phenotypes observed between the *Cactr1*-null and *Camac1*-null strains. It should also be noted that in *S. cerevisiae*, the *ScMAC1* regulon includes six genes, *ScCTR1*, *ScCTR3*, two metal-ductases, *ScFRE1* and *ScFRE7*, and two unidentified ORFs, YFR055w and YIL277w (Gross *et al.*, 2000). This raises the possibility that the phenotypes observed in a *Camac1*-null mutant may result from reduced expression of additional genes in *C. albicans*, such as ferric reductases, that are involved in iron or copper metabolism and not necessarily be due only to reduced expression of *CaCTR1*. The observed anomalies between the two mutant strains also raise the possibility of alternative mechanisms in *C. albicans* for activating copper and iron transport into the cell in response to metal ion starvation.

It has recently been shown that in a wild-type *C. albicans* strain, iron acquisition is dependent upon the availability of copper; with uptake being severely inhibited in copper-depleted conditions (Knight *et al.*, 2002). In the same report, PPD oxidase activity was also shown to be dependent upon copper and this dependency was proposed to be due to the requirement of *CaFet3p*. However, although a *CaFtr1*-null mutant was unable to establish an infection in a systemic mouse model, virulence of a *CaFet3*-null mutant and a *CaC2c2*-null mutant has been shown to be essentially the same as that of a wild-type strain (Eck *et al.*, 1999; Ramanan...
& Wang, 2000; Weissman et al., 2002). Therefore, it appears that there is a paradox with the requirement of multicopper oxidase activity for reductive iron uptake in C. albicans, as deletions in CaFET3 and CaCCC2 would be expected to result in reduced virulence. However, CaCCC2 was isolated and characterized by functional rescue of a corresponding S. cerevisiae mutant and its complete biochemical properties have not been fully elucidated (Weissman et al., 2002). There are also four additional ORFs that are present on the C. albicans genome database with predicted protein products that display significant sequence identity to CaFET3p. These may encode additional multi-copper oxidases that work alongside CaFTR1 in a high-affinity iron transport complex (Eck et al., 1999; Knight et al., 2002). Interestingly, we have now demonstrated that in the absence of CaCtr1p, PD oxidase activity is no longer dependent on copper added to the growth medium. This activity might be attributed to any of the additional ORFs that encode putative multi-copper oxidases that may mobilize internal iron stores in response to copper starvation.

This paper has described important components of a high-affinity iron and copper uptake system that are necessary to enable C. albicans to acquire these essential metals from its various environmental niches. The phenotypes associated with a Cactr1-null mutant indicate an important role of copper uptake for high-affinity iron uptake in C. albicans. These studies have also shown that CaCTR1 is regulated by a copper-sensing transactivator in a manner that appears to be similar to the system described in S. cerevisiae. Future studies on Cactr1- and Camac1-null mutants in infection models will reveal the importance of copper uptake and its regulation for the virulence of C. albicans. These studies will ascertain whether CaCtr1p or CaMac1p are potential drug targets for future therapy of C. albicans infections. The studies described here, and future studies on virulence, will give a greater understanding of the relationship between copper and iron uptake in C. albicans and the role they may play in the survival and proliferation of this organism in the mammalian host.

ACKNOWLEDGEMENTS

We would like to thank Primrose P. E. Freestone for her invaluable help with iron uptake assays, Diana A. Thomas for constructing strain BW57mac1ΔΔ/HIS1 and BW57mac1ΔΔ/HIS1::CTR1 and Peter A Meacock for helpful discussions.

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