The high copper tolerance of *Candida albicans* is mediated by a P-type ATPase

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The pathogenic yeast *Candida albicans* has higher resistance than the baker's yeast *Saccharomyces cerevisiae* to elevated concentrations of copper. To understand the basis of this differential resistance, we performed a functional screen for *C. albicans* genes involved in copper detoxification. Here, we report the isolation of two such genes: a metallothionein, Ca*CUP1*, and a copper-transporting P-type ATPase, Ca*CRP1*. Both genes are induced by extracellular copper. Gene disruptions indicated that the copper extrusion pump is responsible for the unusual resistance of *C. albicans* to copper, whereas the metallothionein is responsible for the residual copper resistance of the Ca*crp1*Δ mutant. We show further that under acidic and anaerobic conditions, such as prevail in the natural niche of *C. albicans*, the digestive tract of animals, Ca*CRP1* function becomes essential for survival in the presence of even very low copper concentrations. These observations suggest that copper in the gastrointestinal tract may present a toxic challenge to which enteric organisms had to adapt.

copper toxicity | metallothionein

Copper, a cofactor of many cellular enzymes, is an essential element. To cope with low-copper environments, yeast (*Saccharomyces cerevisiae*) cells possess a high-affinity copper import system consisting of two copper transporters, Ctr1 and Ctr3 (1, 2). It is likely that the substrate for copper transport is reduced copper, Cu(I). Thus, in aerobic environments, the pathway of copper import also includes plasma membrane-associated cupric reductases that reduce Cu(II) to Cu(I) (3, 4).

Although copper is essential, high copper concentrations are toxic, in large measure because copper catalyzes the synthesis of reactive oxygen species (5). Importantly, however, copper is also toxic under anaerobic conditions, both in bacteria (6, 7) and in yeast (8). Resistance to high copper concentrations is achieved by a variety of mechanisms. In some prokaryotes, copper resistance is mediated by extrusion pumps of the P-type ATPase family of transporters (9–11). In eukaryotes, two major mechanisms of resistance to elevated extracellular copper are shutdown of copper uptake and synthesis of metallothioneins, which function as copper chelators. In *S. cerevisiae*, exposure to high copper concentrations results in down-regulation of the copper transporter genes *CTR1* and *CTR3* and of the cupric reductase *FRE1* (4, 12, 13) as well as in destabilization of the Ctr1 protein (14). Inability to down-regulate these genes results in hypersensitivity to copper (15). High copper concentrations also lead to the induction of small proteins, metallothioneins (16), which tightly bind copper via the thiol groups of cysteine residues arranged in a typical C-X-C sequence. The critical role in copper resistance of the main copper-induced metallothionein in yeast, *CUP1*, is attested by the fact that *cup1*Δ mutants are highly sensitive to copper (17).

Copper-transporting P-type ATPases are found in eukaryotes as well, where they play a major role in intracellular copper transport. Eukaryotic copper-transporting P-type ATPases include the yeast Ccc2 protein (18) and the human Wilson and Menkes disease proteins (19–22). Yeast Ccc2 was genetically shown to be required for growth in low iron concentrations. Its function is to transport copper into a post-Golgi compartment, to allow biogenesis of the multicopper ferric oxidase Fet3. Fet3, in turn, is required for iron uptake (23). The Menkes and Wilson disease proteins are similarly involved in transport of copper to a post-Golgi compartment in mammalian cells. Expression of these proteins in yeast complements the iron import deficiency of the *ccc2*Δ mutant (24–26). In addition, in cultured animal cells exposed to elevated copper concentrations, these proteins are able to migrate to the plasma membrane or to a cytoplasmic vesicular compartment (25, 27–29); in this way, they may increase the resistance of the cell to copper.

Commensal and pathogenic organisms have by necessity adapted to growth—or at least survival—in the specific environments of the animal body. Many parameters of these environments are still unknown. *Candida albicans* is a commensal organism, found in the mouth and gastrointestinal tract of 30–50% of the population. It is also a major fungal opportunistic pathogen, often causing mucosal, cutaneous, or nail infections. Deep-seated life-threatening infection is less common and occurs primarily in immunocompromised or debilitated patients (30). The yeast *S. cerevisiae,* in contrast, is isolated only rarely from patients. One approach to gain better understanding of the survival capacity of pathogenic microorganisms in the animal body would be to study the molecular basis underlying the differential resistance to environmental insults of a pathogenic vs. a nonpathogenic microorganism—for example, *C. albicans* and *S. cerevisiae*. Here, we report that *C. albicans* is unusually resistant to copper, and we identify the molecular basis of this resistance.

Materials and Methods

Plasmid Constructions. Plasmids KB806 (Ca*CRP1*) and KB807 (Ca*CUP1*) are clones from a *C. albicans* genomic DNA library constructed into a 2-μ *URA3* yeast vector (31). The whole KB807 insert (2,538 bp) and 5,023 bp of the 6,094-bp KB806 insert were sequenced on both strands. The KB806 insert was recloned into pRS315 (32) to yield KB815. KB537 (our plasmid collection) carries the yeast *EF1A* coding region under the *GAL1, 10* promoter. Plasmids KB787 and KB788 were used for deleting Ca*CRP1* and Ca*CUP1* from the *C. albicans* genome, respectively. Both contain the Ca*URA3* gene between two direct hisG repeats (33), flanked by the 5′ and 3′ regions of Ca*CRP1* and Ca*CUP1*, respectively, in the pBSIIKS+ vector (Stratagene). KB787 contains a 5′ fragment extending from the beginning of the KB806 insert sequence (−2120 relative to the Ca*CRP1* initiation site) to a *Not*I site introduced by PCR at position + 1 and a 3′ fragment extending from a *Hin*dIII site at position + 3034 to the end of

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Abbreviations: MIC, minimal inhibitory concentration; GFP, green fluorescent protein.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AF193508 and AF193509).

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the KB806 insert (= 942 bp). KB788 contains a 5′ fragment extending from the beginning of the insert of KB807 (−1,324 relative to the Ca*CUP1* initiation site) to a *Bam*HI site introduced by PCR at + 1 and a 3′ fragment extending from an *Nde*I site immediately downstream of Ca*CUP1* to the end of the KB806 insert (= 1,086 bp). KB969 contains a Ca*CRP1-GFP* fusion constructed by PCR by using a yeast codon-modified *GFP* (34).

Yeast Strain Constructions. All yeast transformations were performed by the standard lithium acetate method (35). Strain DTY 113 (*α ura3–50 leu2–3,112 trp1–1 gal1 his cup1Δ61*) (36) was kindly provided by Dennis Thiele (University of Michigan, Ann Arbor, MI). The *ccc2*Δ::*URA3* mutant (YRS7) (23) and its isogenic wild type (YPH252) were kindly provided by Bob Stearman (National Cancer Institute). The *C. albicans* wild-type strains used were CBS562 (Centraal Bureau voor Schimmelcultures, Delft, The Netherlands) and CAF3–1 (33). Deletions were achieved by transforming the CAF3–1 strain with 5 μg of either KB787 or KB788 digested with *Sac*I and *Kpn*I. Transformants representing the desired homologous recombination were identified by Southern blotting. To sequentially delete both alleles of a single gene or multiple genes rare URA^- revertants of the URA^+ transformants that have deleted the *URA3* marker via homologous recombination between the *hisG* repeats were selected on 5-fluoroorotic acid (37). To ascertain that the phenotypes are entirely attributable to the site-directed deletions rather than to secondary genetic changes, each strain was constructed independently at least twice from the starting wild-type strain. KC13 is the double Ca*CUP1* deletant, KC16 is the double Ca*CRP1* deletant, and KC24 is KC13 deleted for both alleles of Ca*CRP1*.

Media; Phenotypic Tests. Yeast extract/peptone/dextrose and synthetic complete media were as described (35), except that the amino acids were added to 0.1 mg/ml each and for *C. albicans*, uridine was used rather than uracil. Sensitivity to copper was tested on plates as indicated or in liquid culture by using synthetic medium supplemented with the indicated amounts of $CuSO_4$. For sensitivity tests in acidic anaerobic conditions, the media were titrated to pH 3 with HCl and supplemented with 10 μg/ml ergosterol/0.5 mg/ml oleic acid (Tween 80). Cu^+ was added as CuCl from a 1 M stock in concentrated HCl. Transcript levels were measured by Northern blotting, followed by hybridization with radioactive probes and quantitation with a phosphorimager. For each sample, the data were normalized to the level of EF1α mRNA by reprobing the membranes with an *EF1A* probe derived from plasmid KB537 (the high homology of the *EF1A* gene between *S. cerevisiae* and *C. albicans*, 90%, allowed us to use the *S. cerevisiae* probe for *C. albicans*). For intracellular copper monitoring, cells grown as indicated were washed four times with ice-cold 50 mM Tris/20 mM EDTA, pH 7.5, and the copper concentration was measured with a Varian SpectraA 250 atomic absorption spectrophotometer equipped with a graphite furnace.

Results

Isolation of *C. albicans* Genes Conferring Copper Resistance in *S. cerevisiae*. The baker's yeast, *S. cerevisiae*, is relatively resistant to copper and is able to grow in up to 2 mM $CuSO_4$ in synthetic medium. We found, however, that *C. albicans* is even more copper resistant and grows in up to 20 mM $CuSO_4$ in synthetic medium (see Fig. 1*A* for a comparison of the growth of both yeasts in the presence of an elevated concentration of copper and Fig. 6*B*, below, for a growth inhibition curve). In rich (yeast extract/peptone/dextrose) medium, *C. albicans* is able to grow even in up to 50 mM $CuSO_4$ (results not shown). To identify the genetic factor determining the high copper tolerance of *C. albicans*, we performed a screen to isolate *C. albicans* genes able to confer increased copper resistance to a highly copper-sensitive *S. cerevisiae* metallothionein mutant. A *C. albicans* genomic library carried on a yeast 2 μ (high-copy) vector (31) was transformed into strain DTY113 (*cup1*Δ), which is normally unable to grow even on 10 μM CuSO4, and the cells were plated on 50 μM $CuSO_4$. Eight independent plasmid clones that conferred to the *cup1*Δ strain the ability to grow on 50 μM $CuSO_4$ were isolated: seven contained overlapping fragments, and one was unique. A representative of the first group and the unique clone were sequenced. Sequence analysis revealed, respectively, an ORF with metallothionein homology, which we called Ca*CUP1*, and an ORF with homology to copper-transporting P-type ATPases, which we called Ca*CRP1* (for *c*opper *r*esistance-associated *P*-type ATPase; see below). Complementation of the copper sensitivity of the *cup1*Δ mutant by these two genes is shown in Fig. 1*B*.

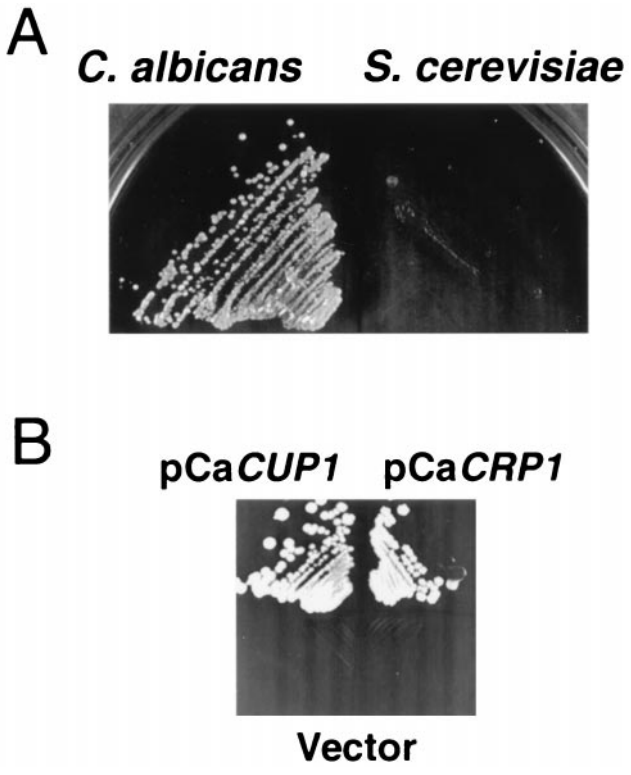

Fig. 1. (*A*) Differential copper resistance of *C. albicans* and *S. cerevisiae*. *C. albicans* strain CAF3–1 and *S. cerevisiae* strain W303 were grown 2 days at 30° on yeast extract/peptone/dextrose supplemented with 12 mM $CuSO_4$. (*B*) Functional complementation of the *S. cerevisiae cup1*Δ copper sensitivity by two *C. albicans* genes. *S. cerevisiae* strain DTY113 (*cup1*Δ) carrying the vector plasmid or plasmid KB807 or plasmid KB806 was grown for 3 days on synthetic complete − URA medium supplemented with 50 μM $CuSO_4$.

The Ca*CUP1* ORF is short, 33 codons, and contains three groups of C-X-C sequences, characteristic of copper metallothioneins (Fig. 2 *Upper*). The Ca*CRP1* ORF is 1,197 codons long and contains all of the signature domains of copper-transporting ATPases (Fig. 2 *Lower*). The highest homology was found with the catalytic domain of the P-type ATPases. Also conserved are the GMXCXXC sequences, found in the amino-terminal domain of this type of proteins (38–40). These sequences are thought to bind free copper or chaperone-bound copper and transfer it to the transmembrane channel domain of the protein. Ca*CRP1* contains three such consensus sequences and, in addition, two CXXC motifs closer to the amino terminus (Fig. 2).

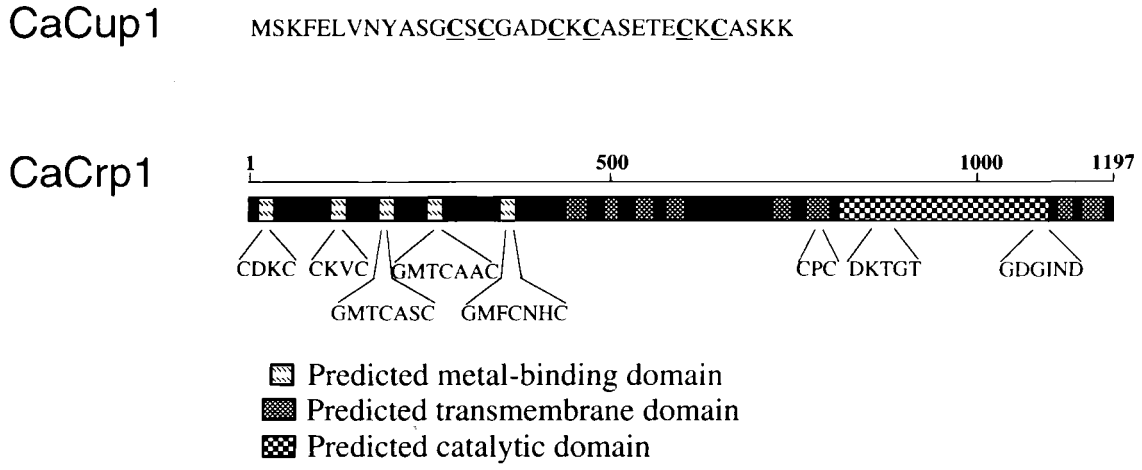

Fig. 2. Sequences of the *C. albicans* proteins CaCup1 and CaCrp1. (*Upper*) CaCup1 protein sequence derived from the DNA sequence. (*Lower*) Schematic depiction of the sequence features of CaCrp1. Eight putative transmembrane segments, centered around positions 460, 505, 550, 590, 740, 785, 1130, and 1165, were predicted on the basis of Kyte–Doolittle hydropathy analysis (41). The five predicted metal-binding motifs start at coordinates 12, 135, 178, 262, and 346. Three highly conserved P-type ATPase motifs within the catalytic domain are also indicated: CPC (position 784), DKTGT (position 829), and GDGIND (position 1060).

Transcriptional Induction of Ca*CRP1* and Ca*CUP1* by Copper. We expected that if Ca*CUP1* and Ca*CRP1* are involved in copper resistance in *C. albicans*, these two genes should be induced by the addition of copper to the medium. This is indeed the case: Northern blot analysis revealed that the addition of 2 mM $CuSO_4$ to an exponentially growing *C. albicans* culture leads to rapid but transient induction of both genes (Fig. 3), similar to the induction of *S. cerevisiae CUP1* (42).

Phenotypic Analysis of Deletions of Ca*CRP1* and Ca*CUP1*. To address directly the role of *CaCUP1* and Ca*CRP1* in copper tolerance, we deleted these genes from the *C. albicans* genome by homologous recombination (see *Materials and Methods*). The Ca*cup1*Δ/Ca*cup1*Δ strain displayed only a slightly increased susceptibility to copper, with a reduction of the minimal inhibitory concentration (MIC) of $CuSO_4$ from 20 mM to 10 mM in synthetic medium. In contrast, disruption of Ca*CRP1* had a dramatic effect on copper sensitivity: the MIC of $CuSO_4$ of the Ca*crp1*Δ/Ca*crp1*Δ strain was reduced to 0.5 mM (Fig. 4). The heterozygotes showed no growth phenotypes on copper. We also tested whether CaCrp1 may be involved in resistance to other heavy metals; however, the Ca*crp1*Δ/Ca*crp1*Δ strain was equally resistant to Zn^{2+}, Cd^{2+}, and Ag^{+} as the wild-type strain (results not shown).

It is likely that the rapid induction of both Ca*CRP1* and Ca*CUP1* by extracellular copper (Fig. 3) reflects the existence of a transcription factor that responds to the presence of copper in the cytoplasm, by analogy with *S. cerevisiae* Ace1 or *Candida glabrata* Amt1 (43–45). The levels of Ca*CRP1* and Ca*CUP1* mRNA could thus serve as a measure of intracellular copper. On the basis of this reasoning, we measured the transcript levels of each of these two genes when the other one is deleted (Fig. 5). In the Ca*crp1*Δ/Ca*crp1*Δ mutant, Ca*CUP1* mRNA levels are

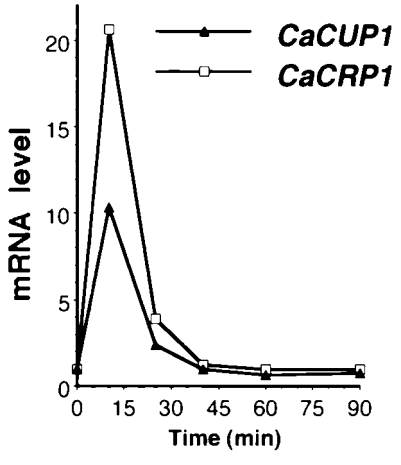

Fig. 3. Induction of Ca*CUP1* and Ca*CRP1* by copper. $CuSO_4$ (2 mM) was added to a logarithmic culture of wild-type *C. albicans* cells (CBS562). RNA was extracted at the indicated times after copper addition, and the mRNAs for Ca*CUP1* and Ca*CRP1* were detected by Northern blotting with specific probes and quantitated with a phosphorimager. EF1α mRNA was used as an internal control to which the data of each sample were normalized. The basal mRNA level was set at 1.

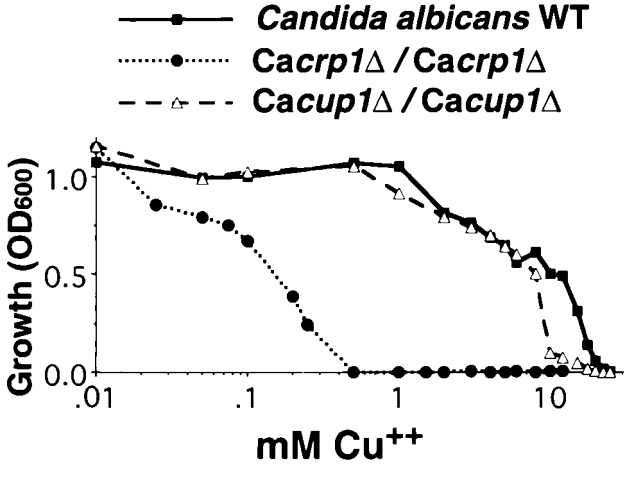

Fig. 4. Growth inhibition by $CuSO_4$ of *C. albicans* strains deleted for both alleles of Ca*CRP1* (KC13) or Ca*CUP1* (KC16). Cultures were grown overnight while shaking in synthetic complete medium supplemented with the indicated concentrations of $CuSO_4$. The optical density of the cultures at 600 nm was measured at the end of the incubation.

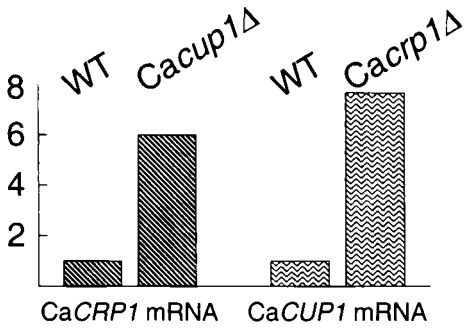

Fig. 5. Enhanced basal expression of Ca*CUP1* and Ca*CRP1* in Ca*crp1*Δ and Ca*cup1*Δ mutants, respectively. mRNA levels in logarithmic cultures of the two mutants and the wild-type strain were measured as described in the legend to Fig. 3. The basal level in the wild type was set at 1.

elevated 7-fold, whereas in the Ca*cup1*Δ/Ca*cup1*Δ strain, Ca*CRP1* mRNA levels were elevated 6-fold. Thus, both genes appear important for maintaining intracellular copper homeostasis, even when extracellular copper levels are low.

The MIC of 0.5 mM seen in the Ca*crp1*Δ/Ca*crp1*Δ strain is still well above that of the *S. cerevisiae cup1*Δ mutant and close to that of the wild-type *S. cerevisiae* (2 mM; see Fig. 6*B*). We asked whether Ca*CUP1* was responsible for this residual copper resistance by constructing a strain deleted for both genes. The Ca*crp1*Δ/Ca*crp1*Δ Ca*cup1*Δ/Ca*cup1*Δ strain was extremely copper sensitive and failed to grow even in 25 μM of $CuSO_4$, close to what is observed for the *S. cerevisiae cup1*Δ mutant (Fig. 6*A*). Thus, the two genes we identified encode the two main copper detoxification factors of *C. albicans*. However, the phenotypes of the individual mutants suggest that Ca*CRP1* has a more prominent role in copper detoxification than Ca*CUP1*, and that it is responsible for the relatively higher copper resistance of *C. albicans* vs. *S. cerevisiae*. To test this further, we transformed a plasmid carrying Ca*CRP1* into a wild-type *S. cerevisiae* strain and measured its resistance to copper. As shown in Fig. 6*B*, the MIC of $CuSO_4$ for the *S. cerevisiae* strain carrying Ca*CRP1* was significantly increased, from 2 mM to 5 mM.

If indeed the function of CaCrp1 is to extrude copper from the cells, then copper would be predicted to accumulate in the mutant cells. We monitored copper accumulation in *C. albicans* wild type vs. the *crp1* double deletion mutant by atomic absorption spectrophotometry. As shown in Fig. 7, the mutant cells grown in normal yeast nitrogen base minimal medium (containing about 0.4 μM $CuSO_4$) already accumulated over four times more copper; after growth overnight in 0.5 mM $CuSO_4$, 40 times more copper had accumulated in the mutant cells.

CaCrp1 Localization. The *S. cerevisiae* copper transporter Ccc2 is located in the trans-Golgi network, where it transfers copper from the cytosol to the lumen (46). Biogenesis of the multicopper ferric oxidase Fet3, which occurs in the trans-Golgi compartment, requires Ccc2. Because Fet3 is required for high-affinity iron transport, the *ccc2*Δ mutant is unable to grow in low iron or in medium containing the iron chelator ferrozine (23). We found that Ca*CRP1* is unable to complement the growth defect of the *S. cerevisiae* ccc2Δ strain on ferrozine (data not shown), suggesting that it does not function in the trans-Golgi compartment. The function of CaCrp1 in copper efflux from the cell suggests that it is located in the plasma membrane. This prediction was tested with a CaCrp1–green fluorescent protein (GFP) fusion. The CaCrp1–GFP fusion is able to complement the copper sensitivity of a *S. cerevisiae cup1*Δ mutant, indicating that it is functional (data not shown).

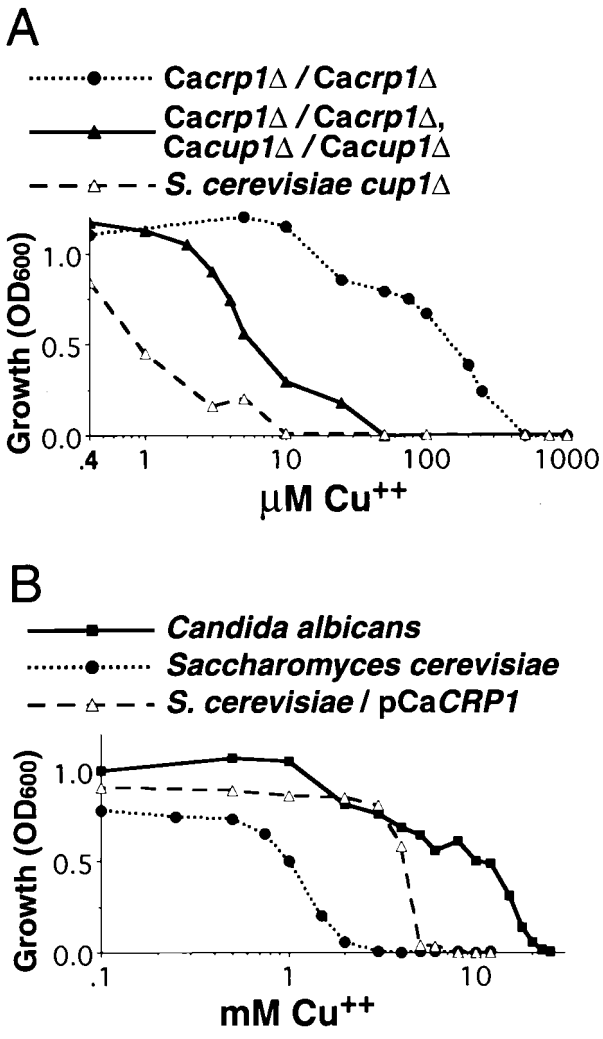

Fig. 6. (*A*) Comparison of growth inhibition by $CuSO_4$ in *C. albicans* strains *crp1*Δ (KC16), *crp1*Δ *cup1*Δ (KC24), and *S. cerevisiae cup1*Δ (DTY113). (*B*) Ca*CRP1* increases the resistance of wild-type *S. cerevisiae* (W303) to copper. The assays were performed as described in the legend to Fig. 4.

Fluorescence microscopy shows that the fusion protein is indeed primarily located in the plasma membrane (Fig. 8). Additional perinuclear staining in many cells, indicative of

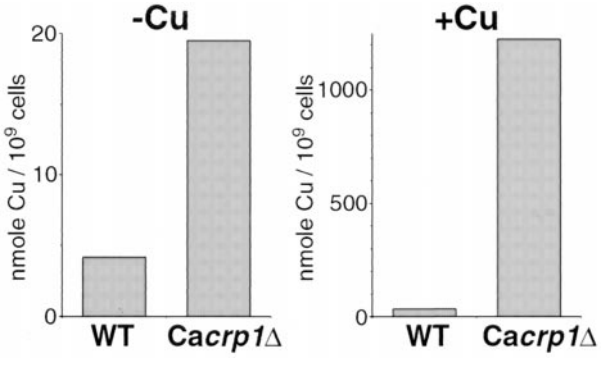

Fig. 7. Copper accumulation in wild-type vs. Ca*crp1*Δ mutant cells. (*Left*) Cells grown overnight in medium without added copper. (*Right*) Cell grown overnight in the presence of 0.5 mM $CuSO_4$.

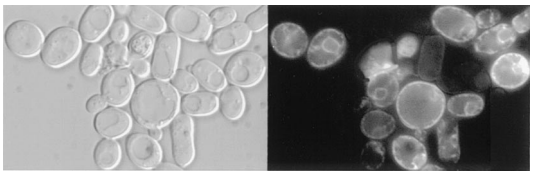

Fig. 8. Localization of a Crp1-GFP fusion protein. KY676 cells (diploid *cup1*Δ/*cup1*Δ) transformed with plasmid KY969 carrying the *CRP1-GFP* fusion were visualized with Nomarski optics (*Left*) or by epifluorescence (*Right*).

endoplasmic reticulum localization, may represent molecules in transit to the plasma membrane.

Copper Toxicity Under Acidic Anaerobic Conditions. *C. albicans* is found normally in the digestive tract of warm-blooded animals. This environment is largely anaerobic and in some parts very acidic. We tested whether the high copper resistance of *C. albicans* is related to these conditions. We found that when the pH of the yeast growth medium, normally 4.5–5.5, is reduced to 3, cells become significantly more sensitive to copper (results not shown). When cells are grown in anaerobic conditions and copper is added in the cuprous form [Cu(I)], the toxicity of copper is increased even more dramatically: even wild-type cells fail to grow in concentrations above 25 μM (Fig. 9). Under these conditions, the Ca*crp1*Δ mutant fails to grow even in 2 μM Cu^+, i.e., at physiological concentrations of copper in the digestive tract (47).

Discussion

C. albicans is estimated to be about 10^8 years removed in evolution from *S. cerevisiae.* During that time, both yeasts have diverged as a result of adaptation to very different environments—the animal body and rotting fruit. The question at the basis of our study is whether comparison between the two organisms can yield insights into the requirements of life in the animal body. The animal host presents numerous challenges for microorganisms attempting to invade and colonize it. An important component of host defenses consists in toxic challenges to the invading microorganism—e.g., the oxidative burst in engulfing macrophages. Can a differential resistance to toxic compounds between the two yeasts be explained by an adaptation to the interaction with the animal body?

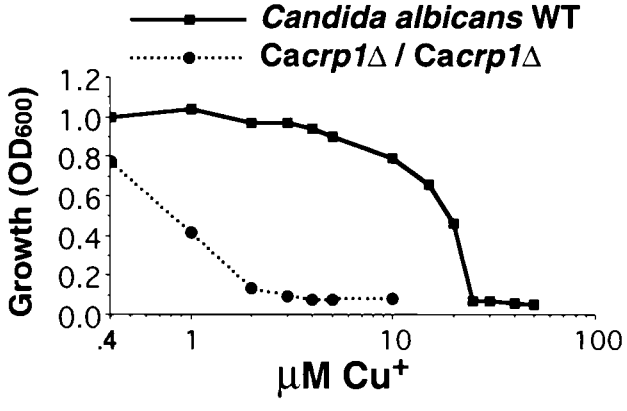

Fig. 9. Growth inhibition by Cu^+ in acidic anaerobic conditions. Cultures of the indicated strains were inoculated into 15-ml screw-capped tubes filled to the rim with synthetic complete medium pH 3, supplemented with ergosterol (10 μg/ml), oleic acid (0.5 mg/ml), and CuCl at the indicated concentrations and incubated at 30°C without shaking for 3 days.

We found that copper is a compound to which *C. albicans* displays increased resistance. We performed a functional complementation screen in *S. cerevisiae* for *C. albicans* genes involved in copper resistance. Two genes were found in our screen, a copper metallothionein and a copper extrusion pump. The derived protein sequence of CaCup1 is identical to the recently reported protein sequence of the main *C. albicans* copper-binding protein (48). The phenotype of a strain deleted of both genes indicates that they represent the two main mediators of copper resistance in *C. albicans*. Of these two genes, Ca*CRP1* plays the more prominent role in copper detoxification and is responsible for the high resistance of *C. albicans* to copper, compared with *S. cerevisiae*. The fact that CaCrp1 is more effective than CaCup1 at detoxification is not surprising, because an extrusion pump functions catalytically, whereas a chelating agent such as metallothionein functions stoichiometrically. Nonetheless, the phenotype of the Ca*cup1*Δ mutant—notably that the basal level of Ca*CRP1* is markedly increased in the Ca*cup1*Δ strain—indicates that Ca*CUP1* plays a role even in the presence of Ca*CRP1*. By analogy with the regulation of copper metallothionein in *S. cerevisiae* and *C. glabrata* and on the basis of the sequence analysis of their promoters (Z.W. and D.K., unpublished data), we assume that Ca*CUP1* and Ca*CRP1* are under the regulation of a copper-responsive transcription factor. Thus, a higher basal level would reflect an elevated cytoplasmic copper concentration. Taken together, our data suggest that CaCrp1 functions in bulk efflux of copper, whereas CaCup1 scavenges residual copper in the cytoplasm.

Copper-transporting P-type ATPases in eukaryotes are implicated in copper transport to intracellular organelles. Both *S. cerevisiae* Ccc2 and the human Menkes and Wilson disease proteins were shown to be located in a *trans*-Golgi compartment, where they deliver copper required for the biogenesis of the ferroxidase Fet3 and of ceruloplasmin, respectively (23–26). Ccc2 does not appear to play a role in copper detoxification. The Menkes and Wilson disease transporters, however, were shown to be able to migrate under certain circumstances to either the plasma membrane or to a vesicular compartment near the plasma membrane, presumably to extrude excess copper (25, 27–29). Thus, it was possible that similarly CaCrp1 can differentially localize according to the copper concentration and carry out both functions. However, we found that, (*i*) CaCrp1-GFP displays plasma membrane localization (Fig. 8) rather than the punctate cytoplasmic localization characteristic of Ccc2 (46); (*ii*) CaCrp1 is unable to complement the iron requirement of a *S. cerevisiae ccc2*Δ mutant (data not shown); and (*iii*) a *C. albicans crp1*Δ/*crp1*Δ mutant is unaffected for growth on low iron (data not shown). In addition, a sequence search against the almost completed *C. albicans* genomic sequence database (http://www-sequence.stanford.edu/group/candida) revealed that the *C. albicans* genome contains a second putative copper-transporting P-type ATPase with higher homology to Ccc2 (39% identity, vs. 29% for CaCrp1; results not shown). Thus, it is possible that this second protein is the functional homolog of ScCcc2 for intracellular iron transport, whereas CaCrp1's only function is in copper detoxification.

Why does *C. albicans*, alone so far among eukaryotes, require a dedicated copper extrusion pump? This type of copper detoxification mechanism is found also in a number of prokaryotes. Strikingly, many of the bacteria having known copper extrusion pumps are commensal or pathogenic enteric organisms (9–11). In the animal body, copper concentration is indeed the highest in the digestive tract, particularly in the stomach and duodenum, but even there it probably does not exceed 10 μM (47). However, we found that under acidic anaerobic conditions, which prevail in that part of the digestive tract, copper becomes much more toxic. The MIC of copper in the Ca*ccc2*Δ mutant under these conditions is 2 μM, suggesting that the existence of a copper-

extruding ATPase in *C. albicans* may have resulted from adaptation to its specific ecological niche, the animal gut.

Why is copper more toxic under anaerobic conditions? In *S. cerevisiae*, exposure to high copper leads to a shutdown of copper import (4, 12–15) and to the increased synthesis of Cup1, which chelates the copper that did enter the cell (16). Because Cup1 acts stoichiometrically, the shutdown of copper import is crucial, because the cell would be unable to cope with continuing copper entry. Mechanisms to inhibit copper import include down-regulation of the *CTR1* gene, which encodes the main copper transporter, as well as destabilization of the Ctr1 protein. We suggest that down-regulation of copper reduction is another important mechanism of shutdown of copper import. Because the substrate of the copper transporter is reduced copper, Cu(I), whereas normally copper is in its oxidized Cu(II) state, cupric reductases on the surface of the cell are an essential component of the copper import pathway (3, 4). Cells exposed to high extracellular copper could rapidly shut down copper import by inhibiting the activity of the cupric reductases, except if the extracellular copper is in the reduced Cu(I) form already. Cu(I) is rapidly oxidized under aerobic conditions but stable under anaerobic conditions. Furthermore, Cu(I) is extremely insoluble at neutral pH but becomes soluble at acidic pH. Thus, it is likely that Cu(I) is prevalent in the stomach. Under these conditions, shutdown of copper import may not be complete, hence the need for a catalytic mechanism of copper detoxification such as a copper extrusion pump.

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