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Three Cell Wall Mannoproteins Facilitate the Uptake of Iron in *Saccharomyces cerevisiae**

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Analysis of iron-regulated gene expression in *Saccharomyces cerevisiae* using cDNA microarrays has identified three putative cell wall proteins that are directly regulated by Aft1p, the major iron-dependent transcription factor in yeast. *FIT1*, *FIT2*, and *FIT3* (for facilitator of iron transport) were more highly expressed in strains grown in low concentrations of iron and in strains in which *AFT1–1^{up}*, a constitutively active allele of *AFT1*, was expressed. Northern blot analysis confirmed that *FIT1*, *FIT2*, and *FIT3* mRNA transcript levels were increased 60–230-fold in response to iron deprivation in an Aft1p-dependent manner. Fit1p was localized exclusively to the cell wall by indirect immunofluorescence. Deletion of the *FIT* genes, individually or in combination, resulted in diminished uptake of iron bound to the siderophores ferrioxamine B and ferrichrome, without diminishing the uptake of ferric iron salts, or the siderophores triacetylfusarinine C and enterobactin. *FIT*-deletion strains exhibited increased expression of Aft1p target genes as measured by a *FET3-lacZ* reporter gene or by Arn1p Western blotting, indicating that cells respond to the absence of *FIT* genes by up-regulating systems of iron uptake. Aft1p activation in *FIT*-deleted strains occurred when either ferrichrome or ferric salts were used as sources of iron during growth, suggesting that the *FIT* genes enhance uptake of iron from both sources. Enzymatic digestion of the cell wall resulted in the release of significant amounts of iron from cells, and the relative quantity of iron released was reduced in *FIT*-deletion strains. Fit1p, Fit2p, and Fit3p may function by increasing the amount of iron associated with the cell wall and periplasmic space.

Although iron is an essential nutrient for virtually every organism, it is also potentially toxic to cells; therefore, the uptake and utilization of iron is tightly regulated. Iron is abundant in the environment, but it is largely present as insoluble oxyhydroxides. Consequently, the bioavailability of iron is exceedingly low. The budding yeast *Saccharomyces cerevisiae* responds to iron deprivation by increasing the expression levels of genes involved in iron uptake. This response is transcriptionally mediated by the major iron-dependent transcription factor in yeast, Aft1p (1, 2). Targets of Aft1p include genes encoding components of the reductive system of iron uptake: the plasma membrane metalloreductases (*FRE1–3*) (3–8), the multicopper ferroxidase (*FET3*) (9–11), and the iron permease (*FTR1*) (12). This system of iron uptake is copper-dependent, and the Aft1p targets *ATX1* and *CCC2* encode proteins involved in the copper loading of Fet3p (13, 14). The transporters Arn1p, Arn2p, Arn3p, and Arn4p are components of the nonreductive, siderophore-iron uptake system, and the genes encoding these proteins are also transcriptionally activated by Aft1p (15–20). Iron salts and low affinity iron chelates (such as ferric citrate) are substrates for the reductive system of uptake and siderophore-iron complexes are substrates for both systems. Siderophores are low molecular weight compounds that can solubilize oxyhydroxides of iron by specifically binding ferric iron with very high affinity. These compounds are synthesized and secreted in the iron-free form by microorganisms, and then the ferric forms are taken up by specific transport systems. *S. cerevisiae* does not synthesize siderophores, but can take up iron bound to different types of siderophores secreted by other species (21, 22).

For iron-containing compounds to gain access to plasma membrane-based uptake systems, the compounds must first penetrate the cell wall and cross the periplasmic space. The yeast cell wall is composed of β-glucans, mannoproteins, and chitin (23–25). It functions in morphogenesis (growth in yeast forms *versus* filamentous forms), adherence (to solid supports), flocculation (adherence to other yeast), and mating. In the absence of the cell wall, spheroplasts rapidly burst as a result of osmotic pressure. The cell wall is a highly dynamic structure and undergoes remodeling during growth, bud emergence, and conjugation. Passage of higher molecular weight molecules is restricted by the cell wall. The porosity of the cell wall changes during different phases of growth, with the highest porosity occurring in exponentially growing cells. The composition of the cell wall mannoproteins changes when cells are grown under anaerobic conditions, reduced temperatures, or in unshaken cultures. One class of cell wall mannoprotein is covalently attached to β-1,6-glucan through a glycosylphosphatidylinositol (GPI)[1] anchor. Analysis of the open reading frames in the *S. cerevisiae* genome has identified numerous putative cell wall proteins, the functions of which remain unknown (26, 27).

We have used cDNA microarrays representing the open

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[1] The abbreviations used are: GPI, glycosylphosphatidylinositol; FOB, ferrioxamine B; FC, ferrichrome; TAFC, triacetylfusarinine C; ENT, enterobactin; BPS, bathophenanthroline disulfonate; HA, hemagglutinin; 5-FOA, 5-fluoroorotic acid.

reading frames of the *S. cerevisiae* genome to identify genes that are transcriptionally activated during iron deprivation and are targets of Aft1p (20). Here we report that three proteins that are transcriptionally regulated by Aft1p are components of the yeast cell wall and function to facilitate the uptake of iron. These genes, designated *FIT1*, *FIT2*, and *FIT3* (for facilitator of iron transport), are also transcriptionally activated in a strain deleted for *YFH1*, the yeast frataxin homologue (28). Strains deleted for *YFH1* exhibit mitochondrial iron accumulation and activation of other Aft1p targets. The *FIT* genes have been suggested to play a role in the uptake or utilization of siderophore-iron. We report that deletion of the *FIT* genes resulted in defects in siderophore-iron uptake, despite increased levels of expression of systems of iron uptake. Growth of *FIT*-deletion strains in media containing either iron salts or iron-siderophore complexes resulted in activation of Aft1p, and the amount of iron associated with the cell wall and periplasmic space was reduced in *FIT*-deletion strains.

EXPERIMENTAL PROCEDURES

Yeast Strains, Media, and Plasmids—Strains were constructed in *YPH499 (MAT**a** ura3–52 lys2–801(amber) ade2–101(ochre) trp1–63 his3–200 leu2–1*) (Yeast Genetic Stock Center, Berkeley, CA). PCR-mediated gene disruption was used to generate deletions of the *FIT* genes (29). The *fit1*Δ*fit2*Δ*fit3*Δ strain was constructed by serial deletion of the *FIT* genes in the following manner. *FIT3* was directly deleted in YPH499 by amplifying the *HISG-URA3-HISG* cassette from the plasmid pMPY-ZAP using the following primers: 5′-CTAAAATGAAATTCT CTTCCGCTTTGGTTCTATCTGCTGTTGCCGCTACCTCACTATAGGG CGAATTGG-3′ and 5′-GATTATTCACATATCATTTACAATAACATGA CGGCAGCAAGGGCAGCACCCTAAAGGGAACAAAAGCTGG-3′. The resulting PCR product was used to transform YPH499, and transformants were screened by PCR. Clones containing the correctly targeted deletion cassette were plated on 5-fluoroorotic acid (5-FOA)-containing plates and 5-FOA-resistant clones selected. For deletion of *FIT2*, the *fit3*Δ strain was transformed with a PCR product amplified from genomic DNA isolated from the strain RG1679 (*MAT**a** ura3 met-15 his3–1 leu2–1 yor382cD*::*KANR*) (Research Genetics) using the following primers 5′-CGAATCTAACTGGGTGCAG-3′ and 5′-CTGCTACATT-GAAGTCACTGAG-3′. Geneticin-resistant clones were selected on YPD plates containing 80 mg/liter G418 (Life Technologies, Inc.) and the transformants screened by PCR to isolate the *fit2*Δ*fit3*Δ strain. For *FIT1*, the following primers were used to amplify the *HISG-URA3-HISG* cassette: 5′-ATTGTCTTCCGCTTTTGTATTATCTGCTATCACC GTTGCCGCTTTAGGCGAGGGAACAAA-3′ and 5′-AGACCCGTACCA GCGGCGATAGAATCAGCCGCACCCGTGTAATTCACGATTTCTATA GGG-3′. *FIT1* was first deleted by transforming BY4741 (*MAT**a** ura3 met-15 his3–1 leu2–1*) (Research Genetics) with the *HISG-URA3-HISG* cassette, and the resulting transformants were screened by PCR. Genomic DNA from the resulting *fit1*Δ strain was used to amplify the locus containing the deletion cassette using the following primers: 5′-GCGCTGTCGCACATTTGGC-3′ and 5′-GGTTGATTCTGCGCATCTG-3′. The *fit2*Δ*fit3*Δ strain was transformed with the resulting PCR product, deletions were confirmed by PCR, and 5-FOA-resistant clones were selected. Strains containing deletions of individual and pairs of *FIT* genes were generated by mating the *fit1*Δ*fit2*Δ*fit3*Δ strain with YPH500 *(MAT*α *ura3–52 lys2–801(amber) ade2–101(ochre) trp1–63 his3–200 leu2–1*), sporulating the diploid, and analyzing random spore clones by PCR. The *HA-FIT1* strain, which expresses a triple copy of the hemagglutinin (HA) epitope inserted into the 25th amino acid position of FIT1, just distal to the signal peptide, was constructed by PCR epitope tagging as described using the plasmid pMPY-3xHA (30) and the following primers: 5′-TCTGCTATCACCGTTGCCGCTTTAGGCGAAAGTATTAC-TACTACGATAAGGGAACAAAAGCTGGAG-3′ and 5′-AGCATCTTGA-GTGACAGTCTTGGTGTAAACATGCCCATTTTTGGTAGCAGTCTCT-AGGGCGAATTGGGT-3′. Integration of the HA epitope was confirmed by PCR and by Western blotting. The strain FET3-HA FTR1-myc, which constitutively overexpresses integrated copies of HA-tagged FET3 and Myc-tagged FTR1 under the control of the phosphoglycerate kinase promoter, was constructed as described (31). Rich medium (YPD) and synthetic defined medium (SD) were prepared as described (32). Defined iron media were prepared as described (33).

Microarrays, Northern, and Sequence Analyses—Microarrays and Northern analyses were performed as described (20). Probes for each of the *FIT* genes were prepared from PCR products corresponding to the open reading frames. Primers designed to amplify each open reading frame were obtained from Research Genetics. The 700 nucleotides located upstream of the *FIT* open reading frames were searched for *AFT1* consensus binding sites using the oligo-analysis program of the Regulatory Sequence Analysis Tools web site (www.ucmb.ulb.ac.be/bioinformatics/rsa-tools). Identified sequences were aligned using MacVector (Oxford Molecular Group). Signal sequences and GPI attachment sequences of the *FIT* genes have been identified previously (27).

Immunofluorescence and Western Blotting—Strains were grown to mid-log phase in defined iron media containing 10 μM ferrous ammonium sulfate to induce the expression of Fit1p. Cells were prepared for immunofluorescence microscopy as described (20). To detect cell wall proteins, the following modifications were performed. Fixed, washed cells were incubated for 10 min in PBS with 10 mM glycine and 0.1% BSA, then incubated with monoclonal antibody HA.11 (Covance) at 1:300 and Cy-3-conjugated donkey anti-mouse antibody (Jackson Immunoresearch) at 1:500 (in the same mixture) for 30 min. Cells were washed three times (10 min each) in PBS after incubation with antibody. The cell pellet was resuspended in 10 μl of Fluoromount G (Southern Biotechnology) and examined by fluorescence microscopy. Western blotting was performed using a 1:5000 dilution of HA.11 as the primary antibody followed by 1:5000 dilution of horseradish peroxidase-conjugated sheep anti-mouse antibody (Amersham Biosciences Inc.). Antibody was detected using enhanced chemiluminescence.

Iron Uptake, β-Galactosidase, and Cell Wall Release Assays—Iron uptake assays were performed as described (20, 34) with the following modifications: Cells were grown in YPD medium overnight and then incubated in YPD supplemented with 0.1 mM bathophenanthroline disulfonate (BPS) and 1 μM $CuSO_4$ (for measurement of ferrous iron uptake) or YPD supplemented with 1 mM BPS (for measurement of siderophore-iron uptake) for 4 h. Supplements were included in the uptake assay buffer and uptake allowed to proceed for 20 min prior to washing. To monitor the expression of *AFT1*-dependent genes, cells transformed with a 2-μm *FET3-lacZ* reporter plasmid (kind gift of D. Winge) were grown to mid-log phase in defined iron media containing 30 μM ferrous ammonium sulfate. β-Galactosidase assays were performed as described (35). To measure the release of iron from cell walls, washed cells were incubated for 1 h with 1 μM ^{55}Fe-ferrichrome in 0.5 M glucose and 50 mM sodium citrate buffer at pH 6.6. Iron-loaded cells were washed twice with 50 mM sodium citrate buffer and once with spheroplasting buffer (1.2 M sorbitol, 20 mM Tris-HCl, pH 7.4). Cells were incubated with different concentrations of Zymolyase-100T (Sigma) in spheroplasting buffer for 10 min at 30 °C. The supernatants and cell pellets were collected after low speed centrifugation in a microcentrifuge (3000 rpm, 4 min), and ^{55}Fe was measured by scintillation counting.

RESULTS

Identification of FIT Genes as Aft1p Targets—cDNA microarrays representing the open reading frames of *S. cerevisiae* were used to identify genes that were transcriptionally activated by growth in iron-poor media and genes that were transcriptionally activated in a strain bearing a constitutively active allele of *AFT1*, *AFT1–1^{up}*. Strains bearing the *AFT1–1^{up}* allele exhibit elevated levels of expression of Aft1p-regulated genes, even under conditions where Aft1p-regulated genes are normally not expressed or expressed at very low levels (1). Three homologous genes that demonstrated elevated mRNA levels in microarrays from iron-poor cultures and the *AFT1–1^{up}* strain were selected for further analysis (Fig. 1*A*). These genes, *YDR534C* (*FIT1*), *YOR382W* (*FIT2*), and *YOR383C* (*FIT3*), exhibited very high levels of induction in microarrays and frequently were the most highly induced genes in the array. Each of these genes contained multiple Aft1p consensus binding sites in the DNA sequences upstream from the open reading frames (Fig. 1*B*). Previously identified Aft1p target genes have exhibited a single consensus binding site in the upstream sequences; the presence of multiple sites in the *FIT* genes could contribute to the extremely high mRNA levels observed in the arrays and in Northern blots (Fig. 1*C*). Northern blot analysis of *FIT1*, *FIT2*, and *FIT3* was performed, and each *FIT* gene exhibited a similar pattern of expression. In a wild-type genetic background, *FIT1*, *FIT2*, and *FIT3* mRNA transcripts were induced 60-, 140-, and

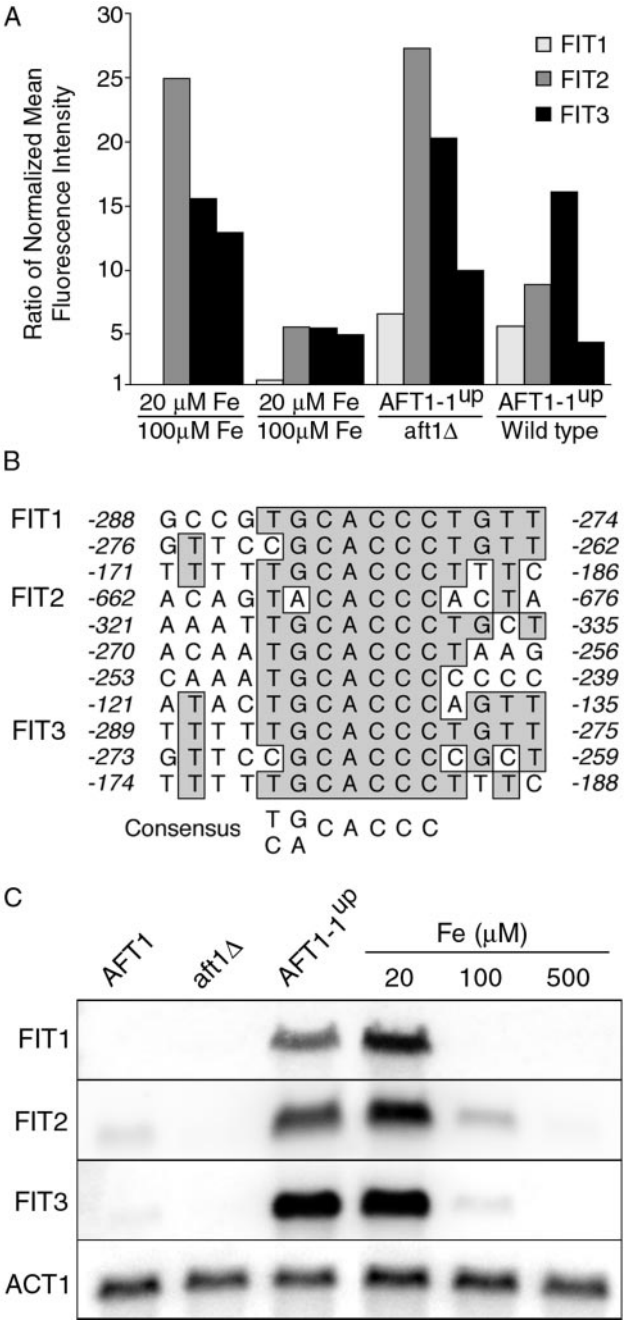

FIG. 1. **Identification of FIT1, FIT2, and FIT3.** *A*, relative mRNA levels of *FIT1*, *FIT2*, and *FIT3* in cDNA microarrays. Data from individual arrays that were performed using the indicated pairs of growth conditions or strains are presented. *Bar height* reflects the ratio of the mRNA levels for each gene. Data for *FIT1* were not obtained in the first array. *FIT3* was represented twice on each array. *B*, AFT1 consensus binding sites in upstream sequences of *FIT* genes. The sequences upstream of the *FIT* open reading frames were searched for AFT1 consensus binding sites. *Boxed* and *shaded letters* indicate identity. *C*, iron- and *AFT1*-dependent expression of *FIT* genes. Total RNA was isolated from congenic strains bearing wild type (AFT1), deleted (aft1Δ), or constitutively active (AFT1–1^{up}) alleles of AFT1 after growth in SD complete medium. Total RNA was also isolated from YPH499 after growth in defined iron medium containing the indicated concentrations of ferrous iron. Northern blot analysis was performed with sequential hybridization of the indicated probes.

230-fold, respectively, by growth in iron-poor media (20 μM iron) as compared with iron-rich media (500 μM iron). The role of Aft1p in the iron-dependent regulation of the *FIT* genes was examined in strains expressing wild type Aft1p, deleted for Aft1p, or expressing Aft1–1^{up}p. Strains were grown in iron-replete synthetic media in which Aft1p-dependent gene expression is normally low. Expression of wild-type Aft1p was associated with very low levels of *FIT* transcripts, and deletion of Aft1p was associated with virtually undetectable levels of *FIT* transcripts. In contrast, Aft1–1^{up}p expression was associated with very high levels of *FIT* transcripts, suggesting that *FIT1*, *FIT2*, and *FIT3* are directly regulated by Aft1p.

Sequence Analysis of FIT Genes—The *FIT* genes are located in the subtelomeric regions of chromosomes IV and XV, and *FIT2* and *FIT3* are part of an unusual cluster of Aft1p-regulated genes (Fig. 2*A*). *FIT2* and *FIT3* occupy adjacent sites and are encoded on opposite strands of the chromosome. They are flanked on either side by *FRE3* and *FRE5*, two members of the *FRE* family of surface metalloreductases, a group of genes that are also regulated by Aft1p and involved in the reductive uptake of iron. The deduced amino acid sequence of *FIT1*, *FIT2*, and *FIT3* (Fig. 2, *B*, *C*, and *D*, respectively) suggested that the proteins were components of the yeast cell wall. Each contains a putative signal sequence at the amino terminus, which targets the proteins for secretion, and a GPI signal for attachment of a GPI anchor. GPI-anchored proteins in the plasma membrane are incorporated into the cell wall by cleavage of the GPI moiety and attachment to β-1,6-glucan (24, 25). The C-terminal sequences of *FIT1*, *FIT2*, and *FIT3*, when fused to a reporter protein, have been demonstrated to direct the reporter protein to the yeast cell wall (26, 27). Furthermore, a version of Fit2p containing the HA epitope distal to the signal sequence was detected in extracts of the cell wall. These sequences, coupled with experimental data, strongly suggest that each of the *FIT* genes is a component of the yeast cell wall.

The cell wall mannoproteins of *S. cerevisiae* are similar in that they tend to be serine- and threonine-rich, be heavily glycosylated, and contain repeated sequences (36). The predicted sequences of the mature *FIT* proteins contain 41–50% serine and threonine residues. *FIT1* and *FIT3* are 61% identical at the amino acid level and share a unique 60-amino acid sequence that is present, as a whole or in part, in four copies in *FIT1* and two copies in *FIT3*. *FIT2* contains two copies of the central portion of this sequence. Additionally, *FIT1* and *FIT3* contain a shorter, 6-amino acid sequence, SA(A/V)ETS, that is present in an array of 11 tandem repeats in *FIT1* and 5 overlapping repeats in *FIT3*.

Cell Wall Localization of Fit1p—To confirm that the Fit proteins were cell wall mannoproteins, we constructed a strain in which the chromosomal copy of *FIT1* contained a triple copy of the HA epitope, cloned in-frame, just distal to the signal peptide. Using this strain, we performed indirect immunofluorescence on cells grown in media containing low amounts of iron to induce the expression of HA-Fit1p (Fig. 3). The fluorescence signal from cells that were fixed, but not subjected to enzymatic digestion of the cell wall or permeabilization, is shown in Fig. 3*A*. HA-Fit1p is clearly located in the periphery of the cell, including the nascent septum in the neck of a budding cell. In contrast, when the cell wall of HA-Fit1p cells was enzymatically removed prior to fluorescent antibody labeling, the fluorescence signal was very low and not detected on the periphery of the spheroplast (Fig. 3*C*). Minimal fluorescence signal was detected in cells that express an HA-tagged version of Fet3p, a protein located in the plasma membrane, when the cell wall was intact (Fig. 3*E*). However, spheroplasted cells expressing Fet3p-HA exhibited a clear plasma membrane pattern of localization (Fig. 3*G*). These data indicated that Fit1p was an integral component of the cell wall, that the majority of the cell-associated Fit1p was incorporated into the cell wall, and that very little Fit1p was present in the plasma membrane.

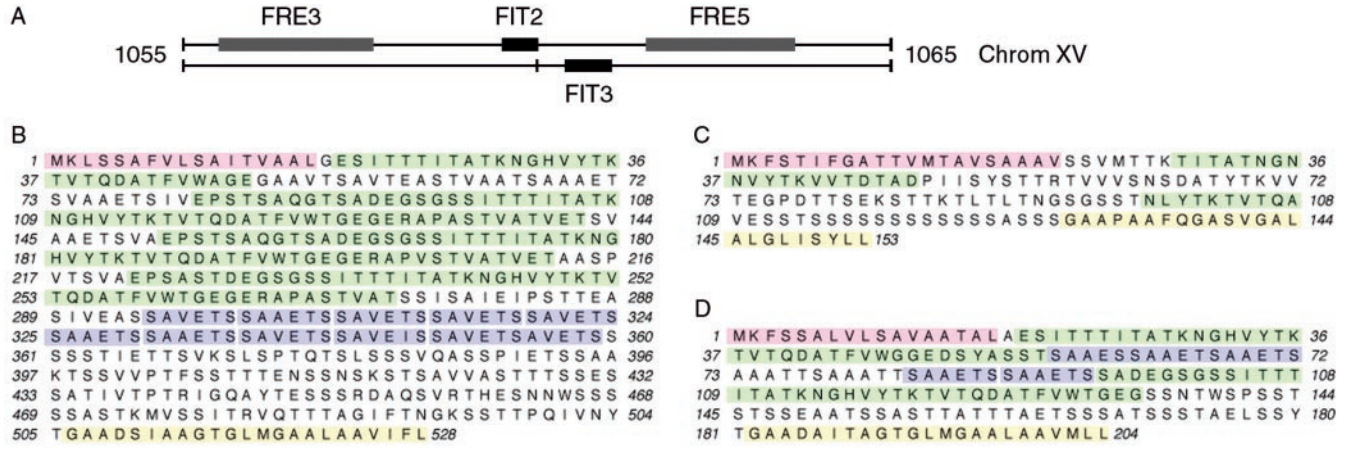

FIG. 2. **Locus and amino acid analysis of *FIT1*, *FIT2*, and *FIT3*.** *A*, cluster of *AFT1* target genes in subtelomeric region of chromosome XV. *B*, analysis of amino acid sequences of *FIT* genes. The deduced amino acid sequences of Fit1p (*B*), Fit2p (*C*), and Fit3p (*D*) are shown. Putative signal sequences (*pink*) and GPI attachment sequences (*yellow*) are highlighted. Long (*green*) and short (*purple*) repeated sequences are also highlighted.

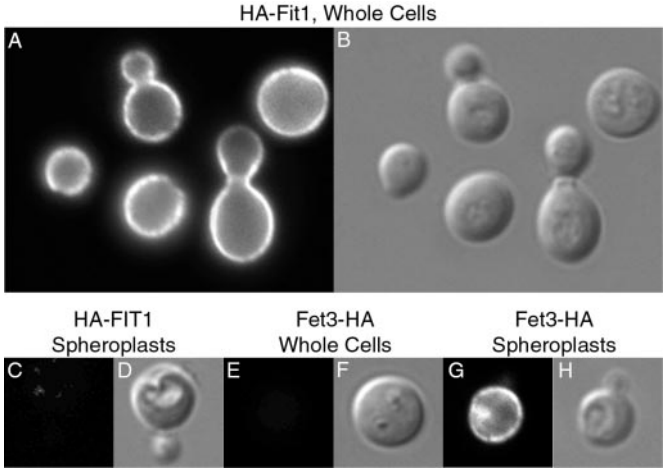

FIG. 3. **Localization of Fit1p to cell wall.** Indirect immunofluorescence microscopy was performed on strains expressing HA-Fit1p (*A–D*) and Fet3p-HA (*E–H*). Strains were grown to mid-log phase in media containing limiting amounts of iron to induce *FIT1* expression. Fixed cells were either directly incubated with primary antibody to detect cell wall protein (*A*, *B*, *E*, and *F*) or were treated with cell wall lytic enzymes for 30 min prior to incubation with primary antibody to detect plasma membrane and intracellular protein (*C*, *D*, *G*, and *H*). Cells were imaged in pairs with fluorescence (*A*, *C*, *E*, and *G*) and differential interference contrast (*B*, *D*, *F*, and *H*) microscopy. HA-11 was used as primary antibody; Cy3-conjugated donkey anti-mouse was the secondary antibody.

Uptake of Iron in FIT-deletion Mutants—The Aft1p-regulated genes that have been functionally characterized have a role in either the reductive or nonreductive uptake of iron. Deletion of the *FIT* genes has been reported to result in subtle defects in growth on media containing the siderophore desferrioxamine B (28). We quantitatively examined the role of the *FIT* genes in the reductive and nonreductive uptake of iron by measuring ferrous iron uptake and ferrioxamine B (FOB)-iron uptake in congenic strains bearing deletions of the *FIT* genes. Cells were grown to mid-log phase in rich medium containing either 100 μM BPS and 1 μM $CuSO_4$ or 1 mM BPS before measurement of iron uptake. BPS is a strong chelator of ferrous iron, but also a weak chelator of copper. Cells grown in 1 mM BPS exhibit induced expression of iron uptake systems, but low levels of ferrous iron uptake because of inactivation of the copper-dependent Fet3p. Inclusion of BPS in the siderophore-iron assay inhibits reductive uptake of iron by chelation of reduced iron and by inactivation of Fet3p without affecting Arnp-dependent siderophore-iron uptake. Using these modified growth and assay conditions, we found that deletion of the *FIT* genes, individually and in combination, did not result in significant defects in high affinity ferrous iron uptake (Fig. 4*A*). However, deletion of the *FIT* genes, individually and in pairs, resulted in an approximately 2-fold reduction in Arnp-dependent FOB-iron uptake (Fig. 4*B*). Surprisingly, deletion of all three *FIT* genes did not result in a defect in FOB-iron uptake. These results were consistently observed and repeated in numerous experiments.

FOB is a siderophore of the hydroxamate class and shares structural features with other hydroxamate siderophores, such as ferrichrome (FC) and triacetylfusarinine C (TAFC) (37). Enterobactin (ENT) is a siderophore of the catecholate class and differs from the hydroxamates both in the coordination site for iron and in that the ferric form carries a −3 charge, whereas the hydroxamates have a neutral charge (38). We questioned whether deletion of the *FIT* genes would impact upon the uptake of iron from other siderophore sources. Uptake of FC-iron, ENT-iron, and TAFC-iron was measured and the results are presented in Fig. 5. Deletion of the *FIT* genes resulted in diminished uptake of FC in a pattern similar to that of FOB (Fig. 5*A*), although, as was the case with FOB, deletion of all three *FIT* genes resulted in FC-iron uptake activity that was similar to that of the *FIT*+ parent strain. In contrast, deletion of the *FIT* genes had no effect on uptake of ENT-iron or TAFC-iron (Fig. 5, *B* and *C*).

Because yeast homeostatically regulate the expression of iron uptake systems through the transcription factor Aft1p, the *FIT*-deletion mutants would be expected to respond to reduced levels of iron uptake by increasing the expression of proteins involved in iron uptake. The preceding experiments were conducted on cells that were grown in media primarily containing ferric iron as simple salts or in complexes with organic acids, but not bound to siderophore. We questioned whether *FIT*-deletion mutants, when grown in media primarily containing siderophore-iron, would adjust the expression of siderophore-iron uptake systems to compensate for the loss of Fit protein activity. When *FIT*+ and *FIT*-deletion mutant strains were grown in synthetic media containing FC, there was very little difference between the strains when the uptake of FC-iron was measured (Fig. 6*A*). These results suggested that the Fit proteins were not critical structural components of the iron uptake systems, but that they might have an indirect impact on the efficiency of iron uptake.

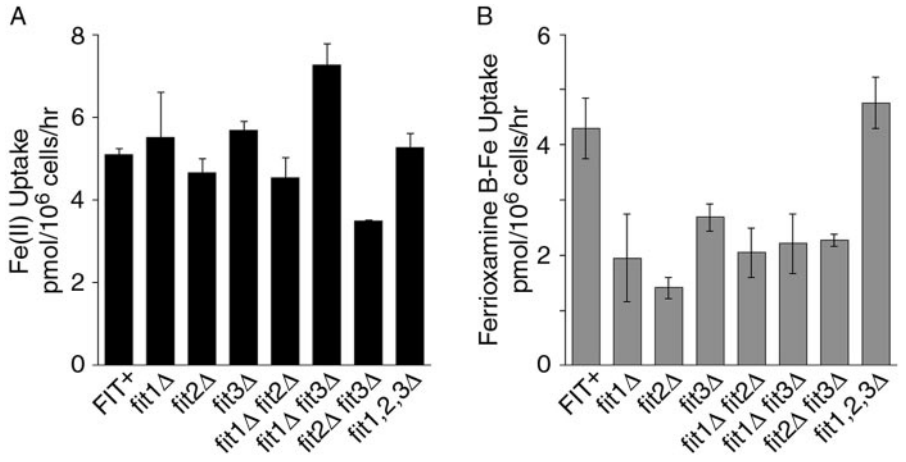

FIG. 4. **Inhibition of ferrioxamine B-iron uptake in *FIT*-deleted strains.** Congenic strains of the indicated genotype were grown in YPD media supplemented with 0.1 mM BPS and 1 μM copper (*A*) or with 1 mM BPS (*B*), and the uptake of 1 μM ferrous ^{55}Fe (*A*) or 1 μM ^{55}Fe-FOB (*B*) was measured. Incubation with 0.1 mM BPS and 1 μM copper induces the expression of high affinity iron uptake systems while preserving the activity of the copper-dependent Fet3p. Incubation with 1 mM BPS alone inhibits Fet3p activity while inducing the expression of the Arnp-dependent siderophore uptake system. Assays were performed in duplicate, and the experiment was replicated three times. Data from a representative experiment are shown; *error bars* indicate average deviation.

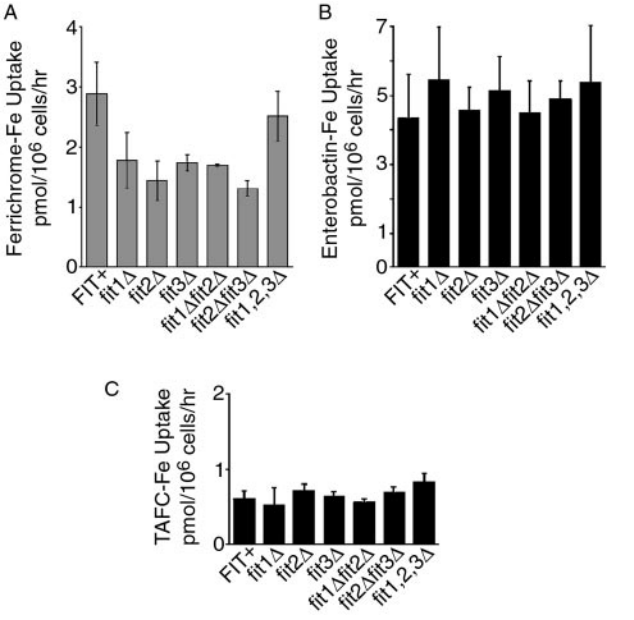

FIG. 5. **Inhibition of ferrichrome-iron uptake in *FIT*-deleted strains.** Congenic strains of the indicated genotype were grown in YPD media supplemented with 1 mM BPS and uptake of ^{55}Fe complexed with ferrichrome (*A*), enterobactin (*B*), and TAFC (*C*) was measured. Assays were performed in duplicate, and the experiment was replicated three times. Data from a representative experiment are shown; *error bars* indicate average deviation.

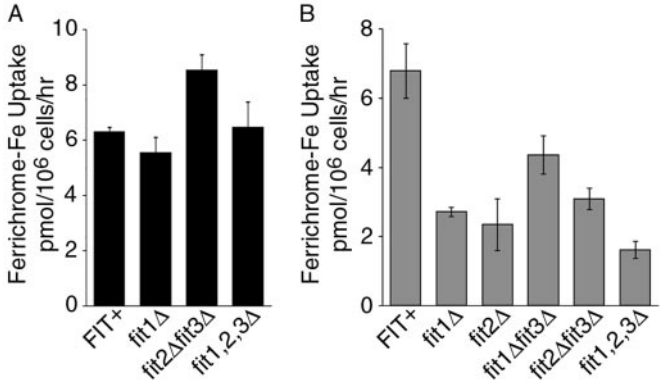

FIG. 6. **Effect of *FIT* deletion on ferrichrome-iron uptake in different growth conditions.** *A*, uptake in strains grown with ferrichrome. Congenic strains of the indicated genotype were grown in defined iron medium supplemented with 10 μM iron and 0.1 μM ferrichrome, and uptake of ^{55}Fe complexed with ferrichrome was measured. *B*, diminished uptake in *FIT*-deleted strains grown in synthetic iron-poor media. Congenic strains of the indicated genotype were transformed with a *FET3-lacZ* reporter plasmid and transformants were grown in defined iron medium containing 30 μM iron. Uptake of ^{55}Fe complexed with ferrichrome was measured. Assays were performed in duplicate, and the experiment was replicated twice. Data from a representative experiment are shown; *error bars* indicate average deviation.

Expression of Systems of Iron Uptake in FIT-deletion Mutants—We hypothesized that, if the Fit proteins functioned to improve the efficiency of iron uptake, then loss of the Fit proteins would result in less efficient iron uptake and a compensatory increase in the expression of systems of iron uptake. We employed two methods to test this hypothesis. First, wild type and *FIT*-deletion strains were transformed with a reporter plasmid bearing the β-galactosidase coding sequences under the control of the *FET3* promoter. This promoter is strongly induced by iron deprivation and is a target for Aft1p (2). To directly compare siderophore iron uptake levels with the expression of Aft1p target genes, these cells were grown in defined iron media containing low concentrations of iron and FC-iron uptake and β-galactosidase activities were measured. FC-iron uptake was lower in the *FIT*-deletion strains, including the *fit1*Δ*fit2*Δ*fit3*Δ strain, than in the congenic parent strain (Fig. 6*B*). When β-galactosidase activity was measured in these cells, the activity was induced 2–3-fold in all of the *FIT*-mutant strains, including the *fit1*Δ*fit2*Δ*fit3*Δ strain (Fig. 7*A*). These data suggested that, although transcription of an Aft1p-target gene (*FET3*) was increased in the *FIT*-deletion strains, these strains did not exhibit a comparable increase in the reductive uptake of ferric iron and they exhibited diminished nonreductive uptake of siderophore iron.

In our second method to evaluate the effects of *FIT* gene deletion on the expression of systems of iron uptake, wild type and *FIT*-deletion strains were transformed with pARN1-HA, a plasmid containing an HA-tagged allele of Arn1p under the control of its own promoter (19). Arn1p is a siderophore-iron transporter with specificity for FC, the expression of which is strongly regulated by iron through Aft1p. Western blot analysis of Arn1p expression levels revealed that deletion of the *FIT* genes resulted in increased expression of Arn1p, and that this increase in expression occurred when cells were grown in media containing either simple salts of iron or FC-iron (Fig. 7*B*). These data suggested that deletion of the *FIT* genes resulted in alterations of the intracellular pools of iron, which led to activation of Aft1p and increased expression of systems of iron uptake. Given that the Fit proteins are located in the cell wall, Fitp-dependent alterations in intracellular pools of iron are likely due to alterations in uptake of iron at the cell surface.

The Role of the Fit Proteins in the Retention of Iron in the Cell Wall and Periplasmic Space—The Fit proteins did not appear to have a role in controlling flocculation or the porosity of the

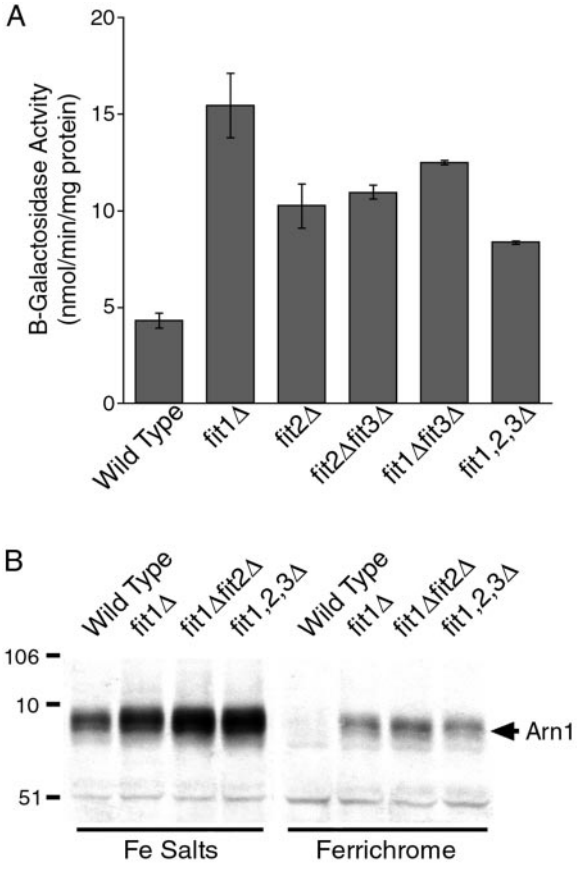

FIG. 7. **Increased expression of systems of iron uptake in strains bearing deletions of *FIT* genes.** *A*, increased expression from a *FET3-lacZ* reporter plasmid. Congenic strains of the indicated genotype were transformed with a *FET3-lacZ* reporter plasmid and transformants were grown in defined iron medium containing 30 μM iron. β-Galactosidase activity was measured as described under "Experimental Procedures." *B*, elevated expression of Arn1p-HA in *FIT*-deleted strains. Congenic strains of the indicated genotype were transformed with pARN1-HA and grown in defined iron medium supplemented with 10 μM ferrous ammonium sulfate (*Fe Salts*, *left*) or 10 μM ferrous ammonium sulfate and 1 μM ferrichrome (*Ferrichrome*, *right*). Arn1 protein was detected by SDS-PAGE and Western blotting.

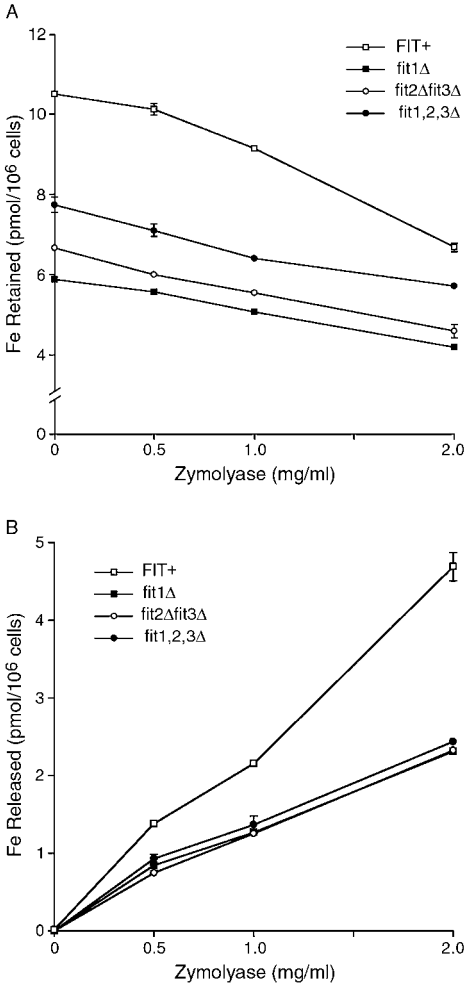

FIG. 8. **Diminished cell wall-associated iron in *FIT*-deleted strains.** Congenic strains of the indicated genotype were grown in YPD medium supplemented with 1 mM BPS, and washed cells were loaded with ^{55}Fe-ferrichrome. Cells were washed and treated for 10 min with the indicated concentration of Zymolyase-100T, and the ^{55}Fe that was retained in the cells (*A*) or released into the supernatant (*B*) was measured as described under "Experimental Procedures."

cell wall (data not shown). Previous work has suggested that low-affinity binding sites for ferric iron exist on the exterior surface of yeast (39). We tested whether the Fit proteins contributed to the retention of iron in the cell wall and periplasmic space by allowing cells to take up labeled iron as ferric-FC, then measuring the retention of the labeled iron within the cells and the release of the labeled iron when the cell wall was partially digested with Zymolyase-100T. Zymolyase is a mixture of β-1,3-glucanase and protease activities that can effect the release of mannoproteins from the cell wall (40). Treatment of iron-loaded, wild type cells with Zymolyase resulted in a dose-dependent release of ~40% of the cell-associated labeled iron into the supernatant fraction (Fig. 8, *A* and *B*). However, *FIT*-deletion mutant strains released significantly lower amounts of iron after Zymolyase treatment than did the wild type strain and no differences were observed between the *fit1*Δ, the *fit2*Δ*fit3*Δ, and the *fit1*Δ*fit2*Δ*fit3*Δ strains. These data indicate that a relatively large proportion of the cell-associated iron is retained in the cell wall and periplasmic space in *S. cerevisiae*, and that deletion of the *FIT* genes results in a decrease in the amount of iron that is retained in the cell wall and periplasmic space. Retention of iron outside the plasma membrane does not occur if cells are incubated with iron at 0 °C, suggesting that cellular metabolic processes are involved in the accumulation of iron.

DISCUSSION

As part of the coordinated response to iron deprivation, *S. cerevisiae* expresses a set of proteins that function in the uptake of iron from a variety of extracellular sources. Here we demonstrate that three proteins that are components of the yeast cell wall are part of this response and that they facilitate the uptake of iron, possibly by increasing the amount of iron associated with the cell wall and periplasmic space. Previous work in characterizing the metal uptake systems of yeast has focused on critical elements of the systems: genes or gene families that when deleted, result in the near complete loss of transport activity. The Fit proteins do not appear to be critical components of the iron uptake machinery; yeast cells can compensate for the absence of these proteins by increasing levels of expression of the other components in the systems of iron uptake. We have directly demonstrated that loss of the Fit proteins results in defects in the Arn-dependent system of siderophore-iron uptake and defects in retention of siderophore-iron in the cell wall. Indirect evidence suggests that the Fit proteins have an effect on the uptake on non-siderophore sources of iron, as well. A strain bearing deletions of *FIT1*, *FIT2*, and *FIT3* had no measurable defect in iron uptake when the cells were grown in iron-chelated, rich media, yet this same strain exhibited a marked defect in FC-iron uptake after

growth in iron-chelated synthetic media. This phenotypic difference may be explained by a greater compensatory increase in the expression of iron uptake systems when the cells were grown in rich media. The Fit proteins may act to enhance the efficiency of the cell's systems of iron uptake by acting as a bridge across the cell wall for iron.

The capacity to express genes that modulate the activity of uptake systems in a more subtle fashion can be important. One example of this subtle modulation is the HFE protein present in mammalian systems of iron uptake (41, 42). Defects in the HFE gene are responsible for the human disease hereditary hemochromatosis. Patients with hereditary hemochromatosis exhibit a subtle increase in the absorption of dietary iron that leads to the slow accumulation of iron in vital organs. Symptoms of the disease are typically not manifest until the fourth or fifth decade of life, but patients frequently develop heart failure, liver failure, and liver cancer. Hence, subtle modulations in iron uptake can have a significant impact on the survival of an organism.

The *FIT* genes were transcriptionally activated by Aft1p, they contain multiple Aft1p consensus binding sites, and they exhibit a greater range of activation than other Aft1p target genes. Conversely, transcript levels of the *FIT* genes are virtually undetectable in an *AFT1*-deletion strain. The *FIT* genes could also be targets for the recently described Aft2p (43). Aft2p is 39% identical to Aft1p and was recently shown to activate the transcription of the Aft1p target, *FET3*.

Deletions of the individual FIT genes and of combinations of FIT genes led to similar defects in the uptake of siderophore-iron and the retention of iron at the surface of the cells. These genes appear to be epistatic, and their protein products may interact within the cell wall to form an active complex or system for iron retention. We found that a surprisingly large amount of iron was associated with the cell wall and periplasmic space. Previous investigators have found that significant amounts of copper are deposited in the cell wall in a sulfide-dependent manner that also depends on active cellular metabolism (44). In addition to facilitating the uptake of iron, the Fit proteins may also contribute to the storage of iron in the cell wall. *FIT* gene expression during periods of moderate iron deprivation could enable cells to store iron to be used in periods of extreme iron deprivation.

Hamada and co-workers (26) identified 53 open reading frames in the yeast genome that potentially encoded GPI-anchored proteins of the plasma membrane and cell wall. More than half of these open reading frames had unknown function. Analysis of the GPI attachment signals indicated that the signals varied greatly in the efficiency with which they directed incorporation of a reporter protein into the cell wall (27). In their studies, the Fit1p sequences directed incorporation much more efficiently than those of Fit2p or Fit3p. Therefore, some Fit2p and Fit3p might also be present on the plasma membrane and in the periplasmic space.

The cell wall is engaged in growth, morphogenesis, adhesion, flocculation, and mating. Here, we add to this list the acquisition and storage of essential nutrients. More than half of the predicted cell wall mannoproteins have unknown function, and as these proteins are functionally characterized the list of cellular processes to which the cell wall contributes will undoubtedly grow.

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