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The Role of the *FRE* Family of Plasma Membrane Reductases in the Uptake of Siderophore-Iron in *Saccharomyces cerevisiae**

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Saccharomyces cerevisiae **takes up siderophore-bound iron through two distinct systems, one that requires siderophore transporters of the ARN family and one that requires the high affinity ferrous iron transporter on the plasma membrane. Uptake through the plasma membrane ferrous iron transporter requires that the iron first must dissociate from the siderophore and undergo reduction to the ferrous form.** ***FRE1*** **and** ***FRE2*** **encode cell surface metalloreductases that are required for reduction and uptake of free ferric iron. The yeast genome contains five additional** ***FRE1*** **and** ***FRE2*** **homologues, four of which are regulated by iron and the major iron-dependent transcription factor, Aft1p, but whose function remains unknown. Fre3p was required for the reduction and uptake of ferrioxamine B-iron and for growth on ferrioxamine B, ferrichrome, triacetylfusarinine C, and rhodotorulic acid in the absence of Fre1p and Fre2p. By indirect immunofluorescence, Fre3p was expressed on the plasma membrane in a pattern similar to that of Fet3p, a component of the high affinity ferrous transporter. Enterobactin, a catecholate siderophore, was not a substrate for Fre3p, and reductive uptake required either Fre1p or Fre2p. Fre4p could facilitate utilization of rhodotorulic acid-iron when the siderophore was present in higher concentrations. We propose that Fre3p and Fre4p are siderophore-iron reductases and that the apparent redundancy of the** ***FRE*** **genes confers the capacity to utilize iron from a variety of siderophore sources.**

Virtually every organism on earth requires iron as an essential nutrient. Although iron is the second most abundant metal in the crust of the earth, the bioavailability of iron can be extremely low. This poor bioavailability occurs because iron is rapidly oxidized in an aerobic environment to the ferric form (Fe(III)),[1] which is poorly soluble in water and forms precipitates of oxyhydroxides. Microorganisms have the capacity to scavenge iron from insoluble precipitates by secreting and taking up siderophores, low molecular weight compounds that bind to Fe(III) with very high affinity and specificity. Siderophores are synthesized and secreted in the iron-free form, which then binds and solubilizes Fe(III) in the extracellular environment. The Fe(III)-siderophore complex is then recognized and selectively taken up by specific transport mechanisms. Many microorganisms synthesize one or a few types of siderophores, yet have the capacity to take up iron from a variety of siderophores secreted by other species of bacteria and fungi (1). Budding and fission yeast appear to be an exception; they neither synthesize nor secrete these compounds (2, 3). *Saccharomyces cerevisiae* can, however, recognize and take up iron from a variety of structurally distinct siderophores (4–10).

S. cerevisiae has two genetically separable systems for the uptake of siderophore-bound iron. One system depends on a family of homologous transporters of the major facilitator superfamily that is expressed as part of the *AFT1* regulon and are termed *ARN1, ARN2* (also *TAF1*), *ARN3* (also *SIT1*), and *ARN4* (also *ENB1*) (6–11). These transporters are expressed in intracellular vesicles. The individual *ARN* transporters exhibit specificity for different siderophores of the hydroxamate and catecholate classes; however, some siderophores, such as rhodotorulic acid, are not substrates of the *ARN* transporters (9). A second system of uptake for siderophore-bound iron depends on the high affinity ferrous iron (Fe(II)) transport complex, which is encoded by *FET3* and *FTR1* and is located on the plasma membrane (12–15). A low affinity Fe(II) transporter encoded by *FET4* is also expressed on the plasma membrane (16). For the siderophore-bound Fe(III) to become a substrate for the Fe(II) transporter, the iron must be both reduced and dissociated from the siderophore. This is accomplished in a single step by the activity of plasma membrane reductase systems, which contain flavocytochromes and have the capacity to reduce siderophore-bound iron (4, 5, 17). *FRE1* and *FRE2* encode plasma membrane metalloreductases that can reduce oxidized forms of both iron and copper (18–23). Strains deleted for *FRE1* exhibit only 10% of the Fe(III)-citrate reductase activity that is inducible in wild-type strains. Deletion of both *FRE1* and *FRE2* results in cells that are completely lacking Fe(III)-citrate reductase activity and fail to grow on iron-poor media. The completed sequence of the *S. cerevisiae* genome revealed the presence of five additional genes with striking similarity to *FRE1* and especially to *FRE2*. Four of these (*FRE3*, *FRE4*, *FRE5*, and *FRE6*) are greater than 35% identical to *FRE2* and are regulated at the transcriptional level by Aft1p (24). The fifth homologue (*FRE7*) is regulated by exogenous copper ions through the Mac1p transcription factor. The functions of these new *FRE* family members have not been identified.

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[1] The abbreviations used are: Fe(III), ferric iron; Fe(II), ferrous iron; FOB, ferrioxamine B; FC, ferrichrome; TAFC, triacetylfusarinine C; ENT, enterobactin; RA, rhodotorulic acid; BPS, bathophenanthroline disulfonate; HA, hemagglutinin; PCR, polymerase chain reaction.

We have investigated the role of the *FRE* family of genes in the uptake of siderophore-bound iron. Although the *FRE* genes appeared to have no role in the *ARN*-dependent uptake of siderophores, they were required for the uptake of siderophore-bound iron through the high affinity Fe(II) transport system. Although *FRE1* and *FRE2* encoded the majority of siderophore reductase activity, Fre3p could specifically facilitate reduction and uptake of iron bound to the trihydroxamate siderophores ferrioxamine B (FOB), ferrichrome (FC), and triacetylfusarinine C (TAFC) and to the dihydroxamate rhodotorulic acid (RA). Fre3p was expressed on the plasma membrane in a pattern consistent with its role in iron uptake through the plasma membrane Fe(II) transport system. Uptake of iron bound to the catecholate siderophore enterobactin (ENT) also occurred through the Fe(II) transport system and required either Fre1p or Fre2p. Expression of Fre4p was sufficient to facilitate the utilization of RA-bound iron when the siderophore was present in higher concentration.

EXPERIMENTAL PROCEDURES

Yeast Strains, Plasmids, and Media—All strains were constructed in YPH499 *MATa ura3-52 lys2-801(amber) ade2-101(ochre) trp1-Δ63 his3-Δ200 leu2-Δ1*. To generate a *FRE1* deletion, strains were transformed with *Bam*HI- and *Hin*dIII-digested pUMG2 and transformants screened by surface ferric reductase activity. For deletion of *FRE2*, a 1.4-kilobase pair fragment of genomic DNA from a *fre1 fre2* double deletion strain (17) was PCR-amplified, and the product was used to transform to histidine prototrophy. Transformants were screened by PCR. PCR-mediated gene disruption was used to generate deletions of the other *FRE* genes (25). The following primers were used to amplify the HISG-URA3-HISG cassette from the plasmid pMPY-ZAP. For *FRE3*, 5′- TGCTTGTCAGGAGCAAGCGCCTCCCCTGCTAAGACAAAAATGTACGGCAAGTTctcactatagggcgaattgg-3′ and 5′-TACCAACTTTGGTATTCTTCAAAGTATTCGATTGCCTTTGCAGGCTTCTCTActaaagggaacaaaagctgg-3′; for *FRE4*, 5′-AGCTTTCGGCCGCAAAGGCCCCACCCAGTAAAACGTCTCTAATAAATACTCActcactatagggcgaattgg-3′ and 5′-AGGCTTGGTATTCCTCCAAGTATTCAATCATTCTTGATGGGTTTCTGATAACctaaagggaacaaaagctgg-3′; for *FRE5*, 5′-AGGTTCTTTAGCAAAACCAGCATCAACTAAGAAAAGAACGCAATGGGACCAGctcactatagggcgaattgg-3′ and 5′-ATTCTGGAATTCCTCCACGTATTCTATCCATTTGTCACCGTGTTCAAGAACTctaaagggaacaaaagctgg-3′; and for *FRE6*, 5′-ACGTGGCTGATATCTTTAACAAAGGCTTTTAATATAAAATTACCACACACTGctcactatagggcgaattgg-3′ and 5′-ACTGATATTCCTCGAAATACTCTATAATTCTCTCTGGGTAACCGAGGAGTTTctaaagggaacaaaagctgg-3′. Deletions were confirmed by PCR, and FOA-resistant clones were selected. Construction of multiply deleted *FRE* mutants was performed by repeated PCR-based gene disruption. Construction of the *ARN* deletion strains and the strain *YPH499 FET3-HA FTR1-Myc* was previously described (10). The FRE3-HA strain, which expresses a triple copy of the HA epitope fused to the carboxyl terminus of Fre3p, was constructed by PCR epitope tagging (26) using the plasmid pMPY-3xHA and the following primers: 5′-CTAGAGAAGCCTGCAAAGGCAATCGAATACTTTGAAGAATACCAAAGTTGGGAACAAAAGCTGGAGCTCCAC-3′ and 5′-AATATATACTTGTGATAGGTAAAATAGTGAGGAAATAAATAAGGTAATTGACTATAGGGCGAATTGGGTACC-3′. Integration of the HA epitope was confirmed by PCR and by Western blotting. The genomic clone of *FRE3-HA* was constructed by isolating genomic DNA from the *FRE3-HA* strain and digesting it with *Hpa*I and *Pst*I. After electrophoresis, DNA fragments from 4.0–5.0 kilobase pairs were extracted (GELase, Epicentre Technologies), ligated into *Pst*I- and *Sma*I-digested Yep351, and transformed into *Escherichia coli* DH5-α. Clones were screened by colony hybridization using a PCR-amplified *FRE3* open reading frame as a probe, and positive clones were analyzed by restriction mapping. Plasmid-encoded expression of Fre3p-HA was confirmed by Western blotting. Rich media (YPD), synthetic defined media (SD), and defined-iron media were prepared as described (27, 28).

Siderophores—Iron-free forms of FC, RA, and the mesylate salt of FOB (Desferal) were purchased from Sigma. Ferric and desferric forms of TAFC and ENT were prepared as described (9, 29). Ferric RA, FC, and FOB were prepared by incubating the desferri-siderophores with an equimolar amount of $FeCl_3$ in 50 mM sodium citrate, pH 6.5, and 5% glucose.

Surface Reductase Assay—Cells were grown to mid-log phase in YPD medium or defined iron media containing 10 μM ferrous ammonium sulfate, and the assay was performed as described (18) with the following modifications. Ferrozine was substituted for bathophenanthroline disulfonate (BPS) as the Fe(II) acceptor, and 1 mM Fe(III)-FC, -FOB, -TAFC, and -RA were the reductase substrates. Absorbance of the cell-free assay mixture was measured at 562 nm.

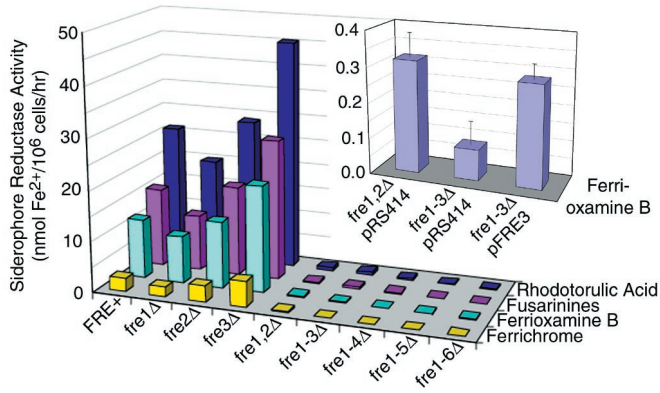

FIG. 1. **Capacity of the *FRE* gene products to reduce Fe(III)-siderophores in *FRE*+ and *fre*− deletion strains.** After culture in rich media or defined iron media containing 10 μM ferrous iron (*inset*), cells of the indicated genotype in the exponential phase of growth were collected, washed, and assayed for Fe(III)- siderophore reductase activity in 1 mM Fe(III)-ferrichrome, -ferrioxamine B, -fusarinine, and -rhodotorulic acid with ferrozine as the Fe(II) acceptor. Absorbance at 562 nm was measured. The experiments were repeated twice, and data from a representative experiment are shown. *Inset,* the *fre1,2,3*Δ and the *fre1,2*Δ strain were transformed with either a high copy number plasmid bearing the *FRE3* gene (pFRE3) or the empty parent plasmid (pRS424) and the Fe(III)-ferrioxamine B reductase activity measured as described. Samples were prepared in triplicate, and the experiment was repeated three times. *Error bars* indicate the S.D.

Plate Assay and Uptake Assay—For the plate assay, modified synthetic complete media were used in which copper and iron were omitted, and 1 μM copper sulfate and 100 μM BPS were added. Trace amounts of iron are supplied by agar (Difco). Plate assays for low concentrations of FC, FOB, TAFC, ENT, and RA were performed as described (10) using 10 μM desferri-FC, -FOB and -TAFC, 5 and 10 μM ferric-ENT, and 15 μM ferric-RA (a concentration with iron-chelating capacity equivalent to 10 μM FC, FOB, TAFC, and ENT). Plate assays for high concentrations of FOB and RA were performed using 100 μM of the desferri-siderophore. Because of ligand exchange between the BPS and the siderophores, the exact concentration of the ferric-siderophore complex in the plate assay is not known. Uptake assays were performed as described (30) in Multiscreen Filtration plates (Millipore) using 1 μM each of ^{55}Fe(III) and desferri-FOB and-ENT.

Immunofluorescence—Strains YPH499 pFRE3-HA and YPH499 FET3-HA FTR1-Myc were grown to mid-log phase in defined iron media containing 5 μM ferrous ammonium sulfate and 1 mM ferrozine to induce the expression of Fre3p. Cells were washed and prepared for immunofluorescence microscopy as described (15). Primary antibody was affinity-purified HA.11 (Babco) at 1:300, and secondary antibody was Cy3-conjugated polyclonal anti-mouse IgG from donkey (Jackson ImmunoResearch) at 1:500. Cells were visualized with a Zeiss 63X/1.4NA objective. Images were acquired with a cooled CCD camera (Cooke) using IP Labs software (Scanalytics).

RESULTS

Reduction of Ferric Siderophores by FRE Reductases—FOB-bound iron can be taken up at the plasma membrane by a reductive mechanism that requires heme (5). The metalloreductase encoded by *FRE1* is a plasma membrane heme protein of the cytochrome *b*-type, and strains deleted for *FRE1* exhibit low Fe(III)-citrate reductase activity (17–19). We tested whether other ferric-siderophores of the hydroxamate class were substrates for cell surface reductases (Fig. 1). In addition to FOB, both the trihydroxamates FC and TAFC and the dihydroxamate RA were substrates for plasma membrane reductases. RA-bound Fe(III), which is only taken up through the Fe(II) transport system, displayed the highest rate of reduction, whereas Fe(III) bound to FC, which is taken up by both

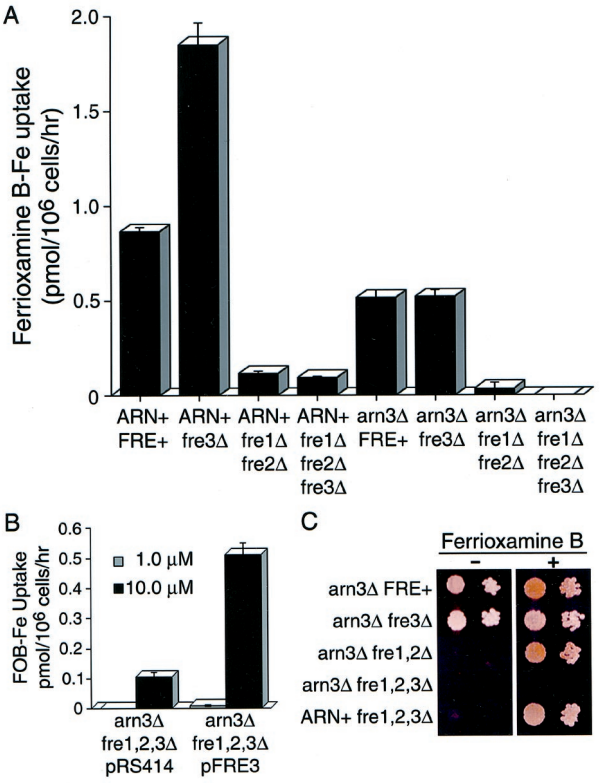

FIG. 2. **Requirement of Fre1p, Fre2p, or Fre3p for reductive uptake of ferrioxamine B-iron.** *A*, absence of FOB-^{55}Fe uptake in a strain deleted for *ARN3*, *FRE1*, *FRE2*, and *FRE3*. Congenic strains of the indicated genotype were grown to the exponential phase in rich media, and uptake of ^{55}Fe bound to 1 μM FOB was measured. *B*, uptake of FOB-^{55}Fe when Fre3p is overexpressed. A strain deleted for *ARN3*, *FRE1*, *FRE2*, and *FRE3* was transformed with either a high copy number plasmid containing the *FRE3* gene (pFRE3) or the parent vector (pRS414) and grown to exponential phase in defined iron media containing 10 μM ferrous ammonium sulfate to induce expression of Fre3p. Uptake of ^{55}Fe in the presence of either 1 μM FOB (*gray bars*) or 10 μM FOB (*black bars*) was measured. *C*, failure of FOB to stimulate growth in yeast deleted for *ARN3*, *FRE1*, *FRE2*, and *FRE3*. Congenic strains of the indicated genotype were plated in serial dilutions on synthetic iron-poor media containing 100 μM BPS (−) or 100 μM BPS and 10 μM desferri-FOB (+). Plates were incubated at 30 °C for 3 days.

the Fe(II) transport system and the Arn1p and Arn3p transporters, displayed the lowest rate of reduction. *FRE1* and *FRE2* encoded 90–98% of the siderophore reductase activity, but there were measurable differences in the siderophore reductase activities of a *fre1*Δ *fre2*Δ strain and a *fre1*Δ *fre2*Δ strain that also carried a deletion of *FRE3* (Fig. 1, *inset*). Deletion of *FRE3* in the *fre1*Δ *fre2*Δ strain resulted in a 73% decrease in the reduction of FOB. This loss of FOB reductase activity was completely restored when the *fre1,2,3*Δ strain was transformed with a plasmid expressing Fre3p, indicating that Fre3p can facilitate the reduction of FOB-iron. These data did not indicate whether the very low level of residual reductase activity associated with expression of Fre3p, Fre4p, Fre5p, and Fre6p could significantly affect iron uptake and utilization by the cell. For this reason, we tested the capacity of *FRE*-deleted strains to take up Fe(II)-FOB and to grow when FOB was the iron source.

The Role of FRE3 in Ferrioxamine B-mediated Iron Uptake and Growth—FOB-bound iron can be transported into the cell by both an Arn3p-dependent system and a Fet3p-dependent system that also involves an Fe(III) to Fe(II) reduction step. We tested the role of *FRE1*, *FRE2*, and *FRE3* in the uptake of FOB-iron in strains both expressing Arn3p and deleted for Arn3p (Fig. 2*A*). When cells were grown in rich media, deletion of *FRE3* alone resulted in an increased uptake of FOB-iron when compared with the congenic parent strain. This effect was consistently reproducible and suggested that deletion of *FRE3* resulted in changes in the intracellular iron pool. Deletion of *FRE1* and *FRE2* resulted in dramatic decreases in FOB-iron uptake, both in an *ARN*+ and an *arn3*Δ background, indicating that Fre1p and Fre2p reductase activities contribute to FOB-iron uptake. Deletion of *FRE3* in addition to *FRE1* and *FRE2* resulted in a small, additional decrease in FOB-iron uptake in both the *ARN*+ and the *arn3*Δ backgrounds, so that there was no detectable FOB-iron uptake in the *arn3*Δ *fre1*Δ *fre2*Δ *fre3*Δ strain. To determine whether Fre3p contributed significantly to FOB-iron uptake, Fre3p was overexpressed from a high copy-number vector in the *arn3*Δ *fre1*Δ *fre2*Δ *fre3*Δ strain (Fig. 2*B*). Fre3p overexpression resulted in a slight increase in uptake when FOB-iron was present at 1 μM and a larger increase in uptake when FOB-iron was present at 10 μM. These data indicated that Fre3p could facilitate the reductive uptake of FOB-iron.

To clarify further the role of *FRE1, FRE2,* and *FRE3* in the utilization of FOB-iron, we examined the growth of *FRE*-deleted strains on iron-limited media containing FOB as an iron source (Fig. 2*C*). We used strains in which *ARN*-dependent FOB uptake was inactivated by deletion of *ARN3*; therefore, iron uptake was dependent on Fet3p and the high affinity Fe(II) uptake system. An *arn3*Δ *fre1*Δ *fre2*Δ strain did not grow in the absence of FOB, but grew as well as the *FRE*+ parent strain in the presence of FOB, indicating that the reductase activities encoded by *FRE1* and *FRE2* are not absolutely required for utilization of FOB-iron. An *arn3*Δ *fre1*Δ *fre2*Δ *fre3*Δ strain, however, failed to grow in the presence of FOB, confirming that Fre3p can facilitate the uptake and utilization of FOB-iron. This result also indicated that endogenous levels of expression of Fre4p, Fre5p, Fre6p, and Fre7p could not support the reductive uptake of FOB-iron. Deletion of *FRE3* alone had no effect on growth on FOB, and transformation of the *arn3*Δ *fre1*Δ *fre2*Δ *fre3*Δ strain with a low copy number plasmid carrying either the *FRE1* or *FRE3* gene completely restored growth on FOB (data not shown). Fre1p, Fre2p, and Fre3p are not required for uptake of FOB through the ARN-dependent system, as an *ARN*+ *fre1*Δ *fre2*Δ *fre3*Δ strain grew well on FOB (Fig. 2*C*).

The Role of Fre3p in the Uptake of Ferrichrome, Triacetylfusarinine C, and Rhodotorulic Acid—Because Fre3p could facilitate the reductive uptake of FOB-iron, we questioned whether structurally related siderophores were also substrates for Fre3p. The iron-binding sites of FC and TAFC are similar in structure to that of FOB in that they each consist of three bidentate hydroxamate ligands that bind Fe(III) in a six-coordinate complex. We examined the capacity of the *FRE* homologues to facilitate growth when FC and TAFC in low concentration (10 μM) were provided as iron sources (Fig. 3, *A* and *B*). Again, these experiments were performed using strains in which the *ARN*-dependent siderophore uptake systems were inactivated by deletion of *ARN1* and *ARN3* to test FC and by deletion of *ARN2* to test TAFC. Deletion of either *FRE3* alone or *FRE1* and *FRE2* together had no effect on FC-mediated growth (Fig. 3*A*). Deletion of *FRE1, FRE2,* and *FRE3* in combination, however, resulted in a failure to grow on FC, indicating that Fre3p can facilitate reductive iron uptake from both FC and FOB. As was the case for FOB, deletion of *FRE1, -2,* and *-3* in an ARN+ background did not impede FC-mediated growth, further supporting the role of the *FRE* genes in siderophore-iron uptake through the Fe(II) transport system but not through the *ARN* system. Similar results were obtained when a strain deleted for *FRE1, -2,* and *-3* was tested for TAFC-mediated growth (Fig. 3*B*), indicating that Fre3p can

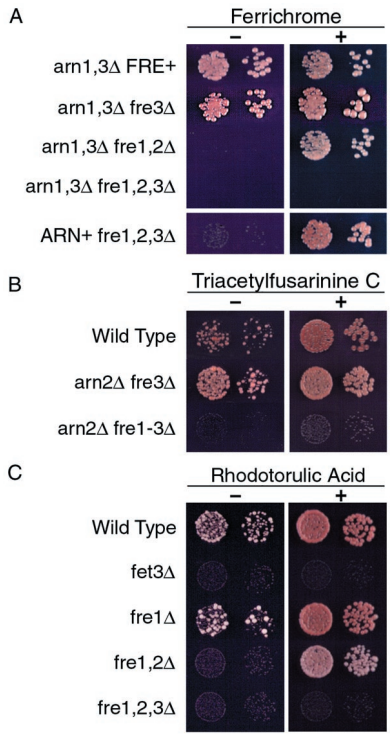

FIG. 3. **Requirement of Fre1p, Fre2p, or Fre3p for growth on ferrichrome, triacetylfusarinine C, and rhodotorulic acid.** Congenic strains of the indicated genotype were plated in serial dilutions on synthetic iron-poor media containing 100 μM BPS and either 0 μM (−) or 10 μM (+) desferri-FC (*A*), desferri-TAFC (*B*), or 15 μM (+) ferric-RA (*C*). Plates were incubated for 4 days at 30 °C.

facilitate reductive iron uptake from each of the three trihydroxamate siderophores tested.

RA-mediated iron uptake in *S. cerevisiae* significantly differs from FOB-, FC-, and TAFC-mediated uptake in that RA is not a substrate for the ARN transporters and RA-iron uptake occurs exclusively through the Fe(II) transport system (9). Also, RA is a dihydroxamate siderophore that coordinates Fe(III) in a RA_3Fe_2 complex (31). Despite these differences, Fre3p also facilitated RA-iron-mediated growth (Fig. 3*C*), as the *fre1*Δ *fre2*Δ*FRE3*+ strain grew as well as the congenic parent strain on ferric-RA, whereas the *fre1*Δ *fre2*Δ *fre3*Δ strain did not grow.

Localization of Fre3p to the Plasma Membrane—Both components of the high affinity Fe(II) transport system, Fet3p and Ftr1p, are expressed on the plasma membrane (10, 13–15). In contrast, the Arn1p, Arn3p, and Arn4p transporters are largely confined to intracellular vesicles that comigrate on density gradients with Pep12p, a protein of the late endosome (9, 10) (data not shown). To determine the cellular localization of Fre3p, we constructed a strain in which the chromosomal copy of *FRE3* carries a triple copy of the HA epitope at the carboxyl terminus. We confirmed that the epitope-tagged Fre3p was functional by expressing the HA-tagged Fet3p from a plasmid in a *FRE3*-deleted strain and observing restoration of FOB-iron uptake to wild-type levels. We grew the Fre3-HAp strain and a strain expressing an HA-tagged version of Fet3p in media containing limiting amounts of iron (to induce Fre3-HAp expression) and performed indirect immunofluorescence (Fig. 4). Fre3p was detected at the periphery of the cell in a pattern that was similar to that of Fet3p, indicating that both Fre3p and Fet3p were expressed on the plasma membrane. Fre3p was not detected in intracellular vesicles. This localization to the plasma membrane was consistent with a role for Fre3p in reductive iron uptake at the plasma membrane through the high affinity Fe(II) system. The absence of detectable Fre3p in intracellular vesicles is also consistent with genetic data indicating that Fre3p is not required for ARN-dependent siderophore uptake.

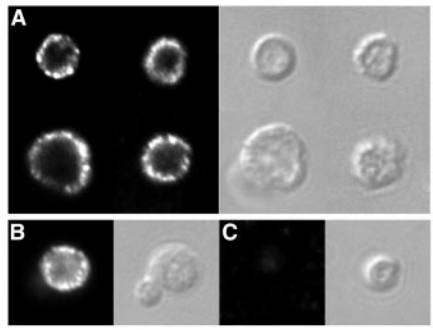

FIG. 4. **Localization of Fre3p to the plasma membrane.** Indirect immunofluorescence microscopy was performed on cells expressing Fre3p-HA (*A*), Fet3p-HA (*B*), and the untagged parent strain (*C*). HA.11 was the primary antibody, and Cy3-conjugated donkey anti-mouse was the secondary antibody. Images are in pairs with fluorescence on the left and DIC on the *right*.

Reductive Uptake of Fe(III)-Enterobactin—ENT is a siderophore of the catecholate class that is synthesized and secreted by species of Gram-negative bacteria, such as *E. coli*. *S. cerevisiae* was recently reported to take up ENT through the *ARN4* transporter (7), but it is not known whether ENT-bound iron can be taken up via a reductive mechanism that requires the high affinity Fe(II) transport system. We examined the uptake of ENT-bound iron in strains bearing a deletion of *ARN4* alone and deletions of all four *ARN* genes, both in strains with intact high affinity Fe(II) transport (*FET3*+) and in strains lacking high affinity Fe(II) transport (*fet3*Δ, Fig. 5*A*). Deletion of *ARN4* in both *FET3*+ and *fet3*Δ backgrounds resulted in decreases in ENT-iron uptake to undetectable levels, confirming that uptake of ENT-iron is facilitated by Arn4p. Uptake of ENT-iron was increased over 10-fold in a *fet3*Δ strain, and this was associated with a large increase in the level of expression of the Arn proteins, especially Arn4p, in the *fet3*Δ strain (data not shown). This increase likely reflects the relative iron deprivation and subsequent Aft1p activation that results from the loss of high affinity Fe(II) uptake in *fet3*Δ strains. These results suggested that the majority of ENT-iron uptake occurs through Arn4p, but whether ENT-iron could also be taken up through a reductive mechanism remained unclear.

To evaluate ENT-iron uptake via the high affinity Fe(II) system, we tested the capacity of ENT to stimulate growth of strains on iron-limited media (Fig. 5*B*). Strains individually deleted for *ARN4* or *FET3* grew as well as the wild-type parent strain in the presence of ENT. Deletion of both *ARN4* and *FET3* resulted in a strain that did not grow in the presence of ENT, indicating that ENT is a substrate for both the *ARN*-dependent and *FET3*-dependent systems of iron uptake. To determine the role of the *FRE* genes in *FET3*-dependent ENT-iron uptake, we tested the capacity of ENT to stimulate growth of *FRE*-deleted strains that were also deleted for *ARN4* (Fig. 5*C*). In this experiment, the *arn4*Δ *FRE*+ and the *arn4*Δ *fre1*Δ strains grew well on iron-limited media in the presence of ENT and very slowly in the absence of ENT. In contrast, the *arn4*Δ *fre1*Δ *fre2*Δ and the *arn4*Δ *fre1*Δ *fre2*Δ *fre3*Δ strains exhibited virtually no growth in the presence or absence of ENT. Expression of *FRE1* from a low copy number plasmid restored ENT-mediated growth to the *arn4*Δ *fre1*Δ *fre2*Δ strain (data not shown), indicating that either Fre1p or Fre2p is also required for the *FET3*-dependent utilization of ENT-iron.

The Role of Fre4p in Rhodotorulic Acid Iron Utilization—Fre4p, Fre5p, and Fre6p did not appear to have a role in the reductive uptake of low concentrations of siderophore-bound

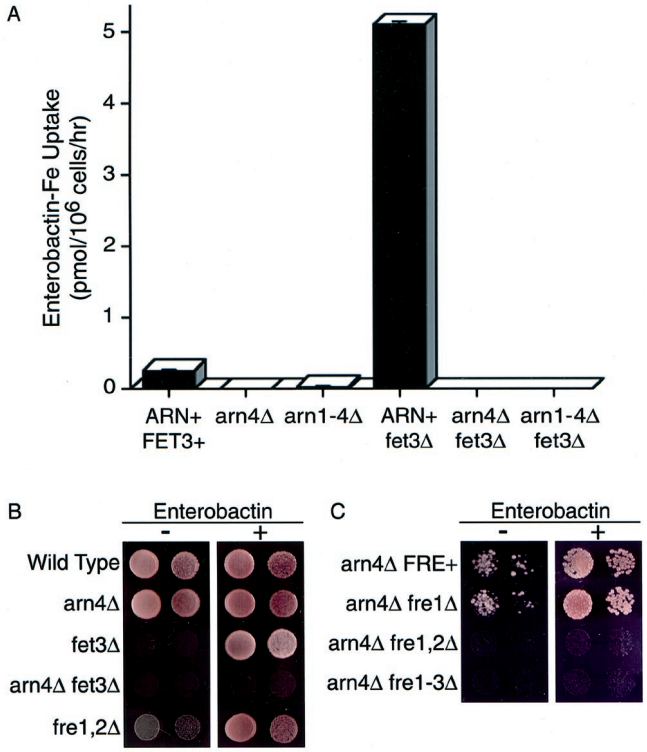

FIG. 5. *A,* uptake of enterobactin-Fe through *ARN4.* Congenic strains of the indicated genotype were grown to exponential phase in rich media and assayed for uptake of ^{55}Fe-enterobactin. *B*, requirement of either *FET3* or *ARN4* for growth on enterobactin; *C*, failure of enterobactin to sustain growth in a strain deleted for *ARN4, FRE1*, and *FRE2.* Congenic strains of the indicated genotype were plated in serial dilutions on synthetic iron-poor media containing 100 μM BPS and either 0 μM (−), 5 μM (+) (*B*) or 10 μM (+) (*C*) ferric-ENT. Plates were incubated for 3 days at 30 °C.

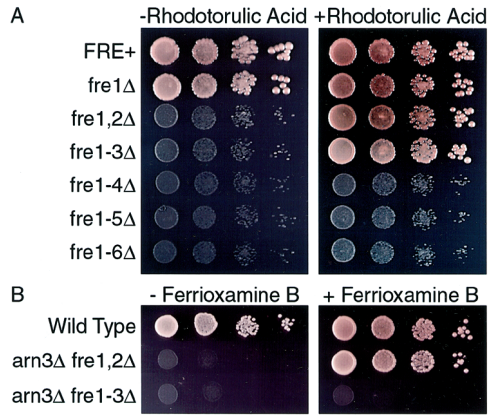

FIG. 6. **Fre4p stimulation of growth in the presence of higher concentration RA but not FOB.** Congenic strains of the indicated genotype were plated in serial dilutions on synthetic iron-poor media containing 100 μM BPS (−*rhodotorulic acid* (*A*), −*ferrioxamine B* (*B*)) and media containing 100 μM BPS and either 100 μM desferri-RA (+*rhodotorulic acid* (*A*)) or 100 μM desferri-FOB (+*ferrioxamine B* (*B*)). Plates were incubated for 4 days at 30 °C.

iron, because endogenous levels of expression of these proteins in *fre1*Δ *fre2*Δ *fre3*Δ strains did not provide sufficient activity to result in growth on low concentrations of siderophore. This apparent lack of function could occur because of the following: 1) *FRE4, FRE5*, and *FRE6* are not expressed as functional enzymes on the plasma membrane; 2) the siderophores we tested were not substrates for Fre4p, Fre5p, and Fre6p; or 3) the tested siderophores were substrates but exhibited lower affinity for Fre4p, Fre5p, and Fre6p. We tested the last hypothesis by examining the growth of *FRE*-deleted stains on a higher concentration of RA and FOB. In Fig. 6, each of the six iron-regulated *FRE* genes was serially deleted, and the resulting strains were tested for the capacity to grow in the presence of 100 μM RA and FOB. Deletion of *FRE1*, *FRE2*, and *FRE3* had no effect on the capacity of a high concentration of RA to stimulate growth on iron-limited plates (Fig. 6*A*). Deletion of *FRE1, FRE2, FRE3*, and *FRE4*, however, resulted in a strain that grew very slowly on iron-limited media in the presence or absence of 100 μM RA. In contrast, a high concentration of FOB did not stimulate growth of the *arn3*Δ *fre1*Δ *fre2*Δ *fre3*Δ strain. These data indicate that Fre4p could specifically facilitate the reductive uptake of RA-iron, but not FOB-iron, through the Fe(II) transport system.

DISCUSSION

Uptake of siderophore-bound iron through the high affinity Fe(II) transport system requires the activity of plasma membrane reductases that are encoded by the *FRE* family of genes. Here we have shown that Fre1p and Fre2p can facilitate the reduction of ferric-siderophores of the trihydroxamate and dihydroxamate classes. This finding was consistent with previous observations that the plasma membrane reductase system of budding yeast exhibits an extremely broad range of substrate specificity that includes both ferric and nonferric electron acceptors (32). We also demonstrated that Fre3p could facilitate the reduction of FOB and that Fre3p and Fre4p could facilitate the reductive uptake and utilization of siderophore-iron, presumably by acting as ferric-siderophore reductases. Although Fre4p could facilitate utilization of RA-iron, we were not able to detect significant Fre4p-dependent RA-iron reductase or uptake activities. The most likely explanation for these observations is that *S. cerevisiae* can grow in the face of extremely low rates of iron uptake, and it is less likely that Fre4p facilitates iron uptake through an activity other than that of a reductase. Fre3p and Fre4p exhibit the most similarity to Fre2p with 72 and 56% identity, respectively, at the amino acid level (24). Fre5p and Fre6p exhibit lesser degrees of similarity to Fre2p (38 and 35%, respectively), and the function of these gene products remains unknown. Although the siderophore reductase activity exhibited by Fre3p is 40-fold lower than that of Fre1p and Fre2p, *FRE3* and *FRE4* mRNA transcripts are present in quantities roughly comparable to those of *FRE1* and *FRE2* (24). This observation suggests that the differences in activity are due less to differences in the rate of transcription and more to differences in the intrinsic properties of the proteins. We observed that overexpression of Fre3p did not result in increased Fre3p-specific reductase activity when compared with the activity associated with endogenous levels of expression. This observation suggests the possibility that the plasma membrane reductase system has additional components that may be present in limiting quantities.

Although Fre1p, Fre2p, Fre3p, and Fre4p could facilitate the reductive uptake of siderophore-iron, they were not functionally interchangeable. Fre3p and Fre4p did not facilitate growth on low concentrations of ferric-ENT, but Fre3p could facilitate growth on low concentrations of di- and trihydroxamates. Fre4p did not facilitate growth in the presence of low concentrations of catecholate siderophore or hydroxamate siderophores nor on high concentrations of the trihydroxamate FOB. Yet Fre4p did facilitate growth on high concentrations of the dihydroxamate RA. These results suggest that the *FRE* reductases differ both in their specificities and affinities for different siderophores. Further evidence of differing specificities is seen in the case of the *FRE3* deletion strain. Deletion of *FRE3* in cells grown in rich media leads to an increased rate of

TABLE I

Comparison of growth stimulation capacity and reduction potential of siderophores

Data from Figs. 2, 3, 5, and 6 are presented. The capacity of the siderophore at a concentration of 10 μM to stimulate growth of strains with the indicated *FRE* genotype was scored (+, wild type growth; −, minimal growth). Growth on plates containing 100 μM RA is reported in parentheses. Reduction potentials (31) are listed in mV. Each reduction potential was measured at pH 7 in dilute aqueous solution *versus* a standard hydrogen electrode. Variations in activity coefficients, due to differences in buffer type or ionic strength, are generally assumed to be <10 mV.

Strain	ENT	FOB	FC	TAFC	RA
			growth stimulation		
fre1Δ	+	+	+	+	+
fre1Δ fre2Δ	−	+	+	+	+
fre1Δ fre2Δ fre3Δ	−	−	−	−	−(+)
			reduction potential		
	−750	−468	−400	−468	−350

uptake of FOB-iron, which might occur if Fre3p facilitated uptake of a significant amount of iron from a specific (but as yet unknown) source. Thus, in the absence of Fre3p, cells would respond to the loss of iron uptake from this specific source by up-regulating remaining systems of iron uptake. An alternative explanation for the increase in iron uptake in the absence of Fre3p is that Fre3p is acting as both a reductase and a sensor of iron. Although externally directed environmental iron sensors have not been reported in budding yeast, a two-component Fe(III) sensor has recently been identified in *Salmonella enterica* (33).

The differences in specificity exhibited by the FRE reductases may be based on differences in the structure of the iron coordination site in the siderophores. Alternatively, differences in specificity may be accounted for by the reduction potentials of the siderophores. In Table I, the capacities of siderophores to stimulate growth of FRE-deleted strains and the reduction potentials (31) of these siderophores are presented. ENT had both the most negative reduction potential and the most stringent reductase requirements, stimulating Arn4p-independent growth only when Fre1p or Fre2p was expressed. Although the data presented here suggest that Fre1p or Fre2p can reduce ENT-iron, ENT, with a reduction potential of −750 mV, is thought to be beyond the range of physiological reductants (31). This may not be the case in an *in vivo* setting, however, as a positive shift in the reduction potential of the siderophore may occur when the pH is lowered, the concentration of the reduced species is very low, or the hydrophobicity of the microenvironment is altered. In contrast to ENT, RA exhibits both the least negative reduction potential and the least stringent reductase requirements, serving as a substrate for Fre1p, Fre2p, Fre3p, and, at higher concentrations, Fre4p. Whether a particular ferric-siderophore can be reduced by one of the FRE reductases may depend more on whether the reductase can generate sufficient reduction potential than on the capacity of the reductase to "recognize" a structural motif.

Reductive uptake of iron at the plasma membrane requires the function of both an Fe(III)-reductase complex (containing a *FRE* gene product and possibly other components (34)) and an Fe(II) transport complex (containing Fet3p and Ftr1p). Fet3p and Ftr1p are physically associated, and their intracellular association is required for efficient expression on the plasma membrane (13–15). Although deletion of the *FRE* genes did not impede the expression of the Fet3p-Ftr1p complex on the plasma membrane,[2] the reductase and transport complexes may be associated as part of a plasma membrane-based iron uptake "machine."

[2] C.-W. Yun, unpublished observations.

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