Characterization and Functional Analysis of the Siderophore-Iron Transporter CaArn1p in Candida albicans*

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Siderophores are small organic compounds with high affinity for ferric iron. Microorganisms commonly acquire iron via siderophore secretion and uptake. Here we report the characterization of the siderophore transporter CaArn1p in the fungal pathogen Candida albicans. Deletion of CaARN1 reduced the ability of C. albicans to use iron bound to the hydroxamate-type siderophore ferrichrome and abolished it when high-affinity iron permease genes (CaFTR1 and CaFTR2) were also deleted, indicating a role of CaArn1p as well as the permeases in ferrichrome-iron uptake. Caarn1 (but not Caftr1ΔCaftr2) assimilated iron from another hydroxamate-type siderophore, ferrioxamine B, suggesting that iron uptake from this compound depends on the permeases, but not on CaArn1p. Northern blot analysis revealed that the transcription repressor CaTup1p repressed CaARN1 expression under iron-replete conditions via the DNA-binding protein Rfg1p. Green fluorescent protein-tagged CaArn1p was observed predominantly in the plasma membrane, with some in the cytoplasm as distinct spots. The number of these spots increased with the increase in ferrichrome concentration, suggesting that CaArn1p internalization might be a mechanism for ferrichrome-iron uptake or for recycling the transporter. Caarn1Δ did not show reduced virulence when injected into the blood stream of mice, implying that CaArn1p is not required for iron uptake along this route of infection.

Iron is an essential nutrient for nearly all organisms because it serves as an obligate component of many indispensable enzymes and other proteins (1). As a general antimicrobial defense mechanism, mammals possess a sophisticated iron-withholding system consisting of high-affinity iron-binding proteins in body fluids, iron uptake proteins on the cell surface, and iron storage proteins inside the cells (1, 2). This system effectively maintains the free iron concentration in body fluid at an extremely low level insufficient for microbial growth. However, microbial pathogens have collectively evolved a diverse repertoire of highly effective iron acquisition mechanisms, some of which appear to be specifically “designed” to defeat the iron-withholding system or to target iron-rich niches of the host (3–6). For example, pathogenic Neisseria species have surface receptors specific for the host extracellular iron-binding proteins transferrin and lactoferrin, turning the two major components of the host iron-withholding system into their iron sources (7, 8). Candida albicans possesses mechanisms for attracting and lysing erythrocytes and utilizing the iron in hemoglobin (9, 10). These iron acquisition schemes constitute important virulence factors. A more commonly used strategy for microorganisms to acquire iron is the secretion of high-affinity iron chelators called siderophores (11–15). Siderophores are able to extract iron from host iron-binding proteins, and the siderophore-iron complexes are then assimilated by the pathogens via specific receptors and transporters. Some microorganisms such as Saccharomyces cerevisiae and pathogenic Neisseria species do not produce their own siderophores, but are able to use the ones secreted by other organisms (7, 16).

C. albicans is currently the most prevalent human fungal pathogen, causing infections in immunocompromised hosts (17). In some patients, C. albicans cells enter the blood stream, establishing fatal systemic infection. How does C. albicans acquire iron for growth in the iron-restricted blood stream and internal organs? We have previously shown that the high-affinity iron permease CaFtr1p is dispensable for C. albicans to establish systemic infection in mice (18). However, there is no evidence that CaFtr1p or its interacting partner oxidase, CaFet3p, can withdraw iron directly from iron-binding proteins of the host. According to studies of iron uptake mechanisms in S. cerevisiae and other organisms, there are at least two possible ways by which C. albicans may dissociate iron from the iron-binding proteins. First, C. albicans produces extracellular ferric reductases, which may convert the bound ferric iron to unbound ferrous iron. The free ferrous iron is then transported into the cells by the high-affinity iron uptake system (1, 19–21). Second, C. albicans may produce siderophores, which directly extract the bound ferric iron. Genes encoding ferric reductases have been cloned from C. albicans (22), and there have been several reports on possible siderophore production by C. albicans (23–25). Either the siderophore-bound iron may be released by the ferric reductases and then picked up by the high-affinity uptake system (18, 26–28), or the siderophore-iron complex is transported into the cell via receptor-mediated mechanisms (28, 29). Thus, it is likely that the siderophore-mediated iron binding may function upstream of and provide free iron to the oxidase-permease iron uptake system. It has been reported that disruption of tonB in Bordetella bronchiseptica and Bordetella pertussis prevents utilization of ferric siderophores, hemin, and hemoglobin as iron sources (30). Thus, it is possible that the siderophore uptake mechanism is related to the use of hemoglobin iron by C. albicans.

Four siderophore transporters (Arn1p, Arn2p/Taf1p, Arn3p/Sit1p, and Arn4p/Enb1p) have been reported in S. cerevisiae, collectively responsible for the uptake of a range of siderophores of different structures and chemical properties (16,

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These proteins belong to the major facilitator superfamily, whose members have been found in many organisms. Currently, one siderophore transporter gene (CaARN1) has been found in *C. albicans*, and its function has been investigated by heterologous expression in *S. cerevisiae*. Ardon et al. (29) reported that CaArn1p expressed in *S. cerevisiae* specifically mediates the uptake of FC1-iron, but does not transport iron bound to FOB. This result seems to contradict the accumulation of fluorescent analogs of both FC and FOB in CaARN1 deletion mutants of either *C. albicans*. The two copies of the CaARN1 gene were sequentially deleted from strains CA14 and CaWYNN2 (Cafr1ΔCafr2Δ) using the URA blaster method (35). The gene deletion strategy is shown in Fig. 1A. PCR primers were designed based on the genome sequence released by the *C. albicans* Genome Sequence Project at Stanford University. Primers 5′-TCTTAAATGCTGGCTTTA-3′ and 5′-GGATCCGATCCGGTTGAGTTAT-3′ amplified a 437-bp 5′-end fragment (nucleotides 344–781), the first base of the HindIII site upstream of the CaARN1 coding region is designated nucleotide 1), and primers 5′-GGATCCGATCCGGTTAATGTTTAT-3′ and 5′-TGCCATATGTTCTCGAGCAG-3′ amplified a 502-bp 3′-end fragment (nucleotides 2703–3205). During the PCR, a BamHI restriction site (underlined in the primer sequences) was added to the 3′- and 5′-ends of the two DNA fragments, respectively. The two DNA fragments were joined at the BamHI site in pGEM-Teasy. Then the hisG-URA3-hisG cassette with compatible ends was inserted at the BamHI site of the above plasmid. The Caarn1Δ::hisG-URA3-hisG cassette was released from the plasmid by NotI digestion, gel-purified, and used to transform CA14 and CaWYNN2 cells by electroporation as previously described (18). Clones of the CaARN1/Caarn1Δ::hisG-URA3-hisG genotype were obtained, and cells were spread onto fluoroacetate acid plates to isolate CaARN1/Caarn1Δ::hisG clones. To disrupt the second copy of CaARN1, a new cassette was constructed in which the hisG tandem repeats were replaced by an 850-bp Escherichia coli chloramphenicol acetyltransferase gene fragment. Also, two new pairs of primers were used to generate the 5′- and 3′-end CaARN1 fragments, which were internal to the ones used in the hisG-URA3-hisG cassette. The primers used were as follows: 5′-TAAACCTATACCTAAGA-3′ and 5′-GGATCCGGTATTATAAAC-3′, which amplified a 413-bp fragment (nucleotides 757–1170), and 5′-GGATCCGATCCGGTATTATAAAC-3′, which yielded a 447-bp fragment (nucleotides 2273–2714). Two independent CaARN1/Caarn1Δ::hisG clones were transformed with the Caarn1Δ::hisG-URA3-cat cassette to obtain Caarn1Δ::hisG-Caarn1Δ::hisG-URA3-cat clones. The URA3 gene was then looped out on fluoroorotic acid plates to produce Caarn1Δ::hisG-Caarn1Δ::hisG-URA3-cat clones. The genotypes of all the gene deletion mutants were verified by Southern blot analysis as exemplified in Fig. 1B.

### Table I: *C. albicans* strains used in this study

| Strain | Genotype | Source |
|--------|----------|--------|
| CA2-1 | URA3/ura3::im434 | Fonzi and Irwin (35) |
| CA14 | ura3::Xmr343/ura3::im434 | Fonzi and Irwin (35) |
| CaWY3 | CaARN1/Canr1Δ::hisG-URA3-hisG | This work |
| CaWY3-1 | Canr1Δ::hisG-URA3-hisG | This work |
| CaWY3-2 | Canr1Δ::cat-URA3-cat/Canr1Δ::hisG | This work |
| CaWY3-3 | Canr1Δ::cat-URA3-cat/Canr1Δ::hisG, C1p10-Canrn1 | This work |
| CaWY3-3.1 | Canr1Δ::cat-URA3-cat/Canr1Δ::hisG, C1p10-Canrn1-GFP | This work |
| CaWY3-3.2 | Canr1Δ::cat-URA3-cat/Canr1Δ::hisG, C1p10-Canrn1-GFP | This work |
| CaWY3-3.3 | Canr1Δ::cat-URA3-cat/Canr1Δ::hisG, C1p10 | This work |
| CaWY3-3.4 | Canr1Δ::cat-URA3-cat/Canr1Δ::hisG, pABS1-Canrn1 | This work |
| CaWY3-3.5 | Canr1Δ::cat-URA3-cat/Canr1Δ::hisG, pABS1-Canrn1-GFP | This work |
| CaWYNN1 | Catup1::hisG-Canfr1::hisG-Canfr1/His3 | This work |
| CaWYNN2 | Catup1::hisG-Canfr1::hisG-Canfr2/His3 | This work |
| CaWY4 | Catup1::hisG-Canfr1::hisG-Canfr2/His3 | This work |
| CaWY4.1 | Catup1::hisG-Canfr1::hisG-Canfr2/His3 | This work |
| CaWY4.3 | Catup1::hisG-Canfr1::hisG-Canfr2/His3 | This work |
| BWP17 | ura3::Xmr343/ura3::Xmr343 | Wilson et al. (37) |
| RZ-1 | rfg1::His3/rfg1::URA3 | Kahlaf and Zilom (46) |
| CaWY5 | rfg1::His3/rfg1::URA3 | This work |
| CaWY6 | Catup1::His3/Catup1::URA3 | This work |
| CaWY7 | nrg1::His3/nrg1::URA3 | This work |

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1 The abbreviations used are: FC, ferrichrome; FOB, ferrioxamine B; BPS, bathophenanthroline sulfonate; GFP, green fluorescent protein.
2 Available at www.sequence.stanford.edu.
C. albicans Siderophore-Iron Transporter CaArn1p

Chromosomal Deletion of CaTUP1, RFG1, and CaNRG1 Genes—CaTUP1, RFG1, and CaNRG1 deletion mutants were all derived from strain BW217 (37) using the same gene deletion strategy. A gene deletion cassette was constructed by flanking a marker gene, CaHIS3 or CaURA3, with two DNA fragments corresponding to the 5′- and 3′-untranslated regions of the target gene, respectively. For sequentially deleting the two copies of a gene, two cassettes containing different selection markers were constructed for each gene. For example, CaTup1Δ::HIS3 and CaTup1Δ::URA3 cassettes were used sequentially to delete the CaTUP1 gene. The following pairs of primers were used to PCR-amplify the 5′- and 3′-flanking DNA fragments of the three genes: the 5′-fragment of CaTUP1, 5′-AAGTCGACATCCATCACTCTGAG-3′ and 5′-ATGGAAGAAGTTGTGTTG-3′; the 3′-fragment of CaTUP1, 5′-AAAA- AATAAGTTGTTGATGAAG-3′; the 5′-fragment of RFG1, 5′-GATTGGGAT- CATCTAC-3′ and 5′-GGCACTACGAATTAAAATGG-3′; the 3′-fragment of RFG1, 5′-ACCTCCACAATATTTCTCACT-3′ and 5′-GCAACTA- TATGGATTAAACC-3′; the 5′-fragment of CaNRG1, 5′-CCACATTGG- ATTATTAC-3′ and 5′-gaactacgaggaagaggaa-3′; and the 3′-fragment of CaNRG1, 5′-GGTTAAATTTGGATGG-3′ and 5′-AGAAAGACCTTGCAAT- GATG-3′. To join the 5′- and 3′-fragments to the selection marker gene, appropriate restriction sites were added to the PCR primers. Transformants were selected on histidine- or uracil-dropout medium according to the selection marker used. Correct deletion of each gene was verified by Southern blotting and examination of phenotypes characteristic of each mutant.

Siderophore-Iron-dependent Growth Assay—Desferri-FC and desferri-FOB were purchased from Sigma. Ferric FC and ferric FOB were prepared by overnight incubation of the desferri siderophore with FeCl₃ at an equal molar concentration as described (16). Agar plates were prepared using the iron-limiting YNB/glucose medium supplemented with 500 μM BPS, followed by the addition of different amounts of siderophore. C. albicans strains to be tested were first grown in YPD medium to saturation. The cells were washed twice with and diluted to a density of 1 × 10⁷ cells/ml in YNB/glucose medium containing 100 μM BPS and grown for an additional 5 h. 10-fold serial dilutions of the cultures were prepared, and cells were spotted onto the agar plates. The plates were incubated at 30 °C for 2 days before photographing.

59Fe Uptake Assay—59Fe uptake assay was performed as described previously (18) with some modifications. C. albicans strains were first grown in YPD medium to saturation and then washed twice with 10 mM EDTA and once with LIM0 medium (18) containing 100 μM BPS. The cells were resuspended in LIM0 medium to a density of 1 × 10⁶ cells/ml and grown for 5 h. Cells were then counted; spun down; and divided into 50-μl aliquots in LIM0 medium, each containing ~2 × 10⁶ cells. 59Fe, FeFC, or FOB + 59Fe were added to each aliquot to a final concentration of 2 μM, and cells were incubated at 30 °C for 30 min. One such aliquot of each strain was kept on ice for 30 min to estimate the background binding of 59Fe to the cell surface. After incubation, cells were washed twice with ice-cold 10 mM EDTA to remove unincorporated 59Fe, spun down, and air-dried briefly before determining the amount of intracellular accumulation of 59Fe using an Amersham Biosciences Count-It 2900 scintillation counter (Model 1528). After subtracting the background binding, the iron uptake was expressed as picomoles of 59Fe/10⁶ cells/min.

Northern Blot Analysis—C. albicans strains were grown in YPD medium at 30 °C to saturation, and each strain was used to inoculate an iron-rich and an iron-limiting medium at a density of 1 × 10⁶ cells/ml. The iron-rich medium was GMM medium supplemented with 200 μM FeCl₃, and the iron-limiting one was GMM medium containing 100 μM BPS. The cells were grown for 8 h before harvesting for the preparation of total RNA. RNA extraction and Northern blotting were performed as described (18). The coding regions of the CaARN1 and CaACT1 genes were PCR-amplified from SC5314 genomic DNA and used as probe.

Western Blot Analysis—Cells were grown to 1 × 10⁶ cells/ml in 5 ml of GMM medium containing either 200 μM FeCl₃ or 100 μM BPS at 30 °C. Cells were spun down by centrifugation at 3000 rpm for 5 min. The cell pellet was resuspended in 200 μl of ice-cold lysis buffer containing 1% Triton X-100, 0.1% SDS, 50 mM Tris (pH 7.2), 1% sodium deoxycholic acid, and one protease inhibitor mixture tablet (Roche Molecular Biochemicals)/25 ml. The cell suspension was transferred to a 2-ml screw-cap tube. An equal volume of glass beads was added, and the cells were lysed using a Mini-Beadbeater (Biospec Products, Inc.) at maximum speed for two cycles of 2-min beating, leaving the sample on ice for 2 min in between. The lysate was spun at maximum speed in an Eppendorf microcentrifuge, and the supernatant was transferred to a new 1.5-ml tube. Protein concentration was determined by the Bradford assay. The proteins were resolved by denaturing polyacrylamide gel electrophoresis and transferred onto Hybond-C nitrocellulose mem-

RESULTS

Identification of the C. albicans Siderophore Transporter Gene CaArn1p—To identify C. albicans siderophore transporter genes, we used ARN1–4 sequences to BLAST-search the C. albicans genome sequence for homologous genes. This search identified one open reading frame conceptually encoding a 604-amino acid polypeptide. Amino acid sequence alignment of this sequence with the known Arnp sequences showed significant homology over the entire alignment, and the highest identity value of 46% was scored with Arn1p. During the course of this study, two other groups published the same gene discovered using similar strategies (28, 29) and demonstrated that the encoded protein indeed functions as a siderophore transporter by heterologous expression in S. cerevisiae. Thus, in this study, we only report the characterization of this gene, CaARN1, in C. albicans.

Chromosomal Deletion of CaARN1—To study the effect of deleting CaARN1 alone and together with genes encoding the high-affinity iron uptake system, we used the URA blaster system (Fig. 1A) to sequentially delete the two copies of the
gene from strain CAI4 and from a Cafr1ΔCafr2Δ mutant (CaWYNR2) that is completely defective in high-affinity iron uptake mediated by the oxidase-permease complex (18). The correct deletion of each copy of the gene was verified by Southern blot analysis (Fig. 1B).


cAarn1Δ Mutant Exhibits a Growth Defect on Iron-limiting Medium Supplemented with Ferric FC—Adron et al. (29) showed that when expressed in S. cerevisiae, CaArn1p is specific for FC-iron, but does not transport FOB-iron. Thus, we chose FC to test the function of CaArn1p in C. albicans and also included FOB for comparison. YNB/glucose medium was prepared by the manufacturer to contain a low amount of iron. However, all the mutant strains (Caarn1Δ, Cafr1ΔCafr2Δ, and Cafr1ΔCafr2ΔCaarn1Δ) were found to grow as well as the wild-type strain on the agar plates prepared using this medium. Thus, we added increasingly higher amounts of the nonpermeable ferrous iron chelator BPS to the medium to determine a concentration at which the wild-type strain exhibited little growth after 2–3 days of incubation at 30 °C unless FC was added. We reasoned that at such a concentration, chelation of ferrous iron by BPS would lower the level of iron uptake through the reductive pathway to a minimum level so that it would not interfere much with the assays for iron uptake mediated by the siderophore transporter. At BPS concentrations between 10 and 50 μM, the Cafr1ΔCafr2Δ mutant exhibited increasingly slower growth (data not shown), whereas the wild-type strain still grew normally. This observation is consistent with the lack of the high-affinity iron uptake system in the mutant strain (18). When the BPS concentration was increased to 500 μM, the wild-type strain exhibited little growth after 3 days of incubation. 5–10 μM ferric FC, ferric FOB, or FeCl₃ was added to this iron-depleted medium, and serially diluted cultures of each strain were spotted onto the agar plates for siderophore-dependent growth assay. Fig. 2 shows that the wild-type strain grew on the plate supplemented with 10 μM FC-iron, but not on the ones supplemented with the same amount of FOB-iron or FeCl₃, demonstrating that C. albicans utilizes FC-bound iron under this condition. Deletion of CaARN1 abolished this growth, and this growth defect was corrected when a copy of CaARN1 was reintroduced into the Caarn1Δ mutant. These results indicate that CaARN1 is responsible for FC-iron utilization. Like the wild-type strain, the Cafr1ΔCafr2Δ mutant also exhibited FC-dependent growth, and this growth was abolished when the CaARN1 gene was deleted, suggesting that FC utilization does not require the high-affinity iron uptake system under the experimental condition used. We noticed that the Caarn1Δ mutant consistently exhibited growth on the plates supplemented with either FC or FOB when the plates were incubated for longer times (data not shown and see below). In contrast, this slow growth was not observed for the Cafr1ΔCafr2ΔCaarn1Δ mutant. This observation suggests that iron chelated by FC and FOB can be used via a route dependent on the oxidase-permease iron uptake system under this condition, albeit much less efficiently.


cAarn1Δ Mutant Is Defective in FC-Iron Uptake—To evaluate iron uptake in a more quantitative manner, we determined the rate of ⁵⁹Fe accumulation in cells of various strains. ⁵⁹Fe was added to a final concentration of 2 μM in the form of either ⁵⁹Fe alone or together with an equal molar concentration of FC or FOB. The results are summarized in Fig. 3. In the Cafr1ΔCafr2ΔCaarn1Δ mutant, which is defective in both high-affinity iron uptake and siderophore transport, there was little iron uptake. Strikingly, the wild-type cells exhibited more than twice the amount of cellular ⁵⁹Fe accumulation when treated with FC + ⁵⁹Fe than with ⁵⁹Fe alone. When FOB was added with ⁵⁹Fe, the wild-type cells also accumulated a significant amount of ⁵⁹Fe to a level —80% of that in cells to which only ⁵⁹Fe was added. When the Caarn1Δ mutant was deleted, the iron uptake from ⁵⁹Fe and FOB + ⁵⁹Fe was nearly unchanged, whereas the uptake from FC + ⁵⁹Fe was markedly reduced to a level similar to that of the uptake from ⁵⁹Fe and FOB + ⁵⁹Fe. These results indicate that, first, CaARN1 is responsible for a large fraction of the total ⁵⁹Fe uptake from FC + ⁵⁹Fe; and that, second, the high-affinity iron uptake system also contributes to the ⁵⁹Fe uptake from FC + ⁵⁹Fe and FOB + ⁵⁹Fe. In the Cafr1ΔCafr2Δ mutant, the iron uptake from ⁵⁹Fe and FOB + ⁵⁹Fe was abolished, indicating that the iron uptake from these sources is entirely mediated by the oxidase-permease high-affinity iron uptake system. In contrast, the iron uptake from FC + ⁵⁹Fe was increased in Cafr1ΔCafr2Δ, suggesting that

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**Fig. 2.** CaARN1 mutants cannot grow on iron-limiting plates supplemented with FC-iron. Approximately 1 × 10⁵ and 1 × 10⁶ cells of each strain were spotted onto each plate. YNB/glucose medium was used to prepare the agar plates. The iron chelator BFS was added to the medium to a final concentration of 500 μM to create an iron-limiting condition. FeCl₃, FC-iron, and FOB-iron were added to different plates as the iron source as indicated at the top. The plates were incubated at 30 °C for 2 days. The C. albicans strains used were CAP2-1 (WT), CaWY3-2 (arn1Δ), CaWY3-3.3 (arn1Δ::ARN1), CaWY3-3.3 (arn1Δ::vector), CaWYN1 (ftr1Δftr2Δ), CaWY4 (ftr1Δftr2Δarn1Δ), and CaWY4.2 (ftr1Δftr2Δarn1Δ::ARN1).

| Concentration (μM) | BFS | FOB | FC | FeCl₃ |
|--------------------|-----|-----|----|-------|
| 500                | +   | +   | +  | +     |
| 10                 | −   | −   | −  | −     |
| 5                  | +   | +   | +  | +     |

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**Fig. 3.** CaARN1 mutants are defective in FC-⁵⁹Fe uptake. Cells were incubated with 2 μM ⁵⁹Fe, FC + ⁵⁹Fe, or FOB + ⁵⁹Fe. The strains included were CAP2-1, CaWY3-2 (arn1Δ), CaWYN1 (ftr1Δftr2Δ), CaWY4 (ftr1Δftr2Δarn1Δ), CaWY4.2 (ftr1Δftr2Δarn1Δ::ARN1), and CaWY4.3 (ftr1Δftr2Δarn1Δ::pASK1-ARN1). The assay was done three times.
the siderophore-iron uptake system may function independently of the oxidase-permease system and is enhanced in the absence of the latter. Reintroduction of CaARN1 into Caftr1ΔCaftr2ΔCaaRN1Δ either as a single copy or on pABSK1 restored iron uptake from Fe + 59Fe, but not from 59Fe and FOB + 59Fe, confirming that CaARN1 is responsible for the uptake of FC-bound iron. The difference in the levels of iron uptake between cells containing a single copy of CaARN1 and cells containing the gene on pABSK1 can be explained by gene dosage effect.

Deletion of CaARN1 Has Little Effect on Virulence in the Mouse Systemic Candidiasis Model—Because the oxidase-permease iron uptake system was shown to be essential for C. albicans growth and virulence in the host (18), we wanted to determine whether CaARN1 is similarly required. We employed the mouse systemic candidiasis model to examine the virulence of the Ura" CaaRN1Δ mutant. Although the Ura" Caftr1ΔCaftr2Δ strain was again found to be avirulent, the CaaRN1Δ mutant exhibited similar levels of virulence and kidney infection as the wild-type strain, suggesting that the CaARN1 gene is not required along this route of infection (data not shown).

Iron-responsive Regulation of CaARN1 Expression—A number of genes responsible for iron uptake are known to be regulated by iron concentration, normally being increased when iron supply is limited and repressed when it is replete (16, 18, 20, 28). Northern blot analysis of CaARN1 mRNA levels in cells grown in iron-depleted and iron-replete media revealed the same pattern of iron-responsive expression (see below). In S. cerevisiae, the transcription factor Aft1p has been shown to activate the expression of genes responsible for high-affinity iron uptake (16, 38, 39). However, so far, an AFT1 homolog has not been found in C. albicans. Recently, Murad et al. (40, 41) used DNA array to profile genes that show higher levels of expression in a C. albicans strain with a deletion of the transcription repressor gene CaTUP1. Interestingly, they found that the expression of CaFTR1 and CaCFL2 was increased by 16- and 61-fold, respectively. They also found significantly elevated levels of expression of these two genes in a strain with a deletion of CaNRG1, which encodes a DNA-binding subunit for the Tup1p-Ssn6p repressor complex (42–44). This result prompted us to examine whether CaTup1p also regulates the expression of CaARN1. Because Rfg1p has also been implicated in mediating Tup1p-Ssn6p binding to DNA (45, 46), we wanted to investigate which of the two proteins, CaNrg1p or Rfg1p (or both), is involved in CaTup1p regulation of CaARN1. For this experiment, we first constructed gene deletion mutants for each of the three genes. The strategy for gene deletion is described under "Experimental Procedures," and the correct gene deletion was verified by Southern analysis (data not shown). As well documented by others (42–46), a common phenotype of Caftr1Δ, CaNrg1Δ, and rfg1Δ mutants is constitutive filamentous growth. Fig. 4 shows that all three mutant strains we tested a single band of ~2.1 kb (sufficiently large to encode the protein) in all RNA samples except the ones prepared from strain CaaRN1Δ, indicating that the detected band represents CaARN1 mRNA. The wild-type cells exhibited a CaARN1 expression pattern typical of many iron-responsive genes, being expressed under iron-depleted conditions, but repressed under iron-replete conditions. In contrast, the Caftr1Δ mutant apparently had lost the transcription repression of CaARN1, which was expressed to a similar level under both conditions, indicating that CaTup1p is responsible for the repression of the CaARN1 gene under iron-sufficient conditions. A similar observation was recently reported by Lesuisse et al. (28). In the CaNRG1Δ mutant, the expression of CaARN1 exhibited the same response to iron concentration as in the wild-type strain, whereas in the rfg1Δ mutant, CaARN1 displayed the same expression pattern as in Caftr1Δ, the loss of repression at high iron concentration. This result indicates that CaTup1p repression of CaARN1 expression is mediated by Rfg1p, but not by CaNrg1p.

Cellular Localization of CaArn1p—In this study, we used GFP tagging to examine CaArn1p localization in living cells. GFP was fused to the C terminus of CaArn1p and expressed from its own promoter. This gene was expressed either as a single copy or on pABSK1. We used Western blot analysis to confirm the expression of a fusion protein of the expected size (~93 kDa) and in an iron-dependent manner in the transformed cells using anti-GFP antibody (Fig. 5A). We then determined the functionality of the protein by testing whether it could rescue the siderophore-dependent growth defect of CaARN1Δ. Fig. 5B shows that the fusion protein, expressed either as a single copy or on pABSK1, fully rescued the FC utilization defect of CaARN1Δ on the iron-depleted plates. Fluorescence microscope examination of the cells grown in iron-limiting medium revealed a bright fluorescent periphery in every cell, either in the yeast or the filamentous form (Fig. 5C), reminiscent of the localization of Ftr1p and Fet3p (16, 18, 27,
The strains used were CAF2-1 (CaWY3-2.5), CaWY3-3.2, Arn1, CaWY3-2 (H9004 WT) and CaWY3-3.5 (H9262). The correct integration was verified by Southern blotting. The growth conditions were as described in the legend to Fig. 2.

C. Cells transformed with either plasmid exhibited a similar membrane localization of CaArn1p. Cells expressing CaArn1p-GFP were grown in the yeast form at 30 °C for 2 h in GMM medium containing 100 μM BPS before visualization by fluorescence microscopy. D, shown is the plasma membrane localization of CaArn1p. Cells expressing CaArn1p-GFP were grown in the yeast form at 30 °C for 2 h in GMM medium containing 100 μM BPS and in the filamentous form at 37 °C for 2 h in GMM medium supplemented with 20% serum and 100 μM BPS before examination by fluorescence microscopy. D, shown is the increase in the intracellular localization of CaArn1p-GFP in cells treated with the FC-iron complex. Cells expressing CaArn1p-GFP were grown in the iron-replete medium containing 100 μM FC-iron for an additional 2 h before visualization by fluorescence microscopy. Fig. 6 shows that there were significantly more intracellular fluorescent spots in the cells treated with FC than in the cells treated with FOB or FeCl3. To further confirm the FC-induced increase in intracellular CaArn1p, we determined whether the number of spots would increase with an increase in FC concentration. Fig. 6 demonstrates that, indeed, the number of intracellular fluorescent spots increased with an increase in FC concentration, which again was not seen in cells treated with FOB or FeCl3 (data not shown).

**DISCUSSION**

In this study, we investigated the properties and functions of the siderophore transporter CaArn1p in *C. albicans*. CaARN1 has been characterized in the heterologous host *S. cerevisiae* (28, 29), which provided very useful information for the design of our experiments to study the gene in its native host. Our study confirmed some previous results, such as the role of CaArn1p in siderophore uptake, its FC specificity, and iron-regulated expression of the gene. In addition, we made some new observations that may shed light on the understanding of CaArn1p function and roles in *C. albicans* virulence.

Comparison of siderophore-iron uptake in mutants with deletions of CaARN1 alone and of both the CaARN1 and CaFTR genes showed that siderophore-iron can be used via both the siderophore transporter and the oxidase-permease iron uptake system. The oxidase-permease system can assimilate iron from both FC and FOB. This route of uptake would require the function of ferric reductases to release iron in ferrous form from the siderophores. The role of ferric reductases in the utilization of siderophore-iron by the oxidase-permease system has been well documented (16, 28, 29, 47). Our results support the conclusion that siderophore-iron uptake occurs through both reductive and non-reductive pathways in yeast. In agreement with the FC specificity of CaArn1p reported by Ardon et al. (29), we also observed that CaArn1p transported FC-bound iron, but had undetectable activity for FOB-bound iron. However, these observations do not agree with the intracellular accumulation of a fluorescent FOB analog reported by Lesuisse.
et al. (28). One explanation could be that the intracellular fluorescence was due to the slow entry of the compound via liquid-phase endocytosis over the 24-h growth of cells before fluorescence microscopy. This slow entry of FOB would not be detected as significant by the 15–30-min $^{59}$Fe uptake assay used by us and by Ardon et al. (29).

Using GFP tagging, we were able to determine CaArn1p localization in living cells. Because the tagged protein could fully correct the defect of Caarn1Δ in using FC-iron, the observed GFP fluorescence pattern should reflect the physiological cellular localization of CaArn1p. In living C. albicans cells, the majority of CaArn1p-GFP was found in the plasma membrane, and a small amount was found in the cytoplasm with a punctate appearance. This localization is apparently different from the predominant localization of Arn1p and Arn3p in intracellular vesicles in S. cerevisiae previously observed by others (16, 47). However, these authors did not exclude the possibility that a small amount of Arn1p and Arn3p could also be localized on the plasma membrane. Although we have not determined the nature of the intracellular vesicles containing CaArn1p in C. albicans, we assume that they are likely to be the same as those containing Arn1p and Arn3p in S. cerevisiae. The two different observations do not necessarily contradict each other. These proteins may be localized in both the plasma membrane and intracellular vesicles in a dynamic equilibrium, which is shifted toward different ends in the two organisms to suit each one’s specific need for siderophore-iron in their respective natural environmental niches. These two organisms are well known to differ in the number of ARN genes, specificity for siderophores, and ability in siderophore production. A similar situation is the ability of C. albicans (but not S. cerevisiae) to shift a dynamic cell cycle-dependent distribution of a pool of actin toward a persistent localization at the hyphal tips when cells switch growth from the yeast form to the filamentous form (48). Then, what is the relationship between the CaArn1p molecules in the two locations? One possibility is that CaArn1p in the intracellular vesicles may come from the internalization of the ones in the plasma membrane. This internalization might be a result of CaArn1p-mediated transport of siderophore-iron complexes into the cells, a mechanism similar to the receptor-mediated uptake of transferrin-iron complexes in mammals (1). Supporting this explanation, we observed an increase in the number of fluorescent spots in cells treated with FC in a concentration-dependent manner, but not in cells treated with FOB or Fe. However, it is equally possible that the receptors are internalized alone as a mechanism to down-regulate CaArn1p levels on the cell surface after translocating iron across the plasma membrane. At this stage, we are unable to distinguish between these two possibilities. Other researchers have also suggested that Arn3p/Sit1p is likely to act as a transporter at the cell surface and be rapidly recycled by internalization (20, 34). These authors mentioned that a mutant completely defective for endocytosis showed normal siderophore uptake, a result strongly challenging the hypothesis of Arnp-mediated internalization as the mechanism for FC uptake. However, the role of endocytosis in FC utilization needs to be tested in C. albicans. Although the mechanism of Arnp-mediated siderophore uptake is still not clear, our observation of a prominent cell-surface localization of CaArn1p strongly suggests that the main site of function for this protein is at the plasma membrane in C. albicans, where it is likely to play a direct role in binding and transporting siderophore-iron. The observation of an FC-induced increase in the intracellular localization of CaArn1p suggests testable hypotheses for the mechanisms of siderophore-iron transport and recycling of the transporter.

The expression of many genes involved in iron uptake is regulated by iron concentration in both S. cerevisiae and C. albicans (16, 18, 20, 36–40). They are normally repressed under iron-replete conditions and activated when the iron supply is limited. In S. cerevisiae, the transcription factor Arp1p activates the expression of genes such as FTR1, FET3, and ARN1–4 (16, 38, 39). Another transcription factor (Arp2p) was recently reported to have a role in the regulation of some iron transport genes (49). However, an AFT homolog does not seem to be present in C. albicans. Currently, it is not clear whether there is any transcription factor in C. albicans that activates transcription in an iron-dependent manner. Recently, evidence, including this study, is accumulating that the global transcription repressor Tup1p is responsible for the repression of at least some iron uptake-related genes in both S. cerevisiae and C. albicans (20, 21, 40). Tup1p associates with Ssn6p to form a general transcription repressor. However, this complex requires additional DNA-binding subunits for its specificity for different promoters (42–44). In C. albicans, two DNA-binding proteins, CaNrg1p and Rfg1p, have been found to mediate CaTup1p repression of target genes (43–46). Therefore, we examined CaARN1 expression in both Cangr1Δ and rfg1Δ mutants to ascertain which one mediates CaTup1p function. We discovered that deletion of CaNrg1p had no discernable effect on the iron-responsive regulation of CaARN1, whereas deletion of RFG1 resulted in loss of repression under iron-replete conditions to the same extent as deletion of CaTUP1. These results indicate that CaTup1p repression of CaARN1 is primarily, if not entirely, mediated by Rfg1p. Because CaNrg1p mediates the repression of CaFTR1 and CaCFL2 in C. albicans (40, 41), it would be interesting to know why the repression of genes for different iron uptake systems are mediated by different DNA-binding proteins.

To determine whether CaARN1 is required for C. albicans virulence, we tested the ability of the Ura+ Caarn1Δ mutant in causing death in mice via intravenous injection. The mutant exhibited a similar level of virulence as its isogenic wild-type strain, suggesting that CaArn1p function is not critically required for iron acquisition during this route of infection. This result indirectly supports our previous report of the essentiality of the oxidase-permease iron uptake system (18), the only other alternative high-affinity iron uptake system known so far in C. albicans. Although the systemic candidiasis model is useful in testing factors important for the pathogen to grow or survive in the bloodstream and internal organs, it does not necessarily reflect the entire natural route of infection. C. albicans mainly colonizes the mucosal surfaces along the gastrointestinal and vaginal tracts. The pathogen is exposed to various environmental niches with limited iron supply and in contact with different resident microbial communities. CaArn1p function may be required for iron uptake in these niches. The use of different infection models is required to unveil the role of siderophore-iron uptake in C. albicans infection.

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REFERENCES
1. Askwith, C. C., de Silva, D., and Kaplan, J. (1996) Mol. Microbiol. 20, 27–34
2. de Silva, D. M., Askwith, C. C., and Kaplan, J. (1996) Physiol. Rev. 76, 31–47
3. Weinberg, E. D. (1984) Physiol. Rev. 64, 105–122
4. Ratledge, C., and Dover, L. G. (2000) Annu. Rev. Microbiol. 54, 881–941
5. Howard, D. H. (1999) Clin. Microbiol. Rev. 12, 394–404
6. Patne, S. M. (1993) Trends Microbiol. 1, 66–69
7. Schryvers, A. B., and Stojilkovic, I. (1999) Mol. Microbiol. 32, 1117–1123
8. Lewis, L. A., Gray, E., Wang, Y. P., Roe, B. A., and Poyer, D. W. (1997) Mol. Microbiol. 23, 737–749
9. Manns, J. M., Mosser, D. M., and Buckley, H. R. (1994) Infect. Immun. 62, 5154–5166
10. Moors, M. A., Stull, T. L., Balnk, K. J., Buckley, H. R., and Mosser, D. M. (1992) *J. Exp. Med.* **175**, 1643–1651
11. Wodkridge, K. G., and Williams, P. H. (1993) *FEMS Microbiol. Rev.* **12**, 325–348
12. Stintzi, A., Barnes, C., Xu, J., and Raymond, K. N. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97**, 10691–10696
13. Rozalska, B., Lisiecki, P., Sadowska, B., Mikuci, J., and Rudniska, W. (1998) *Acta Microbiol. Pol.* **47**, 185–194
14. Byers, B. R., Massaad, G., Barghouthi, S., and Arceneaux, J. E. (1991) *Experimental (Basel)* **47**, 416–418
15. Brem, D., Pelludat, C., Rakin, A., Jacobi, C. A., and Heesemann, J. (2001) *Microbiology (Read.)* **147**, 1115–1127
16. Yun, C. W., Ferea, T., Rashford, J., Ardon, O., Brown, P. O., Botstein, D., Kaplan, J., and Philpott, C. C. (2000) *J. Biol. Chem.* **275**, 10799–10715
17. Girishkumar, H., Yousef, A. M., Chivate, J., and Geisler, E. (1999) *Postgrad. Med. J.* **73**, 151–153
18. Ramanan, N., and Wang, Y. (2000) *Science* **288**, 1062–1064
19. Askwith, C., and Kaplan, J. (1998) *Trends Biochem. Sci.* **23**, 135–138
20. Knight, S. A., Lesuisse, E., Stearman, R., Klausner, R. D., and Dancis, A. (2002) *Microbiology (Read.)* **148**, 29–40
21. Lesuisse, E., Blaiseau, P. L., Dancis, A., and Camadro, J. M. (2001) *Microbiology (Read.)* **147**, 289–298
22. Hammadet, J. E., Williams, P. H., and Cashmore, A. M. (2000) *Microbiology (Read.)* **146**, 869–876
23. Minnick, A. A., Eizember, L. E., McKe, J. A., Delence, E. K., and Miller, M. J. (1991) *Anal. Biochem.* **194**, 223–229
24. Ismail, A., Bedell, G. W., and Lupan, D. M. (1985) *Biochem. Biophys. Res. Commun.* **132**, 1160–1165
25. Ismail, A., and Lupan, D. M. (1986) *Myopathologia* **96**, 109–113
26. Askwith, C., Eid, D., Van Ho, A., Bernard, P. S., Li, L., Davis-Kaplan, S., Sipe, D. M., and Kaplan, J. (1994) *Cell* **76**, 403–410
27. Stearman, R., Yuan, D. S., Yamaguchi-Iway, Y., Klausner, R. D., and Dancis, A. (1996) *Science* **271**, 1552–1557
28. Lesuisse, E., Knight, S. A., Camadro, J. M., and Dancis, A. (2002) *Yeast* **19**, 329–340
29. Ardon, O., Bussey, H., Philpott, C., Ward, D. M., Davis-Kaplan, S., Verroneau, S., Jiang, B., and Kaplan, J. (2001) *J. Biol. Chem.* **276**, 43049–43055
30. Nicholson, M. L., and Beall, B. (1999) *Microbiology (Read.)* **145**, 2453–2461
31. Heymann, P., Ernst, J. F., and Winkelmann, G. (1999) *Biometals* **12**, 301–306
32. Heymann, P., Ernst, J. F., and Winkelmann, G. (1999) *Biometals* **13**, 65–72
33. Heymann, P., Ernst, J. F., and Winkelmann, G. (2000) *FEMS Microbiol. Lett.* **186**, 221–227
34. Lesuisse, E., Simon-Casteras, M., and Labbe, P. (1998) *Microbiology (Read.)* **144**, 3455–3462
35. Forzi, W. A., and Irwin, M. Y. (1993) *Genetics* **134**, 717–728
36. Murad, A. M., Lee, P. R., Broadbent, I. D., Barele, C. J., and Brown, A. J. (2000) *Yeast* **16**, 325–337
37. Wilson, R. B., Davis, D., and Mitchell, A. P. (1999) *J. Bacteriol.* **181**, 168–1874
38. Yamaguchi-Iway, Y., Dancis, A., and Klausner, R. D. (1995) *EMBO J.* **14**, 1231–1239
39. Yamaguchi-Iway, Y., Stearman, R., Dancis, A., and Klausner, R. D. (1996) *EMBO J.* **15**, 3377–3384
40. Murad, A. M., Leng, P., Straffon, M., Wishart, J., Macskill, S., MacCallum, D., Schnell, N., Talibi, D., Marechal, D., Tekaia, F., d'Enfert, C., Gaillardin, C., Odds, F. C., and Brown, A. J. (2001) *EMBO J.* **20**, 4742–4752
41. Murad, A. M., d'Enfert, C., Gaillardin, C., Tournu, H., Tekaia, F., Talibi, D., Marechal, D., Marchais, V., Cottin, J., and Brown, A. J. (2001) *Mol. Microbiol.* **42**, 981–993
42. Braun, B. R., and Johnson, A. D. (1997) *Science* **277**, 105–109
43. Braun, B. R., Head, W. S., Wang, M. X., and Johnson, A. D. (2000) *Genetics* **156**, 31–44
44. Braun, B. R., Kadosh, D., and Johnson, A. D. (2001) *EMBO J.* **20**, 4753–4761
45. Kadosh, D., and Johnson, A. D. (2001) *Mol. Cell. Biol.* **21**, 2496–2505
46. Khalaf, R. A., and Zitomer, R. S. (2001) *Genetics* **157**, 1503–1512
47. Yun, C. W., Tiedeman, J. S., Moore, R. E., and Philpott, C. C. (2000) *J. Biol. Chem.* **275**, 16354–16359
48. Hazan, I., Sepulveda-Becerra, M., and Liu, H. (2002) *Mol. Biol. Cell* **13**, 134–145
49. Blaiseau, P. L., Lesuisse, E., and Camadro, J. M. (2001) *J. Biol. Chem.* **276**, 34221–34226
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