A family of four putative transporters (Arn1p–4p) in Saccharomyces cerevisiae is expressed under conditions of iron deprivation and is regulated by Aft1p, the major iron-dependent transcription factor in yeast. One of these, Arn3p/Sit1p, facilitates the uptake of ferrioxamine B, a siderophore of the hydroxamate class. Here we report that ARN family members facilitate the uptake of iron from the trihydroxamate siderophores ferrichrome, ferrichrome A, and triacetylfusarinine C. Uptake of siderophore-bound iron was dependent on either the high-affinity ferrous iron transport system or the ARN family of transporters. The specificity of each siderophore for individual transporters was determined. Uptake of ferrichrome and ferrichrome A was facilitated by both Arn1p and Arn3p. Uptake of triacetylfusarinine C was facilitated by Arn2p, although small amounts of uptake also occurred through Arn1p and Arn3p. In contrast to the trihydroxamates, uptake of iron from the dihydroxamate rhodotorulic acid occurred only via the high-affinity ferrous iron system. Epitope-tagged Arn1p was expressed in intracellular vesicles in a pattern that was indistinguishable from that of Arn3p, whereas Ftr1p, a component of the high-affinity ferrous system, was expressed on the plasma membrane. These data indicate that S. cerevisiae maintains two systems of siderophore uptake, only one of which is located on the plasma membrane.

Iron is the second most abundant metal in the earth’s crust; despite the abundance of iron, acquisition of this essential nutrient poses significant challenges for most organisms. The bioavailability of iron is very low in an aerobic environment because iron is present as relatively insoluble ferric hydroxides; therefore, organisms have had to develop strategies to solubilize and acquire this metal. Most bacteria and fungi synthesize, secrete, and take up at least one type of siderophore and yet also have the capacity to take up siderophores secreted by other organisms (1). Siderophore-mediated iron uptake has been well studied in prokaryotes (2), but much less is known about this process in eukaryotes (3). Although Saccharomyces cerevisiae does not secrete siderophores, it is capable of taking up siderophore-bound iron (4, 5), and a specific transport system for the hydroxamate-type siderophore ferrioxamine B (FOB) has recently been described (6, 7).

Budding yeast take up FOB-iron through two separate, high-affinity systems as follows: 1) a plasma membrane-based system in which the FOB-bound Fe(III) is reduced to ferrous iron (Fe(II)), released from the siderophore, and then transported by the high-affinity Fe(II) transport system encoded by FET3 and FTR1 (5, 7–9); and 2) a system requiring ARN3/SIT1 (6, 7). ARN3 encodes one of a family of four highly homologous, iron-regulated putative transporters of the major facilitator superfamily of transporters. ARN1–4 were identified through cDNA microarrays representing the genome of Saccharomyces cerevisiae (7); they are transcriptionally activated under conditions of iron deprivation and are under the control of the major iron-dependent transcription factor, AFT1 (10, 11). ARN3 is required for the uptake of FOB-bound iron when the high-affinity Fe(II) uptake system is inactivated. Arn3p is located in intracellular vesicles similar to the late endosomal/pre-vacuolar compartment, suggesting either that Arn3p rapidly cycles to the plasma membrane or that FOB is delivered to Arn3p through an endocytic mechanism. We determined whether the ARN family of transporters could facilitate the uptake of iron bound to other hydroxamate-type siderophores that are secreted by fungi, specifically, ferrichrome (FC), ferrichrome A (FC-A), triacetylfusarinine C (TAF), and rhodotorulic acid (RA).

Here we report that FC-, FC-A-, and TAF-bound iron was taken up by both the FET3-dependent system and ARN-dependent systems, whereas RA-bound iron was taken up solely through the FET3-dependent system. FC and FC-A uptake was facilitated specifically by both Arn1p and Arn3p, whereas TAF was primarily facilitated by Arn2p and to a lesser extent by Arn1p and Arn3p. Epitope-tagged Arn1p was localized to intracellular vesicles and was resistant to digestion by extracellular proteases. These data suggested that Arn1p and Arn3p were expressed in the same intracellular, late endosomal compartment.

**Experimental Procedures**

**Yeast Strains, Plasmids, and Media**—The construction of congenic ARN1 deletion strains in YPH499 was described previously (7). The ARN1-HA strain was constructed by polymerase chain reaction epitope tagging (12) using the following primers: 5'-ATTTTGGATTGTGTTTGA-AAAAACTCTCATCAAAAATCTCATTAAAAGGGGAGAAGAAAAAGCT- GGAATGAC3' and 5'-AATAATATGGGTGAAGTTGATCAAGCC AATCATGCAACGTAAACATCAACCTATAGGGCGAATTGGGTACC- AATCATGCAACGTAAACATCAACCTATAGGGCGAATTGGGTACC-
3'. Integration of the HA epitope was confirmed by polymerase chain reaction and Western blotting. Construction of the strain YPH499 FET3-3-HA FTR1-myc was described previously (7). The plasmid pMET-Arn13MyC was provided by J. Kaplan. It is a high copy number plasmid in which Arn1p is expressed with a carboxyl-terminal Myc epitope under the control of the MET25 promoter (13). Rich media (YPD) and defined media (SD) were prepared as described (14). Media with defined iron content were prepared as described (15).

Isolation and Purification of Siderophores—N\(^{-}\)-Acetylfusarinines (fusigens) and partially acetylated fusarinines were extracted from minimal iron growth media as described previously (16), except that cultures were grown at an initial pH of 5.0 (17). N,N,N\(^{-}\)-triacytethylfusarinine C (TAFc) was selectively extracted from the aqueous mixture using benzene:chloroform (1:1, v/v). Alternatively, ferric TAFc was isolated by selective extraction from mixtures of ferric-N\(^{-}\)-acytethylfusarinines, using n-butyl alcohol (18), and the iron was removed with 8-hydroxyquinoline (19). The TAFc was lyophilized and sealed in glass under partial vacuum for storage. The purity of the TAFc was determined as the neutral species on two-dimensional paper electrophoresis as follows: 1) pH 4.9, pyridine:acetic acid:water (16:10:960, v/v) (20), and 2) pH 1.9, 4% formic acid buffer. As further evidence of its purity, a sealed sample of TAFc was hydrolyzed in 1 M NH\(_4\)OH for 72 h at 30 °C, and ammonium was removed by evaporation to dryness. Upon pH 4.9 electrophoresis, the hydrolysate was found to contain the three anions corresponding to the expected N\(^{-}\)-acytethylfusarinines. Ferrichrome and rhodotorulic acid were purchased from Sigma. Ferrichrome A was isolated from cultures of Ustilago sphaerogena (21) and recrystallized from water:methanol (99:1, v/v). The purity of ferrichrome and ferrichrome A were confirmed by electrophoresis.

Plate Assays and Uptake Assays—Plate assays for FC, FC-A, TAFc, and RA were performed as described (7) using 10 μM of the desferri form of FC and TAFc, 10 μM ferric FC-A, and 10 or 100 μM desferri-RA. Uptake assays were performed as described (22) in multiscreen filtration plates (Millipore) using 1 μM each of \(^{55}\)Fe(III) and the desferri form of either FC or TAFc. Kinetic assays were performed identically to the uptake assays, using a 60-min incubation, except that the concentration of \(^{55}\)Fe(III)-siderophore complex varied from 0.2 to 50 μM. Kinetic data were analyzed using EnzymeKinetica (Trinity Software).

Immunofluorescence, Protease Treatment, and Western Blotting—Immunofluorescence was performed as described (9) with the following modifications. Cells carrying pMET-Arn13MyC were transferred to methionine-free SD medium for 2 h prior to fixation, and affinity-purified monoclonal antibody 9E10 at 1:500 was used as the primary antibody. Protease treatment and Western blotting were performed as described (7).

**RESULTS**

The Role of ARN1, ARN3, and FET3 in Ferrichrome-mediated Growth and Iron Uptake—S. cerevisiae has been reported to exhibit uptake of iron bound to ferrirocinc, a hydroxamid siderophore of the ferrichrome group (6). We tested whether FC or FC-A could function as an iron source in budding yeast and whether the high-affinity Fe(II) transport (FET3-dependent) system or the ARN-dependent system was required (Fig. 1). Both wild type and arn1Δ strains grew slowly on iron-limited media, whereas fet3Δ strains did not grow (Fig. 1A). When FC was added to iron-limited media both arn1Δ and fet3Δ strains grew well, indicating that S. cerevisiae can utilize FC as an iron source. Deletion of all four ARN genes in a fet3Δ strain resulted in no detectable growth when FC was the iron source. This result indicated that budding yeast required either an ARN-dependent system or a FET3-dependent system for the uptake of FC-iron. To determine which of the ARN transporters was required for utilization of FC-iron, we examined the capacity of FC to support the growth of fet3Δ strains also deleted for each of the ARN genes, singly and in pairs (Fig. 1B). Deletion of any single ARN gene had no effect on growth, but deletion of both ARN1 and ARN3 resulted in no detectable growth on FC. Identical results were obtained when FC-A was substituted for FC (data not shown), indicating that both Arn1p and Arn3p can facilitate the uptake of FC- and FC-A-iron as well. These data do not, however, indicate the relative rates of uptake of FC and FC-A through Arn1p and Arn3p. We measured the rate of FC-\(^{55}\)Fe uptake in strains deleted for FET3 and the ARN genes, singly and in pairs (Fig. 2A). Deletion of ARN1 had minimal effect, whereas deletion of ARN3 resulted in a 66% decrease in the accumulation of FC-iron. Deletion of both ARN1 and ARN3 resulted in a decrease in FC-iron uptake to undetectable levels. The kinetics of FC-iron uptake through Arn3p and Arn1p were determined individually by measuring the rate of uptake of FC-iron in the presence of increasing concentrations of substrate. These measurements were performed in an ARN3+arn1Δ arn2Δ arn4Δ fet3Δ strain (Fig. 2, B and C) and in an ARN1+arn2Δ arn3Δ arn4Δ fet3Δ strain.
Iron-poor media (Fig. 3, A). Again, FET3+ strains grew slowly, and FET3-deleted strains exhibited no growth on iron-poor media. When TAFC was present, both the fet3Δ strain and the arn1-4Δ strain grew well, whereas the fet3Δ arn1-4Δ strain exhibited no growth. These data indicated that, as was the case for FC, FC-A, and FOB, TAFC can supply iron to budding yeast through either a FET3-dependent or ARN-dependent system. In Fig. 3B, we tested the capacity of the individual ARN genes to confer growth to strains lacking high-affinity Fe(II) transport (fet3Δ) when TAFC was the iron source. When ARN2 was deleted, yeast grew very slowly and exhibited a markedly reduced colony size. This also occurred when ARN2 was deleted in combination with either ARN1, ARN3, or ARN4, suggesting that ARN2 facilitates the uptake of TAFC-iron but that other ARN genes also have the capacity to facilitate the uptake of TAFC-iron. Only when ARN2 was deleted in combination with ARN1 and ARN3 did yeast exhibit no growth on TAFC, indicating that Arn1p and Arn3p can take up TAFC-iron, albeit at a reduced rate that is limiting for growth.

Yeast grew more slowly on TAFC as an iron source than with FC as an iron source. To determine the biochemical basis for this observation, we measured the rate of uptake of TAFC-iron in strains bearing deletions of the ARN genes that were also lacking high-affinity Fe(II) uptake (fet3Δ) (Fig. 4A). In an ARN+ strain, the rate of uptake of TAFC-iron was much lower than that of FC-iron (0.09 versus 4.1 pmol/10^6 cells/h). When ARN2 was deleted, uptake of TAFC-iron was only slightly detectable above background. TAFC-iron uptake through Arn1p or Arn3p could not be accurately measured, again indicating that TAFC-iron is taken up predominantly through Arn2p. We determined the kinetics of TAFC-iron uptake in an ARN2-arn1Δ arn3Δ arn4Δ fet3Δ strain and found that whereas the affinity of Arn2p for TAFC-iron was similar to the affinities of Arn1p and Arn3p for FC (K_m = 1.6 × 10^{-6} versus 0.9 × 10^{-6} and 2.3 × 10^{-6} M, respectively), the capacity of Arn2p was lower than that of Arn1p (V_max = 0.27 versus 0.7 pmol/10^6 cells/h) and much lower than that of Arn3p (V_max = 6.8 pmol/10^6 cells/h). The net result of these kinetic differences is that FC-iron is much more efficiently taken up by yeast than is TAFC-iron.

Utilization of Rhodotorulic Acid—Budding yeast have been reported to take up iron bound to the peptide-linked, dihydroxamate siderophore RA through a reductive mechanism (24). That is the Fe(III) that is bound to RA is first reduced to Fe(II), then released from the RA, and finally taken up through a Fe(II)-specific transport system. We tested the genetic requirements for utilization of RA-iron by plating yeast on iron-poor media containing no RA, 10 \mu M RA, or 100 \mu M RA (Fig. 5). Wild type yeast and a strain bearing deletions of all four ARN genes demonstrated a only a very slight increase in colony size when grown on iron-poor media supplemented with RA. In contrast, strains bearing deletions of FET3 exhibited no growth on 10 or 100 \mu M RA. By themselves, these data did not confirm that RA was utilized by yeast nor did they indicate a requirement for the FET3-dependent, high-affinity Fe(II) transport system. However, an FET3+ strain bearing deletions of the surface reductases encoded by FRE1 and FRE2 failed to grow on iron-poor media and yet grew well in the presence of 100 \mu M RA. Taken together, these data confirm that RA-bound iron can be utilized by budding yeast and that the high-affinity Fe(II) transport system is required, but Fre1p and Fre2p are not the reductases involved in the release of iron from RA.

Localization of Arn1p to Intracellular Vesicles—Although the high-affinity Fe(II) transport system is located in the plasma

C. C. Philpott and C.-W. Yun, unpublished observations.
membrane, Arn3p is primarily located in intracellular vesicles (7, 9, 25, 26). We performed indirect immunofluorescence to determine whether other ARN family members were located in intracellular vesicles. Arn1p was detected in yeast carrying an episomal copy of Myc epitope-tagged ARN1 under the control of a methionine-regulatable promoter. We confirmed that the Myc-tagged version of Arn1p was functional by expressing Arn1p-Myc from a high copy number plasmid and observing complementation of the growth defect on FC of an arn1Δ arn3Δ fet3Δ strain. Expression levels of Arn1p-Myc from the MET25 promoter were similar to the endogenous levels of expression of Arn1p-HA and ARN3p-HA in strains in which the chromosomal copy of ARN1 or ARN3 carried a triple copy of the hemagglutinin (HA) epitope at the carboxyl terminus. Arn1p-Myc was detected in multiple, small intracellular vesicles (Fig. 6, A and B); this pattern of fluorescence was identical to that seen for Arn3p-HA (Fig. 6, C and D). In contrast, a Myc epitope-tagged version of Ftr1p, a component of the high-affinity Fe(II) transport system, was detected primarily on the plasma membrane (Fig. 6, E and F).

Indirect immunofluorescence indicated that Arn1p was primarily located intracellularly; to determine whether a small fraction of Arn1p was expressed on the plasma membrane, we tested whether Arn1p-HA was sensitive to digestion by external proteases. Intact cells expressing Arn1p-HA or Fet3p-HA were treated with Pronase before being lysed and analyzed by Western blotting (Fig. 6). The amount of intact Fet3p-HA was greatly reduced by protease treatment (lanes 9 and 10), as would be expected for a protein on the plasma membrane. In contrast, protease treatment did not change the amount of intact Arn1p-HA present in cells (lanes 5 and 6), although Arn1p was completely digested in the presence of protease and detergent (lanes 7 and 8). These data support the conclusion that, although the high-affinity Fe(II) transport system is expressed on the plasma membrane, the ARN transporters are mainly expressed in an intracellular organelle.

DISCUSSION

Uptake of siderophore-bound iron in budding yeast was accomplished through two genetically separable systems as follows: one that requires an intact high-affinity Fe(II) transport system and one that requires transporters of the ARN family. This was initially demonstrated for the hydroxamate siderophore FOB; here we have shown that these systems also operate to take up iron from FC, FC-A, and TAFC. Substrates have been identified for three of the four ARN family members as follows: Arn1p transported FC, FC-A, and to a minor extent, TAFC; Arn2p transported TAFC; and Arn3p transported FOB, FC, FC-A, and to a minor extent, TAFC. Interestingly, Arn1p and Arn3p exhibit the greatest similarity in terms of substrate specificity, although Arn1p and Arn2p exhibit the greatest sequence homology of these transporters (53% identity at the amino acid level) (7).

Two of the Arn transporters, Arn1p and Arn3p, have been localized to intracellular vesicles. These vesicles comigrate on density gradients with those containing the endosomal protein Pep12p (7), suggesting that siderophore uptake could occur in the compartment variously referred to as the late endosome/pre-vacuole/post-Golgi (27, 28). Why siderophore transport might occur intracellularly is not clear, but the mechanism by which transport is energized may be involved. The members of the major facilitator superfamily of transporters do not rely on density gradients with those containing the endosomal protein Pep12p (7), suggesting that siderophore uptake could occur in the compartment variously referred to as the late endosome/pre-vacuole/post-Golgi (27, 28). Why siderophore transport might occur intracellularly is not clear, but the mechanism by which transport is energized may be involved. The members of the major facilitator superfamily of transporters do not rely on hydrolysis of ATP for energy coupling; rather, transport is driven by chemiosmotic gradients (29). The late endosome is

3 C.-W. Yun, unpublished observations.

4 J. Kaplan, personal communication.
acidic, with a proton gradient generated by components of the vacuolar-ATPase (30). This proton gradient may provide the energy required for siderophore uptake through the Arn\text{p} transporters.

In Gram-negative bacteria, the outer membrane siderophore receptors are highly specific and can discriminate between the different ferric hydroxamates (2). Our data indicated that the hydroxamate transport systems of budding yeast exhibited far less specificity. One possible explanation for this apparent lack of specificity is that Arn\text{p}1, Arn\text{p}2, and Arn\text{p}3 specifically recognize the structure coordinating the bound iron. Siderophores form six-coordinate, octahedral complexes with ferric iron (23).

In siderophores of the hydroxamate class, each hydroxamate group forms a bidentate ligand; a single siderophore with three hydroxamate groups forms a hexadentate ligand, which satisfies the requirement for a six-coordinate complex. This hexadentate, trihydroxamate-F\text{e}^(III) structure is shared by FC, FC-A, FOB, and TAFC. Arn\text{p}1 and Arn\text{p}3 were shown to transport FC and FC-A, which are cyclic hexapeptides containing three N\text{d}-acylated hydroxy-\text{L}-ornithine residues plus either three glycines (FC) or one glycine and two serines (FC-A), the latter also containing the acidic trans-\text{b}-methylglutaconyl group on the three \text{d}-N-acyl residues (3, 23). Although FC and FC-A have significant structural differences, they are more similar to each other than they are to a third transport substrate of Arn\text{p}3, FOB. FOB consists of a linear chain of three peptide-linked hydroxamic acids, terminating in a free amino group (31, 32). TAFC is a cyclic, hexadentate, trihydroxamate, but differs from FC, FC-A, and FOB in that the hydroxamic acid residues are joined by ester linkages (16). Arn\text{p}2 was the primary transporter for TAFC, whereas FC, FC-A, and FOB were not substrates for Arn\text{p}2. If Arn\text{p}1 and Arn\text{p}3 specifically recognized the peptide-linked, trihydroxamate-ferric iron complex, then the coprogens, another peptide-linked, trihydroxamate class of siderophores (23, 33), would be predicted to be transport substrates for Arn\text{p}1 and/or Arn\text{p}3.

Arn\text{p}4, which exhibits the least sequence similarity to the other ARN genes, does not appear to have a role in the transport of the hydroxamate class of siderophores. Recent work has indicated that Arn\text{p}4 transports ferric enterobactin, a hexadentate, ester-linked siderophore of the catecholate class that is secreted by many species of bacteria.\textsuperscript{5} RA, which is not a

\textsuperscript{5} G. Winkelmann, personal communication.
A and immunofluorescence microscopy was performed on cells expressing through the required for the uptake of FC, FC-A, and TAFC-bound iron were not required for uptake of RA-bound iron, neither are they shown that the surface reductases encoded by requires reduction of the iron-siderophore chelate. We have rous iron transport system. Implicit in this observation is a the siderophores described here through the high-affinity fer-
and forms an Fe2RA3 complex with Fe(III) (34, 35).

substrate for the Arn transporters, differs from the other siderophores in that it is a tetradentate, dihydroxamate ligand
and is a tetradentate, dihydroxamate ligand substrate for the Arn transporters, differs from the other sid-

FIG. 6. Localization of Arn1p to intracellular vesicles. Indirect immunofluorescence microscopy was performed on cells expressing Arn1p-Myc (A and B), Arn3p-HA (C and D), Ftr1p-Myc (E and F), or the untagged parent strain (G and H). Monoclonal 9E10 (A, E, and G) or HA.11 (C) was the primary antibody, and Cy-3-conjugated donkey anti-mouse was the secondary antibody. Panels are in pairs as follows: fluorescence on the left, and Nomarsky on the right. I, resistance of Arn1p to extracellular proteases. Congenic strains expressing wild type levels of Arn1p-HA or overexpressing Fet3p-HA and the untagged parent strain were grown to mid-log phase in YPD media. Cells were then incubated in buffer alone (C), with Pronase (P), with Triton (T), or Pronase and Triton (P+T) on ice prior to lysis. Lysates were analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting with HA.11 antibody. Intact Arn1p-HA (small arrowhead) and intact Fet3p-HA (large arrowhead) are indicated. Molecular masses in kDa are indicated on the left.

Future experimentation will reveal whether any of these reductases are involved in siderophore-mediated iron uptake.

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Note Added in Proof—Winkelmann and co-workers (Heyman, P., Ernst, J. F., and Winkelmann, G. (1999) Biometals 12, 301–306) have recently reported the identification of ARN2/TAF1 as a triacetylfusari-
nine C transporter.

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Siderophore-Iron Uptake in *Saccharomyces cerevisiae*: IDENTIFICATION OF FERRICHROME AND FUSARININE TRANSPORTERS

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