A High-Affinity Iron Permease Essential for *Candida albicans* Virulence

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Microbial pathogens must compete with the iron-withholding defense systems of their host to acquire this essential nutrient. Here, two high-affinity iron permease genes, *CaFTR1* and *CaFTR2*, were isolated. *CaFTR1* expression was induced under iron-limited conditions and repressed when iron supply was sufficient, whereas the expression of *CaFTR2* was regulated in a reversed manner. Mutants lacking *CaFTR1* but not *CaFTR2* exhibited a severe growth defect in iron-deficient medium and were unable to establish systemic infection in mice. Thus, CaFTR1-mediated iron-uptake mechanism constitutes a virulence factor of *Candida albicans* and may be a target for the development of anti-*Candida* therapies.

Candida albicans is the cause of most fungal infections in immunocompromised patients, and systemic candidiasis may have fatal consequences (*1*). Pathogenicity of *C. albicans* has been attributed to several factors that enable the pathogen to damage and penetrate tissues, to escape host immune systems, and to establish systemic infection (*2–4*). However, little is known about how *C. albicans* acquires certain essential nutrients that are highly limiting in the host. One such nutrient is iron, which functions as an obligate cofactor of enzymes in many biological processes, such as DNA replication and electron transport (*5*). Mammals have developed an elaborate iron-withholding defense system against microbial infections, which collectively create an environment with virtually no free iron (*6–8*). Possession of specialized iron-uptake systems is intimately related to virulence of bacterial pathogens, such as *Haemophilus*, *Neisseria*, and *Yersinia* (*9–11*).

To study iron transport in *C. albicans* and its relevance to pathogenesis, we screened a subtracted cDNA library (*12*) for genes differentially expressed in cells grown in an iron-limited medium and investigated their importance for virulence in mice. We isolated two related genes, *CaFTR1* and *CaFTR2*, which are homologous to the high-affinity iron permease gene *FTR1* of *Saccharomyces cerevisiae* (*13*). *CaFTR1* and *CaFTR2* encode two proteins consisting of 381 and 382 amino acids, respectively, sharing 83% identity with each other and ~54% identity with FTR1. CaFTR1 and CaFTR2 contain multiple putative transmembrane domains and potential iron binding sites at positions similar to those in FTR1 (*14*).

We first used Northern blot analysis to examine *CaFTR1* and *CaFTR2* expression in response to iron concentration change in the *C. albicans* strain SC5314 (*15*). A *CaFTR1*-specific probe detected a transcript of ~1.66 kilobases (kb), and a *CaFTR2*-specific probe detected one of ~1.4 kb (Fig. 1A). Both transcripts are sufficiently large to encode the proteins. Expression of the two genes was distinctly regulated. The highest amount of *CaFTR1* transcript was detected in cells grown in LIM1, and the amount decreased with the increase of iron concentration and was hardly detectable in cells grown in LIM1000 (*12*). The response of *CaFTR2* transcript level was the opposite of that of *CaFTR1* transcript, exhibiting the highest transcription level in cells grown in LIM1000 and a negligible level in cells grown in LIM1. From this differential regulation of the two genes, we would predict CaFTR1 to be a major iron permease used by *C. albicans* to grow in iron-deficient environments.

To determine whether CaFTR1 and CaFTR2 were functionally equivalent to FTR1, we tested whether they could rescue the iron-dependent growth defect of a *S. cerevisiae ftr1* strain (*16*). *CaFTR1* and *CaFTR2* were expressed from an *S. cerevisiae* centromeric vector under the control of a galactose-inducible promoter. The *ftr1* cells transformed with either *CaFTR1* or *CaFTR2* exhibited little growth on the low-iron plate containing glucose in contrast to the considerable growth on the plate containing galactose (Fig. 1B). Transformants carrying the vector alone could not grow on the low-iron plate even in the presence of galactose. All transformants grew equally well on the iron-sufficient plates containing either glucose or galactose, that is, under conditions in which the low-affinity iron transporter functions (*17*). We also determined ^{59}Fe uptake by these transformants (*18*). The *ftr1* cells transformed with the vector exhibited no intracellular iron accumulation when $^{59}FeCl_3$ was supplied at 2 μM, a concentration in which only high-affinity permeases are active [(*17*) and Fig. 1C]. Introduction of either *CaFTR1* or *CaFTR2* restored the iron uptake to ~30 and 60% of the amount exhibited by the wild-type cells. Previous studies showed that FTR1 function requires interaction with a ferrous oxidase FET3 (*13*). Heterologous expression of the *Schizosacchacromyces pombe* iron permease gene *FIP1* alone in an *S. cerevisiae ftr1* mutant could not correct the iron uptake defect (*19*). Our result suggests that the two *C. albicans* permeases may be able to interact with FET3 to constitute a partially functional iron transporter. This possibility is supported by the much higher identity in amino acid sequence between CaFTR and FTR1 (~54%) than between FIP1 and FTR1 (~43%). We also observed that the *ftr1* mutants transformed with either *CaFTR1* or *CaFTR2* exhibited no increased iron uptake, when the culture medium contained 1 mM $FeCl_3$ (*20*), a concentration that would repress *FET3* expression (*19*).

To elucidate the respective roles of *CaFTR1* and *CaFTR2*, we constructed gene-deletion mutants for *CaFTR1* or *CaFTR2* or both genes from the *C. albicans* strain CAI4 (*14*). In the process of deleting the *CaFTR1* gene, we consistently found three copies of the gene in the genome, concluding that that is likely the natural status of the gene. We first evaluated the growth of all the *Caftr* strains in iron-limiting medium. The wild-type SC5314 and all the Ura3$^+$ *Caftr* strains (containing one *CaURA3* copy at the gene disruption locus) were first grown in SD200 to saturation and then diluted to 1×10^4 cells/ml in LIM0. *Caftr1*, *Caftr2*, and *Caftr1 Caftr2* strains were able to grow at a rate similar to that of SC5314 to near saturation in this iron-deprived medium. However, when each culture, after reaching saturation, was diluted again to 1×10^4 cells/ml in LIM2 and LIM200, *Caftr1* and *Caftr1 Caftr2* strains failed to grow in LIM2 but grew as well as SC5314 in LIM200 (Fig. 2A). The *Caftr2* strain grew normally in both media. Other researchers have also demonstrated the necessity of multiple passages of yeast cells in iron-deprived medium for the manifestation of growth inhibition (*21*, *22*), presumably because the cells must first exhaust the intracellular iron stores accumulated during growth in iron-sufficient media. Reintroduction of *CaFTR1* on a *C. albicans* autonomous-replicating plasmid pABSK1 (*23*) into *Caftr1* and *Caftr1 Caftr2*

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strains restored their growth in LIM2. Thus, strains lacking *CaFTR1* but not *CaFTR2* were unable to grow in the iron-limiting medium.

To determine whether the *Caftr* strains were defective in high-affinity iron uptake, we assayed $^{59}FeCl_3$ accumulation in cells that had been grown in an iron-limited medium (Fig. 2B). Iron accumulations in the *Caftr1* and *Caftr1 Caftr2* strains were reduced to near background level. In contrast, the *Caftr2* cells accumulated an amount of ^{59}Fe similar to that of SC5314. Reintroducing *CaFTR1* on pABSK1 into the *Caftr1* and *Caftr1 Caftr2* strains resulted in an accumulation of ^{59}Fe nearly twice that in SC5314 cells, likely because of the ability of the plasmid to exist in multiple copies. Introducing the plasmid without the *CaFTR1* gene had no effect. Thus, CaFTR1 was responsible for nearly all of the high-affinity iron-uptake activity in the cells grown under iron-limiting conditions.

The *CaFTR1* gene was essential for *C. albicans* to grow in an iron-deficient medium. Was it also required for *C. albicans* to grow in vivo? We inoculated mice with various *Caftr* mutants to examine their lethality and colonization of kidneys. Again, the $Ura3^+$ strains were used because $Ura3^-$ strains have reduced virulence (*24*). An inoculum of 1×10^6 cells of SC5314 or the *Caftr2* strain resulted in 100% mortality within 4 days of injection (Fig. 2C). In contrast, all of the animals inoculated with *Caftr1* or *Caftr1 Caftr2* strains survived to the 42-day end point. Transformation of *CaFTR1* on pABSK1 into *Caftr1* or *Caftr1 Caftr2* cells fully restored the lethality. The kidneys from two mice of each group were removed on day 2. Microscopic examination revealed that the kidneys from mice inoculated with strains lacking *CaFTR1* appeared normal, whereas the tissues from mice injected with strains containing a functional *CaFTR1* gene (or genes) showed lesions filled with both hyphal and yeast cells (Fig. 2D). The colony-forming units from animals of the former group were $1.4 \pm 0.26 \times 10^4$ ($n = 8$) per gram of kidney in contrast to $3.8 \pm 1.81 \times 10^6$ ($n = 6$) from the animals of the latter group. Thus, *CaFTR1* was indeed essential

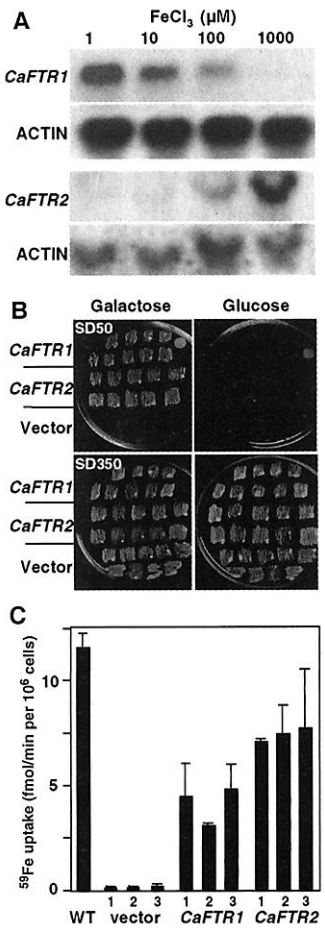

Fig. 1. Iron-regulated expression and iron permease activity of *CaFTR1* and *CaFTR2*. (**A**) Iron-regulated expression of *CaFTR1* and *CaFTR2* in *C. albicans*. (**B**) Correction of iron-dependent growth defect of *S. cerevisiae ftr1* mutant by *CaFTR1* and *CaFTR2*. *S. cerevisiae ftr1* mutant was transformed with a vector expressing either *CaFTR1* or *CaFTR2* under the control of Gal1-10 promoter or the vector alone. Multiple transformants were picked and patched onto low- (SD50) and high-iron (SD350) plates containing either glucose or galactose. The plates were incubated for 4 days at 30°C. (**C**) High-affinity iron uptake. Wild-type (WT) and *ftr1* strains transformed with *CaFTR1*, or *CaFTR2*, or the vector (three clones each) were examined for high-affinity iron uptake.

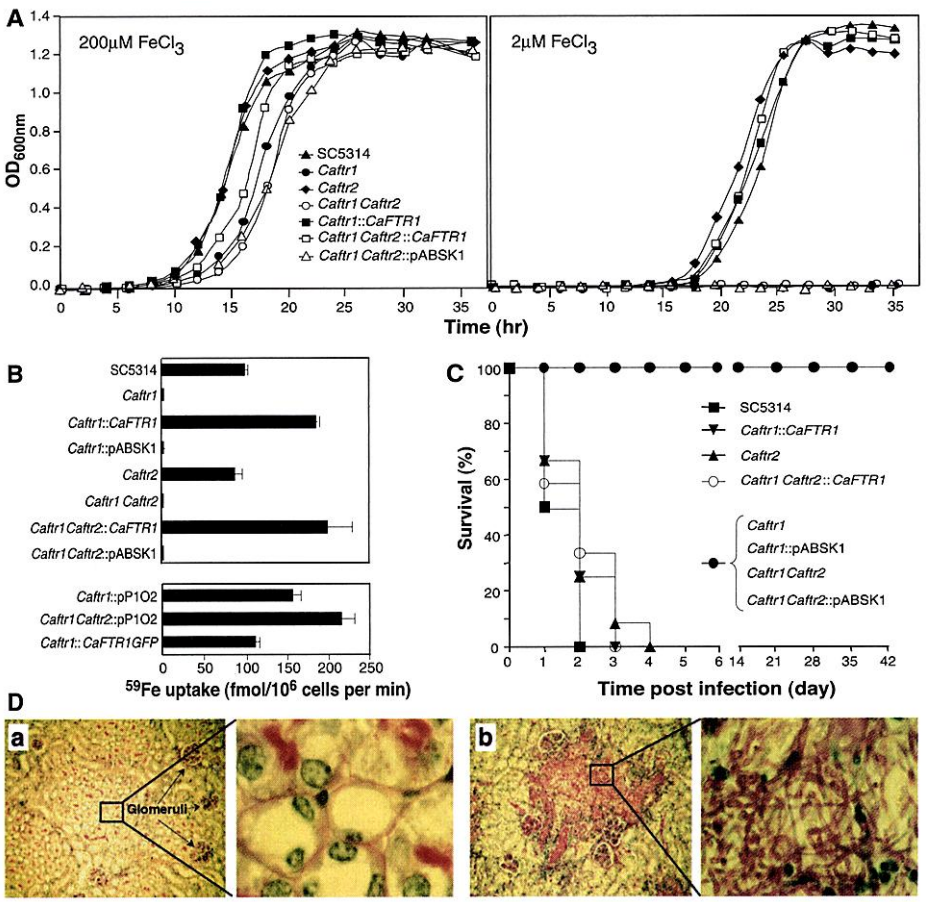

Fig. 2. Phenotypes of *Caftr* strains. (**A**) *Caftr1* strains fail to grow in iron-limited medium. All the strains were grown in uridine-dropout LIM2 and LIM200 media and monitored by measuring OD_{600} at 1.5-hour intervals. The strains are SC5314, *Caftr1*, *Caftr2*, *Caftr1 Caftr2*; the *Caftr1* and *Caftr1 Caftr2* strains transformed with pABSK1 expressing *CaFTR1* (*Caftr1*::*CaFTR1* and *Caftr1 Caftr2*::*CaFTR1*); and the same mutants transformed with the vector pABSK1. (**B**) *Caftr1* strain is defective in high-affinity iron uptake. The same strains used in the growth assay were subjected to the iron-uptake assay. The assay was also conducted on the *Caftr1* and *Caftr1 Caftr2* strains transformed with pABSK1 expressing *CaFTR2* under the control of *CaFTR1* promoter (*Caftr1*::pP1O2 and *Caftr1 Caftr2*::pP1O2) and on the *Caftr1* strain transformed with pABSK1 expressing GFP-tagged CaFTR1 (*Caftr1*::*CaFTR1-GFP*). (**C**) Strains lacking *CaFTR1* were avirulent. Twelve mice per group were injected with early stationary-phase yeasts of a *C. albicans* strain (1×10^6 viable cells per mouse in 0.2 ml of phosphate-buffered saline) grown in SD200-uridine and monitored for survival over a 42-day period. (**D**) Strains lacking *CaFTR1* did not colonize the kidney of injected mice. The kidney sections were stained with periodic acid–Schiff's stain. The photomicrographs show corresponding cortical regions. (a) A representative appearance of tissues from mice infected with the strains lacking *CaFTR1*; (b) a representative appearance of tissues infected with strains carrying functional *CaFTR1*.

for *C. albicans* to grow and to cause systemic infection in the host. Other possible iron-uptake mechanisms, if there are any, are either not sufficient for the organism to acquire iron in vivo or they function in a CaFTR1-dependent manner.

The *CaFTR2* gene was not required for *C. albicans* to grow in iron-deficient conditions. Could CaFTR2 function as a high-affinity iron permease in *C. albicans* when expressed? *CaFTR1* gene promoter was used to control the expression of *CaFTR2* from pABSK1 (*25*). The *Caftr1* and *Caftr1 Caftr2* strains transformed with this plasmid accumulated iron in amounts comparable to that in the same mutant cells transformed with the plasmid expressing *CaFTR1* (Fig. 2B, plasmid pP1O2), suggesting that CaFTR2 may function as high-affinity iron permease in *C. albicans*. However, its function was not required for *C. albicans* to grow in highly iron-limiting environments, consistent with the lack of *CaFTR2* expression under similar conditions. The presence of a pair of functionally similar but differentially regulated genes suggests that these genes evolved to function in different environments. *C. albicans* also possesses a pair of pH-regulated genes, *PHR1* and *PHR2*. They are maximally expressed at distinct pH and play different roles in morphogenesis (*26*).

The iron-transport function of CaFTR1 requires it to reside on the cell surface. To determine its cellular localization, CaFTR1 was tagged with the green fluorescent protein (GFP) at its COOH-terminus and expressed from the *CaFTR1* promoter on pABSK1 (*27*). *Caftr1* cells transformed with this plasmid exhibited high amounts of iron uptake, suggesting a functional fusion protein (Fig. 2B, *Caftr1*::*CaFTR1-GFP*). The transformants were then grown in either SD50 or SD1000 to exponential phase. Horse serum (10%) was added to a separate set of cultures to induce filamentous growth. Cells of both yeast and filamentous forms grown in SD50 were visualized to have a bright fluorescent

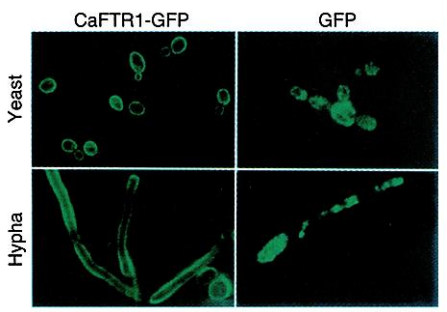

Fig. 3. Cell surface localization of CaFTR1. The Ura3$^-$ *Caftr1* was transformed with pABSK1 expressing either a CaFTR1-GFP fusion or GFP alone. The transformants were grown in SD50 to exponential phase.

periphery (Fig. 3), consistent with localization to the plasma membrane. The cells expressing the GFP alone showed intracellular fluorescence. Cells grown in SD1000 exhibited only faint disfused background fluorescence (*28*).

Thus, *CaFTR1* is required for *C. albicans* to acquire iron in iron-deficient environments both in vitro and in vivo. Deletion of the *CaFTR1* gene abolished the pathogen's capacity to establish systemic infection in mice. Among all the strategies speculated to be important for *C. albicans* to acquire iron in vivo (*29*), *CaFTR1* appears to play a central role and represents a potential target for the development of anti-*Candida* therapies.

References and Notes

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12. *C. albicans* cells were normally grown in YPD medium (2% yeast extract, 1% peptone, and 2% glucose) or SD medium. One liter SD contains 6.7 g yeast nitrogen base without amino acids (Difco), 0.8 g complete supplement mixture (CSM) yeast medium or CSM-ura (without uracil) (Bio101), 20 g glucose, 50 mM MES (pH 6.1) (*13*). Limited-iron medium (LIM) was prepared, and iron was removed as described (*30*, *31*). To prepare SD medium containing limited or sufficient iron, SD was first treated by adding 1 mM ascorbic acid and 1 mM ferrozine. $FeCl_3$ was added to LIM or SD to prepare LIMx and SDx, where *x* is the micromolar concentration of iron. For growing Ura3$^-$ strains in SD, 50 μg/ml uridine was added. From *C. albicans* strain (ATCC 10259), two cultures grown in LIM2 and LIM1000 were used to construct a subtracted cDNA library to enrich genes differentially expressed in LIM2 by using the polymerase chain reaction (PCR) Select Subtracted cDNA Library kit (Clontech). Sequencing the library clones identified two overlapping cDNA of 160 and 210 base pairs (bp). The cDNA was used to probe a *C. albicans* lambda genomic library. Two DNA fragments of ~5.7 and 5.4 kbp, encoding *CaFTR1* and *CaFTR2*, respectively, were isolated and sequenced using a DNA sequencer (Applied Biosystems, model 377).
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15. Strain SC5314 was grown in SD200 to saturation and then diluted to OD_{600} 0.05 in LIM supplemented with 1, 10, 100, and 1000 μM $FeCl_3$. The cells were grown at 30°C to OD_{600} 0.8 to 1.0 for total RNA preparation by using Trizol reagent (LTI). RNA (25 μg) was separated on a 0.22 M formaldehyde and 1.2% agarose gel, and blotted onto Hybond-N nylon membrane (Pharmacia-Amersham). The coding regions of *CaFTR1* and *CaFTR2* were PCR-amplified and ^{32}P-labeled as probes. Blots were washed in 0.2× standard saline citrate with 0.1% SDS for 1 hour at 65°C.
16. *S. cerevisiae* haploid strain CRY2α (*can1-100, ade2-1, his3-11, 15, leu2-3, 112, trp1-1, ura3*) was used to construct the *ftr1* strain (*14*).
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18. Iron-uptake assay was done as described (*17*). Cells were grown in minimal medium containing galactose and 50 μM $FeCl_3$ for 8 hours before the assay. $^{59}FeCl_3$ (2 μM) was used in a 10-minute assay at 30°C. Radioactivity was counted by a LKB Compugamma counter (model 1282), and the amount bound to the cells kept on ice was subtracted as background.
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23. An ~3770-kb Cla I fragment containing *CaFTR1* coding region and ~1.3 kb of both 5′ and 3′ flanking sequences from the genomic clone were inserted at the corresponding sites of plasmid pABSK1 (gift from H. Chibana, University of Minnesota) containing a *CaURA3* gene and the multiple cloning site from pBluescript (Stratagene). The resulting pABFTR1 was transformed into the Ura3$^-$ *Caftr1* strain and transformants selected on SD200-uridine plates.
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25. To construct a plasmid expressing *CaFTR2*, primers 5′-tcatacgtcgattaataa-3′ and 5′-gcggatcctgttaatgtttatttttt-3′ amplified *CaFTR1* promoter [nucleotides (nt) −1292 to −1, the first base of the start codon ATG is nt 1] from pABFTR1 with a Bam HI site added on the 3′ end. The PCR fragment was cut with Cla I (nt −1287) and Bam HI and inserted between the corresponding sites in pABSK1 to yield pP1. The *CaFTR2* coding region and 400-bp 3′ flanking sequence (nt 1 to 1479) was amplified by using a high-fidelity PCR kit (Roche) from the genomic clone with Bam HI and Xba I sites on the 5′ and 3′ ends, respectively. This PCR fragment and pP1 were cut with Bam HI and Xba I and ligated to yield pP1O2.
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27. The *CaFTR1* gene promoter and the coding region (nt −1310 to 1143) were PCR-amplified with Xho I and Xba I sites added to the 5′ and 3′ ends, respectively. The PCR fragment was cut with Xho I and Xba I and inserted between the corresponding sites in pABSK1 to yield pNPO1. A 700-bp fragment (nt 1147 to 1847) downstream from *CaFTR1* stop codon was PCR-amplified with Xba I and Sac II sites attached to the 5′ and 3′ ends, respectively. Both this PCR product and pNPO1 were digested with Xba I and Sac II and ligated to yield pNOE1. The GFP coding sequence (*32*) was PCR-amplified with Xba I sites attached to both ends, and fused in frame with *CaFTR1* at the Xba I site in pNOE1. The plasmid was transformed into Ura3$^-$ *Caftr1* cells and clones selected on SD200-uridine plates. The transformants were grown in either SD50 or SD1000 to exponential phase for fluorescence microscopy (Leica).
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