Identification of a *Candida albicans* Ferrichrome Transporter and Its Characterization by Expression in *Saccharomyces cerevisiae*

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*Saccharomyces cerevisiae* can accumulate iron through the uptake of siderophore-iron. Siderophore-iron uptake can occur through the reduction of the complex and the subsequent uptake of iron by the high affinity iron transporter Fet3p/Ftr1p. Alternatively, specific siderophore transporters can take up the siderophore-iron complex. The pathogenic fungus *Candida albicans* can also take up siderophore-iron. Here we identify a *S. cerevisiae* siderophore transporter, CaArn1p, and characterize its activity. CaARN1 is transcriptionally regulated in response to iron. Through expression studies in *S. cerevisiae* strains lacking endogenous siderophore transporters, we demonstrate that CaArn1p specifically mediates the uptake of ferrichrome-iron. Iron-ferrichrome and gallium-ferrichrome, but not desferri-ferrichrome, could competitively inhibit the uptake of iron from ferrichrome. Uptake of siderophore-iron resulting from expression of CaARN1 under the control of the MET25-promoter in *S. cerevisiae* was independent of the iron status of the cells and of Aft1p, the iron-sensing transcription factor. These studies demonstrate that the expression of CaArn1p is both necessary and sufficient for the non-reductive uptake of ferrichrome-iron and suggests that the transporter may be the only required component of the siderophore uptake system that is regulated by iron and Aft1p.

Iron is an essential element for all eukaryotes and most prokaryotes. Its importance in biology lead to the evolution of multiple uptake mechanisms that would satisfy the requirements for the metal in both single cell and multicellular organisms. Under conditions of iron starvation most microorganisms secrete siderophores, low molecular weight organic molecules that bind extracellular iron (1). Siderophores are chemically heterogeneous and there are specific transport systems for different siderophores (2, 3). In some instances a transport system may take up more than one siderophore. Many microorganisms secrete more than one siderophore, and in addition to utilizing their own siderophores they may take up iron complexes of siderophores secreted by other microorganisms.

The budding yeast, *Saccharomyces cerevisiae*, does not secrete siderophores, yet it can use siderophore-iron. Siderophore-iron uptake can be accomplished by either extracellular reduction and subsequent uptake of the iron by the high affinity iron transport system Fet3p/Ftr1p or by the uptake of siderophore-iron complexes by specific transporters belonging to the major facilitator super family (4, 5). The hydroxamate-type siderophores ferrioxamine B, triacyl fursarincine C, and ferrichrome are taken up by the *S. cerevisiae* siderophore-iron transporters Arn1p, Arn2p, and Arn3p (5–8). Arn4p was found to be specific for the catecholate-type bacterial siderophore enterobactin (9).

Iron uptake mechanisms are highly conserved among yeast. *Schizosaccharomyces pombe* and *Candida albicans* have a high affinity uptake system that is homologous to the Fet3p/Ftr1p transport system of *S. cerevisiae* (10–12). Unlike *S. cerevisiae*, the pathogenic fungus *C. albicans* can secrete siderophores (13). To date no specific siderophore uptake system in *C. albicans* has been identified. Based on studies of siderophore-iron utilization in *S. cerevisiae*, we hypothesized that *C. albicans* may have siderophore transporters. In this paper, we demonstrate the existence of a specific siderophore transporter in *C. albicans*, CaArn1p, encoded by the ORF* CaYHL040C*, which is orthologous to the ARN1 siderophore transporter in *S. cerevisiae*. Genetic analysis in *C. albicans* is difficult because it lacks a sexual cycle and exists as a diploid. *S. cerevisiae* has been used successfully as a tool to characterize *C. albicans* genes. The elemental iron transport systems from other yeast species retain function when expressed in *S. cerevisiae* (10–12). Characterization of CaARN1 in *S. cerevisiae* shows that the *C. albicans* siderophore transporter has high specificity for ferrichrome and that expression of the transporter is necessary and sufficient for siderophore-iron transport.

**EXPERIMENTAL PROCEDURES**

*Yeast Strains, Plasmids, and Media—*Yeast strains used in this study are listed in Table I. The following primers were used to construct pmetaCaARN1 (5'-CGGATCCCGATGACATCTTACGAG-3' and 5'-CGGATCCGATGACATCTTACGAG-3'), pmet-flagCaARN1 (5'-CGGATCCCGATGACATCTTACGAG-3'), and pmet-CaARN1-myec (5'-CGGATCCCGATGACATCTTACGAG-3').

1 The abbreviations used are: ORF, open reading frame; PCR, polymerase chain reaction; gel electrophoresis; BPS, baphothephenanthroline sulfonate; CM, synthetic media; FOB, ferrioxamine B; Fe, ferrichrome; Ga, gallium; HA, hemagglutinin.

2 The nomenclature for the *C. albicans* siderophore transporter, CaYHL040C, is based upon homology to the *S. cerevisiae* ARN1 ORF YHL040C. The *C. albicans* ORFS have not been assigned to specific chromosomes.
mRNAs on Northern blots correlated with the lengths expected from
Detector kit (Kirkegaard & Perry Laboratories, Inc.). The size of the
5 actin probe was generated using 5 °TCCTATTAAACAGCTACTCTTTTCTTC-3

uptake activity was expressed as pmol of 59Fe/min/106 cells.

° generated using a C. albicans CAI-4 primers 5°CTCTTACCAG-3°/H11032

TC with FeCl3 or Ga(NO3)3 at a ratio of 1.0:0.9. All
Sigma. Siderophore complexes were prepared by overnight incubation
of desferri-siderophores with FeCl3 or Ga (NO3)3. All

ATCTTACCAG-3°/H11032

TRP1

HIS3

TRP1

HIS3

LEU2

URA3

Y9 Δαf1

MATa, ura3-52 lys2-801 (amber) ade2-101 (ochre) trp1-D63 his3-D200
leu2-31, af1- TRP1

MATa, ura3-52 lys2-801 (amber) ade2-101 (ochre) trp1-D63 his3-D200
leu2-31 S635: HISS3

Δαf3 Δαrn1,2,3,4

MATa, ura3-52 lys2-801 (amber) ade2-101 (ochre) trp1-D63 his3-D200
leu2-31 S635: HISS3 Δαrn1: HIS2 Δαrn3: HIS2 Δαrn4: HIS2 HISS3

Wild type BY4741

MATa his3-Δ1 leu2-Δ0 met15-Δ0 ura3-Δ0

BY4741 Δαf1

MATa his3-Δ1 leu2-Δ0 met15-Δ0 ura3-Δ0 af1- KanMX4

BY4742 Δαf2

MATa his3-Δ1 leu2-Δ0 met15-Δ0 ura3-Δ0 af2- KanMX4

BY4741 Δαf1 Δαf2

MATa his3-Δ1 leu2-Δ0 met15-Δ0 ura3-Δ0 af1- af2- KanMX4

A gift from Julian Rutherford,
University of Utah

Table I
Strain name Genotype Reference
C. albicans CAI-4

URA3: imm434/URA3: imm434

(35)

C. albicans CAI-4

URA3: imm434/URA3: imm434

(35)

S. cerevisiae

Y9 Δαf1

MATa, ura3-52 lys2-801 (amber) ade2-101 (ochre) trp1-D63 his3-D200
leu2-31, af1- TRP1

MATa, ura3-52 lys2-801 (amber) ade2-101 (ochre) trp1-D63 his3-D200
leu2-31 S635: HISS3

Δαf3 Δαrn1,2,3,4

MATa, ura3-52 lys2-801 (amber) ade2-101 (ochre) trp1-D63 his3-D200
leu2-31 S635: HISS3 Δαrn1: HIS2 Δαrn3: HIS2 Δαrn4: HIS2 HISS3

Wild type BY4741

MATa his3-Δ1 leu2-Δ0 met15-Δ0 ura3-Δ0

BY4741 Δαf1

MATa his3-Δ1 leu2-Δ0 met15-Δ0 ura3-Δ0 af1- KanMX4

BY4742 Δαf2

MATa his3-Δ1 leu2-Δ0 met15-Δ0 ura3-Δ0 af2- KanMX4

BY4741 Δαf1 Δαf2

MATa his3-Δ1 leu2-Δ0 met15-Δ0 ura3-Δ0 af1- af2- KanMX4

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ATCTTACCAG-3° and 5° CGGAATTCCTATTAATTCGAAGTCTCC-5°/H9262

Y9 Δαf3

MATa, ura3-52 lys2-801 (amber) ade2-101 (ochre) trp1-D63 his3-D200
leu2-31, af1- TRP1

MATa, ura3-52 lys2-801 (amber) ade2-101 (ochre) trp1-D63 his3-D200
leu2-31 S635: HISS3

Δαf3 Δαrn1,2,3,4

MATa, ura3-52 lys2-801 (amber) ade2-101 (ochre) trp1-D63 his3-D200
leu2-31 S635: HISS3 Δαrn1: HIS2 Δαrn3: HIS2 Δαrn4: HIS2 HISS3

Wild type BY4741

MATa his3-Δ1 leu2-Δ0 met15-Δ0 ura3-Δ0

BY4741 Δαf1

MATa his3-Δ1 leu2-Δ0 met15-Δ0 ura3-Δ0 af1- KanMX4

BY4742 Δαf2

MATa his3-Δ1 leu2-Δ0 met15-Δ0 ura3-Δ0 af2- KanMX4

BY4741 Δαf1 Δαf2

MATa his3-Δ1 leu2-Δ0 met15-Δ0 ura3-Δ0 af1- af2- KanMX4

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C. albicans (CAI-4) genomic DNA was used as a template to amplify
ORFs CaYHL040C by PCR using 92°C, 40 s, 55°C, 60 s, and 68°C for 3
conditions. The PCR products were digested with BamHI and
EcoRI and cloned behind a MET25 promoter in the vectors pTF62
(LEU2) or pTF63 (URA3). All constructs were verified by DNA sequenc-
ing. pARN1-HA and pmetARN1-HA were as described (6).

Cells were grown in YPD (1.0% yeast extract, 2.0% peptone, 2.0%
glucose), CM (a synthetic medium of yeast nitrogen base, amino acids,
and glucose), CM deficient in specific amino acids, CM made iron
limited by the addition of 50 μM bathophenanthroline sulfonate (BPS)
(14) or CM made iron replete by addition of 50 μM FeCl3.

Siderophore Plate Growth—The siderophores ferrioxamine B (FOB)
(Desferal®), ferrichrome (Fc), and rhodotorula acid were obtained from
Sigma. Siderophore complexes were prepared by overnight incubation
of desferri-siderophores with FeCl3 or Ga(NO3)3 at a ratio of 1.0:0.9. All
siderophore complexes were filter-sterilized (0.2 μM filter) (15, 16).

For plate assays, iron-limiting CM was used in which BPS was added
to a final concentration of 50 μM, 100 μl of Fe-siderophore complexes
were added to a final concentration of 0.5 μM, and the plates allowed to
dry at 30°C overnight. Yeast were grown to mid-log phase and 10-fold
dilutions spotted onto plates and grown for 2 or 3 days at 30°C.

Iron Uptake Assay—Iron transport assays were performed as de-
scribed (17) with the following modifications. Briefly, 2 × 107 cells were mixed with 59Fe supplied as 59FeCl3 or 59Fe-siderophore. Cells were
incubated at 30°C for 15 min, placed on filters (Whatman GF/C), and
washed with EDTA-containing buffer to remove unincorporated iron.
The filters were air-dried, and associated radioactivity determined. The
uptake activity was expressed as pmol of 59Fe/min/106 cells.

Western Blot Analysis—Cells were grown to mid-log phase, collected
by centrifugation, and washed. Spheroplasts were made and Dounce
homogenized, and a postnuclear supernatant obtained. A crude mem-
brane fraction was isolated by centrifugation at 15,600 × g for 30 min.
Protein concentrations were determined, and equal amounts run on
10% SDS-polyacrylamide gel electrophoresis. Samples were transferred to
nitrocellulose, probed with anti-FLAG (M2 Sigma), (1:2000) followed by goat anti-mouse horse-
radish peroxidase (Jackson Immuno Research Laboratories Inc,
1:10,000). Western blots were developed using the chemiluminescence
reaction reagent Renaissance (PerkinElmer Life Sciences) as per the
manufacturer’s instructions.

Northern Blot Analysis—Total RNA was isolated using standard
techniques (18). All samples were isolated from mid-log phase cultures
grown in defined iron media containing either 50 μM FeCl3 (‘high iron’) or
50 μM BPS (‘low iron’). A biotinylated CaARN1 probe was PCR-
generated using a C. albicans (CAI-4) genomic prep as a template,
primers 5°-CGGATCCGGCATGACTTACCGACG-3° and 5°-CGGAATTCCTATTAATTCGAAGTCTCC-3° with PCR conditions 92°C
for 40 s, 55°C for 60 s, and 68°C for 3 min. A biotinylated C. albicans
actin probe was generated using 5°-TGCGGATGAGCAGGTCTCC
and 5°-GCTCTGAAATCTTTCGTTACC. The blots were developed using DNA
Detector kit (Kirkegaard & Perry Laboratories, Inc.). The size of the
mRNAs on Northern blots correlated with the lengths expected from
the calculated sequences.

RESULTS

C. albicans has been shown to use different hydroxamate
siderophores for its growth on plates (19). To determine
whether C. albicans has a specific siderophore transport sys-
tem, we measured the uptake of 59Fe-siderophore complexes in
C. albicans. Wild type cells were able to take up 59Fe from both
59Fe-Fc and 59Fe-FOB (Fig. 1a) in a concentration dependent
manner. The apparent Km of uptake was 1.88 μM and Vmax 3.20
pmol/min/106 cells for Fe and 5.71 μM and 2.40 pmol/min/106
cells, respectively, for FOB, values that are similar to that seen in
S. cerevisiae (6). Both siderophores were found to promote
cell growth (data not shown).
In *S. cerevisiae* siderophore-iron is accumulated by two mechanisms (4, 5). The first involves reduction of siderophore-iron complexes by cell surface reductases and uptake of iron by the high affinity iron transport system. A second mechanism for siderophore-iron utilization results in the uptake of siderophore-iron complexes by specific transport systems. To determine whether *C. albicans* also employs both mechanisms we examined the effect of a non-permeable Fe(II) chelator on siderophore-iron-mediated transport. Reduction of siderophore-iron complexes leads to the formation of Fe(II), which is the substrate for the high affinity iron transport system and chelation of Fe(II) prevents iron uptake. Incubation of *C. albicans* with the impermeable Fe(II) chelator BPS results in the complete inhibition of 59Fe uptake when cells are incubated with 59Fe(III)Cl3 in citrate buffer (Fig. 1b). This same concentration only effects a 50% reduction in the uptake of 59Fe(III)-Fc. Uptake of 59Fe-FOB is completely inhibited by the addition of BPS. These results suggest that a significant component of Fe-Fc uptake does not occur by reduction at the cell surface, whereas uptake of Fe-FOB only occurs by reduction followed by transport of elemental iron.

*S. cerevisiae* has a family of siderophore transporters (5). We questioned whether *C. albicans* also had an orthologous siderophore transport system. Inspection of the *C. albicans* genome at 5x coverage (sequence.stanford.edu/group/candida/) revealed one ORF that demonstrated a high homology to the *S. cerevisiae* siderophore transporters (*ARN1–4*) (Fig. 2). This ORF was on the 33,181 base pair contig 4–3057 and was from base pairs 15,417–13,606 on the reverse strand. The amino acid identity between the putative *C. albicans* ARN, CaYHL040C, and the yeast ARNs ranged between 28–46%, with *ARN1* showing the highest identity and homology (46 and 63%, respectively). Primers were designed, and CaYHL040C was amplified using PCR from a *C. albicans* genomic DNA prepara-
The hypothesis that the No band was observed under high iron conditions. This finding supports the hypothesis that the iron-sensing transcription factor Aft1p (5). If the CaARN1 is regulated by iron, then we might expect that its transcription would be iron-regulated, as are other known components of the S. cerevisiae ARN1 family. To study the function of CaARN1 in S. cerevisiae siderophore transporters, we cloned CaARN1 under the regulation of the MET25 promoter and expressed it in a Δfet3Δarn1–4 strain of S. cerevisiae. These cells are unable to take up siderophore iron either through the high affinity iron transport system or the ARN family of siderophore transporters and were used previously to characterize S. cerevisiae siderophore transporters (5). The Δfet3Δarn1–4 cells can not grow on BPS plates supplemented with siderophores (5). Transformation of this strain with a plasmid containing a MET25-regulated CaARN1, permitted cell growth on media supplemented with Fe-Fc, but not supplemented with Fe-POB (Fig. 4) or Fe-rhodotorulic acid (data not shown). Both N-terminal and C-terminal epitope-tagged CaARN1 constructs also permitted growth on Fe-Fc.

To further characterize the iron uptake activity of cells transformed with CaARN1, uptake studies were conducted using 59Fe-Fc. In Δfet3Δarn1–4 cells, the uptake of 59Fe-Fc was significantly higher in cells transformed with the methionine-regulated CaARN1 constructs than in cells transformed with vector alone. The rate of iron accumulation in cells expressing CaArn1p (under the control of the MET25 promoter) was much greater than Δfet3 cells expressing the native ARNs under iron-limited conditions, which should result in their maximal induction (Fig. 5a). There were no significant differences in 59Fe-Fc uptake between cells expressing the epitope-tagged protein (5 FLAG or 3′Myc) and the non-tagged CaArn1p. Addition of methionine to the growth media reduced the rate of 59Fe-Fc uptake (Fig. 5b), although not to baseline levels. Western analysis of cells expressing CaArn1p showed regulation of CaARN1 expression (Fig. 5c). At high levels of methionine (560 μg/ml), no CaArn1p was detected by Western blot, although 59Fe-Fc uptake was still observed. This uptake may be due to the lack of complete repression of the MET25 promoter, particularly in high copy vectors.

We utilized pmetCaARN1-expressing cells to define the specificity of Fe recognition and transport. Addition of desferri-Fc to 59Fe-Fc-containing media did not inhibit uptake of 59Fe-Fc (Fig. 6). This result indicates that the transporter must recognize a structural difference in the Fe once it has bound iron. It has been reported that there is little conformational difference between Fe- and Ga-complexed hydroxamate siderophores (21). Both metals are bound in the trivalent state, although unlike Fe-Fc, Ga-Fc complexes cannot be reduced. Both Fe-Fc and Ga-Fc exhibited a similar concentration-dependent inhibition of 59Fe-Fc uptake (Fig. 6). Mineral Ga, supplied as Ga(NO)₃, had no effect on the uptake of 59Fe-Fc. The inhibition of 59Fe-Fc uptake by Ga-Fc was transient, as cells washed free of Ga-Fc showed no subsequent inhibition of 59Fe-Fc uptake (data not shown). These results suggest that Ga-Fc competes with Fe-Fc for a recognition site on CaArn1p. Ferrichrome synthetic analogues are recognized and taken up by the Ustilago maydis ferrichrome uptake system (15, 16). These analogues were not taken up by pmet-CaARN1-transformed Δfet3Δarn1–4 cells, as ascertained by growth, radiotracer experiments, and fluorescence microscopy (data not shown). These results demonstrate that the activity of CaArn1p is sensitive to alterations in siderophore structure.

Ferrichrome Uptake in Iron-depleted and Iron-replete Cells—
The high affinity iron transport system, comprised of Fet3p and Ftr1p, is regulated at the level of transcription by Aft1p (22). Expression of FET3 and FTR1 by iron-independent promoters showed that these proteins mediated iron transport even when cells were iron-replete (10). These results indicate that the only two surface proteins required for high affinity iron transport are Fet3p and Ftr1p. Because the S. cerevisiae siderophore transporters are also regulated by Aft1p, we asked whether other proteins regulated by Aft1p are required for siderophore transport. The S. cerevisiae strain Δfet3Δarn1–4 was transformed with plasmids containing S. cerevisiae ARN1 under its own promoter, pmetARN1 or pmetCaARN1. Cultures were grown under high iron or iron-limiting conditions for 6 h and then assayed for 59Fe-Fc uptake (Fig. 7a). The 6-h incubation period is long enough to permit expression of Aft1p-regulated genes (14). As expected, cells expressing Arn1p under the control of its native promoter, showed Fe-Fc uptake when grown under iron-depleted conditions but not under iron-replete conditions. Expression of either ARN1 or CaARN1 under the control of the MET25 promoter led to a high rate of Fe-Fc uptake regardless of whether cells were grown in high or low iron media. The rate of iron uptake was similar in cells expressing either the S. cerevisiae ARN1 or the C. albicans ARN1. These results suggest that once expressed, the siderophore transporter can function independently of cellular iron or other genes whose transcription is dependent on the Aft1p transcription factor. This conclusion is further supported by measuring 59Fe-Fc uptake in Δaft1 cells transformed with pARN1, pmetARN1, or pmetCaARN1 (Fig. 7b). There is little iron taken up by Δaft1 cells transformed with a plasmid that has ARN1.
under the control of the Aft1p-sensitive promoter. The same cells transformed with the indicated plasmids were plated in serial dilutions on CM containing 50 µM BPS and 0.5 µM Fe-FOB or Fe-Fc. Plates were incubated at 30 °C for 2 days and colonies examined for growth.

When the ARNs are expressed from their endogenous promoters there is little measurable siderophore iron transport in the absence of AFT1. Siderophore iron, however, can support the growth of S. cerevisiae /H9004 aft1 cells (Fig. 8). Recently, a homologue of AFT1, termed AFT2, has been identified, and it appears to share some functions with AFT1 (23). To determine whether AFT2 played a role in siderophore iron transport, we examined a /H9004 aft2 strain for its ability to grow on siderophore iron. Deletion of both AFT1 and AFT2 results in a lack of growth on Fe-Fc. In the absence of the Arnp’s or Fet3p there is no growth on Fe-Fc (Fig. 8 and Ref. 6). These results suggest that in S. cerevisiae AFT2 may permit a low level of expression of ARN1.

DISCUSSION

Siderophore-mediated iron accumulation is an important mechanism of iron uptake for many organisms including bacteria, fungi, and plants (2, 25). Numerous studies have shown that siderophore transport systems are virulence factors in microorganisms (26–28). S. cerevisiae, which does not secrete siderophores, has multiple transport systems that can utilize siderophores secreted by other organisms (5–9, 29). C. albicans, a pathogenic fungus of major medical importance, can secrete...
are cultured in methionine-deficient media with 50 μM FeCl$_3$ (iron replete) or 50 μM BPS (iron-depleted). Cells were grown to mid-log, and uptake of $^{59}$Fe-Fc was measured.

**Fig. 7. Arn1p-mediated $^{59}$Fe-Fc uptake in iron-replete or iron-depleted conditions.** *S. cerevisiae Δfet3, Δfet3Δarn1-4 (a) or *S. cerevisiae Δfet1 (b) transformed with ARN1, pmetARN1, or pmetCaARN1 were cultured in methionine-deficient media with 50 μM FeCl$_3$ (iron replete) or 50 μM BPS (iron-depleted). Cells were grown to mid-log, and uptake of $^{59}$Fe-Fc was measured.

**Fig. 8. Siderophore-independent growth in Δfet1, Δfet2, or Δfet1Δfet2 strains of *S. cerevisiae.* Indicated strains were grown to mid-log and then plated in serial dilutions on CM, CM-BPS, or CM-BPS with added siderophore iron. Plates were incubated for 2 days at 30 °C, and colonies examined for growth.
release. An answer to this question will affect the development of toxic siderophores. A release of siderophore iron while the siderophore is bound to the surface of the transporter, as opposed to being transported into the cell prior to iron release, would affect the classes of toxic compounds that might be made. Our results suggest that whatever the mechanism of siderophore iron release, it is highly similar for both C. albicans and S. cerevisiae, and perhaps for other fungi as well.

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