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The *FET4* Gene Encodes the Low Affinity Fe(II) Transport Protein of *Saccharomyces cerevisiae**

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Previous studies on Fe(II) uptake in *Saccharomyces cerevisiae* suggested the presence of two uptake systems with different affinities for this substrate. We demonstrate that the *FET3* gene is required for high affinity uptake but not for the low affinity system. This requirement has enabled a characterization of the low affinity system. Low affinity uptake is time-, temperature-, and concentration-dependent and prefers Fe(II) over Fe(III) as substrate. We have isolated a new gene, *FET4*, that is required for low affinity uptake, and our results suggest that *FET4* encodes an Fe(II) transporter protein. FET4's predicted amino acid sequence contains six potential transmembrane domains. Overexpressing *FET4* increased low affinity uptake, whereas disrupting this gene eliminated that activity. In contrast, overexpressing *FET4* decreased high affinity activity, while disrupting *FET4* increased that activity. Therefore, the high affinity system may be regulated to compensate for alterations in low affinity activity. These analyses, and the analysis of the iron-dependent regulation of the plasma membrane Fe(III) reductase, demonstrate that the low affinity system is a biologically relevant mechanism of iron uptake in yeast. Furthermore, our results indicate that the high and low affinity systems are separate uptake pathways.

Although iron is an abundant element, its availability can often limit the growth of an organism because the oxidized form of the metal, Fe(III), is extremely insoluble at neutral pH. Therefore, organisms require efficient mechanisms to obtain enough iron to support cell growth. Two basic strategies of iron uptake have been identified in many organisms (for review, see Ref. 1). One strategy involves the use of Fe(III) chelators, called siderophores, that are secreted by some bacteria, fungi, and plants. These chelators bind extracellular Fe(III); the Fe(III)-siderophore complex is then taken up by the cell via specific transport systems. The yeast *Saccharomyces cerevisiae* does not appear to secrete its own siderophores (2). This eukaryotic microbe uses a second strategy involving Fe(II)-specific transport systems. First, an Fe(III) reductase located in the plasma membrane reduces extracellular Fe(III) to Fe(II), which is then taken up by the cell (3–6). This mechanism of iron uptake has also been identified in some bacteria (7, 8), other fungi (9, 10), and many plant species (11, 12). Mammalian cells may use a similar mechanism of iron uptake. The iron-binding protein transferrin and the transferrin receptor found on the cell surface provide iron to many different cell types in mammals. Many studies have suggested that a plasma membrane Fe(III) reductase reduces transferrin-delivered Fe(III) to Fe(II) either in endocytic vesicles or at the cell surface (13–16). This Fe(II) may then be transported into the cell via Fe(II)-specific transport systems (17–23). Although Fe(II) uptake systems are common in nature, only one gene that encodes an Fe(II) transporter, the *feoB* gene from *Escherichia coli*, has been isolated and characterized (24).

Biochemical analysis of iron uptake in yeast suggested the presence of two Fe(II) uptake systems. One system has high affinity for iron (apparent K_m = 0.15 μM), is Fe(II)-specific, and requires the *FET3* gene (6, 25). *FET3* encodes a multi-copper oxidase that may drive high affinity Fe(II) uptake by a group translocation mechanism in which transported Fe(II) is oxidized back to Fe(III) during the uptake process (25, 26). As described in this report, mutations in the *FET3* gene have made possible a biochemical analysis of low affinity Fe(II) uptake. Furthermore, we have developed a genetic screen to identify genes involved in low affinity uptake. This paper describes the isolation and characterization of one such gene, *FET4*. The *FET4* gene encodes the low affinity Fe(II) transporter and represents the first eukaryotic Fe(II) transporter to be characterized at the molecular level.

EXPERIMENTAL PROCEDURES

Strains and Culture Methods—Strains used included DY1455 (*MATα ade2 can1 his3 leu2 trp1 ura3*), DY1456 (*MATa ade6 can1 his3 leu2 trp1 ura3*), DEY1394 (*MATα ade6 can1 his3 leu2 trp1 ura3 fet3–2::HIS3*), DDY33 (*MATα ade2 can1 his3 leu2 trp1 ura3 fet4–1::LEU2*), DEY1419 (*MATa/α ade2/+ade6/+can1/can1 his3/his3 leu2/leu2 trp1/trp1 ura3/ura3 fet3–2::HIS3/fet3–2::HIS3*), DDY2 (*MATa can1 leu2 his3 trp1 ura3 fet3–2::HIS3*), DDY4 (*MATa can1 his3 leu2 trp1 ura3 fet3–2::HIS3 fet4–1::LEU2*), W103 (*MATa ura3 ino1 fre1–1*) (5), and DEY1421–5C (*MATa trp1 ura3 fet3–2::HIS3 fre1–1*). DEY1421–5C was isolated as a haploid segregant from a DEY1394 × W103 diploid strain. Yeast cells were grown in 1% yeast extract, 2% peptone supplemented with either 2% glucose (YPD) or 2% galactose (YPgal). These media were made iron-limiting by the addition of an Fe(II) chelator, bathophenanthroline disulfonate (BPS),[1] to the stated concentrations. Cells were also grown on synthetic defined (SD, 0.67% yeast nitrogen base without amino acids) medium supplemented with 2% glucose and any necessary auxotrophic requirements. Plasmids were selectively removed from yeast strains using 5-fluoroorotic acid (27). Sporulation of diploid strains and tetrad dissections were performed as described (28). Cell number in liquid cultures was determined by measuring the optical density of cell suspensions at 600 nm (A_{600}); these values were converted into cell numbers with a standard curve.

Fe(III) Reductase and Fe(II) Uptake Assays—Assays of Fe(III) reductase and Fe(II) uptake activities were performed as described (6) except that ^{55}Fe was used rather than ^{59}Fe and cell-associated radioactivity

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) L34837.

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[1] The abbreviations used are: BPS, bathophenanthroline disulfonate; SD, synthetic defined medium; bp, base pair(s).

was measured by liquid scintillation counting. All assays were performed on exponentially growing cells harvested at culture A_{600} values of between 1 and 4. Where indicated, iron was supplied as Fe(II) by adding sodium ascorbate (1 mM) to the uptake assay solution. Stock solutions for metal competition experiments were prepared by dissolving the chloride salt of each metal into LIM-EDTA (6) at a concentration of 100 mM. These stocks were then diluted into the assay solutions to the designated final concentration before the cells were added. Due to its limited solubility, a $PtCl_2$ stock was prepared as described (6).

Cloning, DNA Sequence Analysis, and Disruption of the FET4 Gene—*E. coli* and yeast transformations were performed using standard methods (29, 30). DEY1394 was transformed with a plasmid library containing yeast cDNAs inserted under the control of the *GAL1* promoter in pRS316-GAL1 (31). Approximately 4,000 Ura⁺ transformants were isolated and plated onto YPgal agar plates supplemented with 200 μM BPS. Eight independent transformants were isolated that formed larger colonies on this medium than the untransformed parent strain, DNA was prepared from each, and these plasmids were then transformed into *E. coli* TOP10F′ (Invitrogen Corp.). Plasmid DNA was isolated and partially sequenced using the T7 primer from the vector to determine the relationship of the various inserts in these plasmids. DNA sequence analysis was performed as described by Borson *et al.* (32). The eight plasmids each contained a cDNA derived from the same gene, and the insert of one plasmid, pCB1, was sequenced on both strands. A series of nested deletions were produced by the combined action of exonuclease III and nuclease S1 (33) and sequenced using oligonucleotide primers derived from vector sequences adjacent to the cDNA insertion site. Data base comparisons and hydropathy analysis were performed using UWGCG and DNA Strider Version 1.0 software, respectively (34, 35).

A disruption allele of *FET4*, *fet4–1::LEU2*, was constructed by subcloning a *SacI-KpnI* fragment from pCB1 into Bluescript SK⁺ (Stratagene Cloning Systems) to generate pSK⁺FET4. This subcloned fragment contains the entire *FET4* cDNA insert. A *LEU2* fragment was prepared for insertion into the *FET4* gene by polymerase chain reaction of *LEU2* from YEp351 (36) using flanking oligonucleotide primers synthesized with *PstI* sites at their 5′ termini (primer sequences were 5′-AACTGCAGGTTAACTGTGGGAATACTCAGG-3′ and 5′-AACTGCAGTTCTTGAGGGAACTTTCACCA-3′). The resulting polymerase chain reaction fragment was purified from an agarose gel (Prep-A-Gene, Bio-Rad), digested with *PstI*, and inserted into *PstI*-digested pSK⁺FET4 to generate pSK⁺ fet4–1::LEU2. To verify that this allele retained no *FET4* function, the *BamHI-SacI* fragment containing the disruption allele was subcloned back into pRS316-GAL1. Plasmid pSK⁺ fet4–1::LEU2 was digested with *SacI* and *BamHI* and used to replace the chromosomal locus in a homozygous *fet3* mutant diploid (DEY1419) and a wild type haploid strain (DY1455) by single-step gene transplacement (37). Correct transplacement of the *FET4* alleles was demonstrated by Southern blot hybridization. The *fet3* single mutant (DDY2) and *fet3 fet4* double mutant (DDY4) strains are haploid segregants of a DEY1419 transformant. DDY33 is a *fet4* mutant generated by transformation of DY1455.

RESULTS

Low Affinity Fe(II) Uptake Is FET3-independent—Mutations in the *FET3* gene eliminate high affinity Fe(II) uptake (25). To examine the role of *FET3* in low affinity uptake, we assayed this activity in wild type and *fet3* mutant cells over a broad range of Fe(II) concentrations. As observed previously (5, 6), uptake in wild type cells was biphasic (Fig. 1*A*), suggesting the presence of separate high and low affinity systems. The high affinity system was saturated for substrate at a concentration of 1 μM Fe(II). An increase in uptake rate attributable to the low affinity system was observed at concentrations greater than 5 μM. These results suggest that the activity of the low affinity system can be estimated in wild type cells by subtracting the high affinity system's contribution (*i.e.* the uptake rate at 1 μM) from the rate observed at higher concentrations (Fig. 1*A*, *open triangles*). In the *fet3* mutant strain assayed at low concentrations (<1 μM), little Fe(II) uptake activity was detected (Ref. 25 and Fig. 1*A*). At higher Fe(II) concentrations, uptake rates assayed in the *fet3* mutant strain were similar in magnitude to the low affinity activity observed in the wild type strain. These data suggest that low affinity uptake was unaffected by the *fet3*

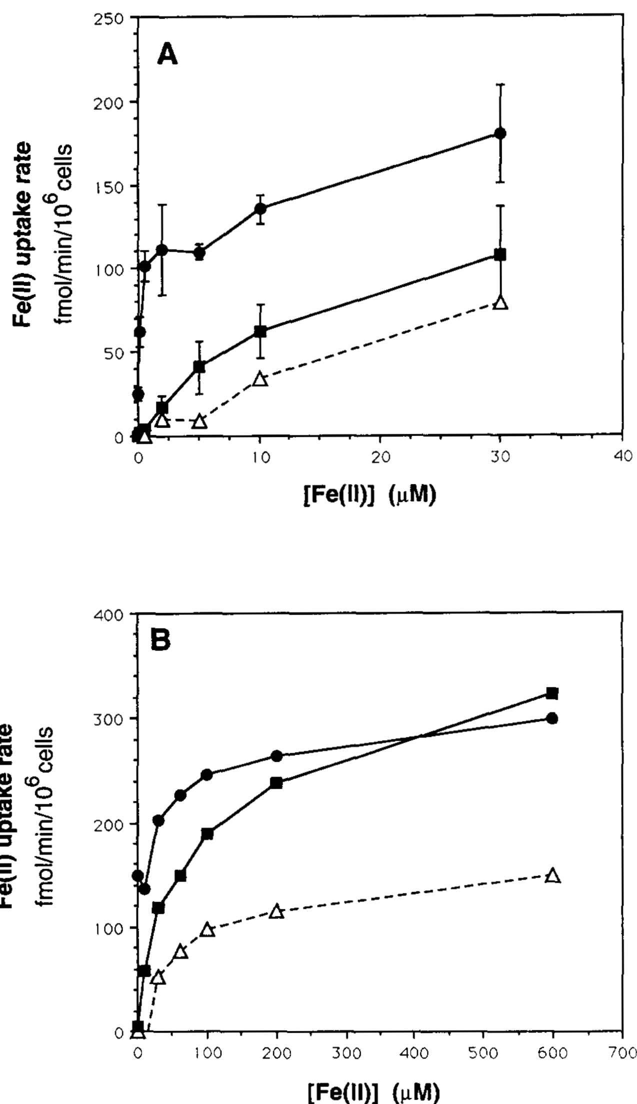


FIG. 1. **Concentration dependence of Fe(II) uptake by wild type and *fet3* cells.** YPD-grown wild type (DY1455, *closed circles*) and *fet3* (DEY1394, *closed squares*) cells were assayed for Fe(II) uptake rates over a range of concentrations. The *dashed lines* marked with *open triangles* represent the accumulation of Fe(II) in wild type cells at 1 μM subtracted from the accumulation of Fe(II) supplied at the designated concentration. *A*, Fe(II) uptake rates assayed at 0.015–30 μM; *B*, Fe(II) uptake rates assayed at 1–600 μM. Each point represents the mean of two separate experiments each performed in duplicate. The standard deviations for each experiment was <10% of the mean.

mutation. Furthermore, they indicate that the activity of the low affinity system can be measured directly in *fet3* mutant cells.

When Fe(II) uptake rates were determined at concentrations up to 600 μM, the low affinity activity appeared saturable in both wild type and *fet3* mutant cells and had similar apparent K_m values (~30 μM) in these two strains (Fig. 1*B*). The observed plateau could have resulted from incomplete reduction of Fe(III) because of insufficient ascorbate in the assay, but a 10-fold increase in ascorbate concentration (10 mM) had no effect on the apparent K_m of the low affinity system (data not shown). Therefore, we conclude that the plateau effect is caused by saturating levels of the Fe(II) substrate. The low affinity activity in wild type and *fet3* mutant cells did differ in their V_{max} values. The estimated V_{max} of the low affinity system in the wild type strain was 143 fmol/min/10^6 cells, while the V_{max} in the *fet3* mutant was more than twice that value (312 fmol/min/10^6 cells).

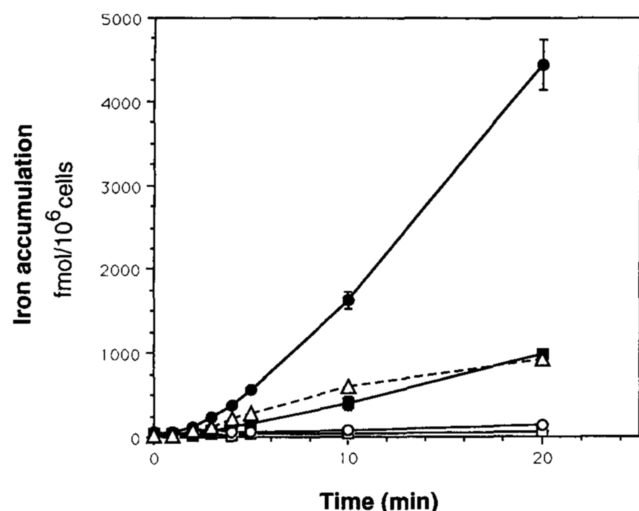


FIG. 2. **Time- and temperature-dependence of Fe(II) uptake by wild type and *fet3* cells.** YPD-grown wild type (DY1455, *circles*) and *fet3* (DEY1394, *squares*) cells were assayed at 10 μM Fe(II) for iron accumulation over time. Each point represents the mean of two separate experiments each performed in duplicate. Cells were assayed at either 30 °C (*closed circles* and *squares*) or 4 °C (*open circles* and *squares*) The *dashed line* marked with *open triangles* represents the wild type accumulation of Fe(II) at 1 μM (not shown) subtracted from the accumulation of Fe(II) supplied at 10 μM measured at each time point. The *error bars* represent ± 1 S.D.

Metal accumulation by yeast cells is often delineated into two separate mechanisms. First, cations can adsorb to negatively charged groups on the cell wall or plasma membrane in a very rapid, temperature-independent process. Alternatively, uptake of the cation across the plasma membrane occurs time and temperature dependently. To assess the mechanism of iron accumulation by the low affinity system, we examined its time and temperature dependence. In wild type cells, little Fe(II) accumulated at 4 °C (Fig. 2). At 30 °C, following an initial lag of 2–3 min, cell-associated iron increased in a near linear fashion for 20 min. To estimate the contribution of the low affinity system to the overall accumulation in wild type cells, we subtracted the accumulation measured at 1 μM from that measured at 10 μM (Fig. 2, *open triangles*). The *fet3* mutant strain also accumulated little iron at 4 °C but showed a linear increase in cell-associated iron over time at 30 °C. The accumulation of iron in *fet3* cells was similar in magnitude to the estimated low affinity activity in wild type cells. These data demonstrate that iron accumulation by the low affinity system is both time- and temperature-dependent and suggests that this accumulation is caused by uptake rather than adsorption of the metal to the cell surface.

Transport Specificity of Low Affinity Uptake—To determine if the low affinity system prefers Fe(II) over Fe(III) as substrate, iron uptake was measured in cells in which the plasma membrane Fe(III) reductase had been mutationally or pharmacologically inactivated. First, we determined whether a mutation in the *FRE1* gene, encoding the Fe(III) reductase, inhibited uptake by the low affinity system (Table I). The *fre1-1* allele decreased the reductase activity to less than 10% of the *FRE1* levels. The iron uptake rate in *fre1* mutants was also low when iron was supplied to either *FET3* or *fet3* cells as Fe(III). Uptake rates in *fre1* strains were restored to nearly *FRE1* levels when extracellular Fe(III) was reduced to Fe(II) by adding ascorbate. Second, we took advantage of the observation that Pt(II) is an inhibitor of the Fe(III) reductase (6). In a *fet3* mutant strain, the rate of Fe(III) reduction was decreased by Pt(II) to approximately 20% of the untreated control rate (Table I). Iron uptake was also inhibited by Pt(II) when iron was supplied as Fe(III).

TABLE I
Dependence of the low affinity system on Fe(III) reduction

YPD-grown cells were assayed for Fe(III) reduction rate and iron uptake rate at 30 μM in the absence or presence of 1 mM sodium ascorbate. A, effects of the *fre1-1* mutation on reductase and iron uptake activity. Strains used were DY1455 (wild type), DEY1394 (*fet3*), W103 (*fre1*), and DEY1421-5C (*fet3 fre1*). B, effects of Pt(II) on reductase and iron uptake activity. DEY1394 cells were assayed for reductase activity and uptake activity in the absence or presence of 10 μM $PtCl_2$. All values represent the mean of two experiments each performed in duplicate.

Strain	Fe(III) reduction rate[a]	Iron uptake rate[b]	
		-Ascorbate	+Ascorbate
A. Wild type	216 ± 33	117 ± 13	154 ± 17
fet3	231 ± 13	52 ± 1	130 ± 8
fre1	3 ± 2	6 ± 1	111 ± 8
fet3 fre1	19 ± 2	4 ± 1	112 ± 3
Addition	**Fe(III) reduction rate[a]**	**Iron uptake rate[b] -Ascorbate**	**+Ascorbate**
B. None	260 ± 90	82 ± 8	116 ± 2
10 μM Pt(II)	50 ± 10	5 ± 1	90 ± 7

[a] pmol/min/10^6 cells (mean ± S.D.).
[b] fmol/min/10^6 cells (mean ± S.D.).

Reducing Fe(III) to Fe(II) with ascorbate nearly restored the uptake rate in the presence of Pt(II) to untreated control levels. These results demonstrate that Fe(II) is preferred over Fe(III) as substrate for transport by the low affinity system.

To assess the ability of the low affinity system to transport other substrates, we tested several divalent transition metals with ionic radii similar to that of Fe(II) (0.74 Å) for their ability to inhibit accumulation of iron by the low affinity system in *fet3* mutant cells. The concentration of competitor metals in these assays was 50 times higher than the iron concentration. The addition of Zn(II), Mn(II), or Sr(II) did not reduce accumulation of iron by the low affinity system (Table II). While Ni(II) was moderately effective at inhibiting accumulation, Co(II) and Cd(II) were each found to be potent inhibitors of the low affinity system. Both metals were also found to inhibit iron accumulation when iron was supplied as Fe(II), indicating that Fe(III) reduction was not the inhibited step (data not shown). Furthermore, the decrease in iron accumulation was probably not caused by metal toxicity because control experiments detected no loss of cell viability resulting from Co(II) or Cd(II) exposure.

The inhibition of iron accumulation by Co(II) and Cd(II) is not necessarily because uptake is inhibited. These metals might limit iron accumulation by increasing membrane permeability or by disrupting some intracellular process that prevents Fe(II) efflux. To test if iron retention is affected by Co(II) and Cd(II), we took advantage of the fact that wild type cells can accumulate Fe(II) through either the low or the high affinity system. If these metals were inhibiting Fe(II) retention, they would interfere with Fe(II) accumulation by either of these pathways. However, neither Co(II) nor Cd(II) had a significant effect on iron accumulation by the high affinity system (Table III). These data suggest that inhibition of the low affinity system by Co(II) and Cd(II) is mediated at the level of uptake and not iron retention.

Isolation of the FET4 Gene—Strains bearing a mutation in the *FET3* gene grow poorly on iron-limited media because of a defect in high affinity uptake. To identify other yeast genes involved in iron uptake, we developed a genetic screen to isolate those genes that, when overexpressed, increase the growth rate of a *fet3* mutant on iron-limited media. This screen was performed with a plasmid library in which yeast cDNA inserts were expressed under the control of the galactose-inducible *GAL1* promoter (31). This library was transformed into a *fet3* mutant strain, transformants were isolated and plated onto a

TABLE II
Effect of divalent transition metals on iron uptake by the low affinity system

YPD-grown DEY1394 cells were assayed for iron uptake rate at 10 μM Fe(III) in the absence or presence of 500 μM of the indicated divalent cation. Inhibition is shown as the percent of the uptake rate observed in the untreated control. All values represent the mean of two experiments each performed in duplicate.

Addition	Ionic radius	Iron uptake rate[a]	Percent of control
	Å		
None		22 ± 0.5	100
Zn(II)	0.74	24 ± 2	109
Mn(II)	0.80	20 ± 3	91
Sr(II)	1.12	24 ± 3	109
Ni(II)	0.69	14 ± 1	64
Co(II)	0.72	1 ± 1	5
Cd(II)	0.97	3 ± 1	14

[a] fmol/min/10^6 cells (mean ± S.D.).

TABLE III
Effects of Co(II) and Cd(II) on low and high affinity uptake

Low affinity uptake was measured in YPD-grown DEY1394 at 10 μM Fe(II). High affinity uptake was measured in YPD-grown DY1455 at 2 μM Fe(II). All values represent the mean of two experiments each performed in duplicate (±1 S.D.).

Addition	Low affinity system	High affinity system
	fmol/min/10⁶ cells	
None	22 ± 1	138 ± 4
200 μM Co(II)	2 ± 1	126 ± 35
200 μM Cd(II)	5 ± 1	157 ± 6

galactose-containing medium (YPgal) made iron-limiting by the addition of 200 μM BPS. BPS is a chelator that binds iron in the medium and prevents its uptake by yeast cells (6). From 4,000 independent transformants, eight transformants were isolated that formed larger colonies on this medium than the *fet3* parent strain. For all eight, this improved growth required expression of the plasmid's cDNA insert; suppression was not apparent on an iron-limited medium that contained glucose (YPD + 200 μM BPS) in which the *GAL1* promoter is inactive. Furthermore, when the plasmids were removed from these strains, galactose-dependent suppression of the *fet3* phenotype was no longer observed. Partial sequence analysis of the cDNA inserts from these eight plasmids indicated that each was derived from the same gene. We have designated this gene *FET4* (for Fe(II) transport).

Effect of FET4 Over-expression on Fe(III)-Reductase and Fe(II) Uptake Activities—The improved growth of cells overexpressing the *FET4* gene on iron-limited media suggested that this high level of expression might increase the activity of one or more component of the yeast iron uptake system. Therefore, we measured Fe(III) reduction and Fe(II) uptake rates in a *fet3* mutant and a wild type strain. These cells, bearing the vector (pRS316-GAL1) or a *FET4*-expressing plasmid (pCB1), were grown on either glucose- or galactose-containing media. Overexpression of *FET4* had no effect on reductase activity in either strain (data not shown). In Fe(II) uptake assays conducted with 30 μM Fe(II), a concentration near the apparent K_m for low affinity Fe(II) uptake, no difference in uptake rate was observed between pCB1- and vector-bearing cells grown on glucose. However, *FET4* overexpression on galactose increased the rate of Fe(II) uptake in the *fet3* mutant by 8-fold compared with the vector control (Fig. 3*A*). This increase in uptake rate was not dependent on the *fet3* mutation; a 2–3-fold increase was also observed in wild type cells. The increased rate of Fe(II) uptake observed in strains overexpressing *FET4* could result from restored high affinity activity. Therefore, we assayed the rate of Fe(II) uptake in these strains at 1 μM. A 4-fold increase

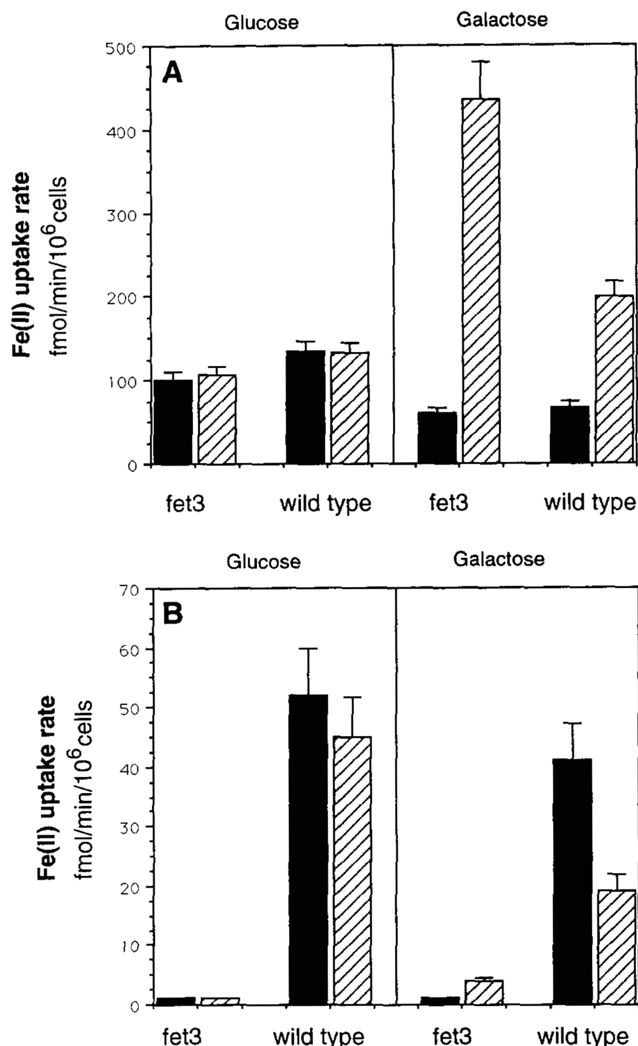


FIG. 3. **Effect of *FET4* overexpression on low and high affinity uptake.** Wild type (DY1456) and *fet3* (DEY1394) cells bearing either the vector (pRS316-GAL1) (*filled bars*) or pCB1 (*hatched bars*) were grown to stationary phase in SD medium lacking uridine. These cells were then inoculated into YP media containing either 2% glucose or 2% galactose and grown 18 h prior to assay. *A*, effect of *FET4* overexpression on low affinity uptake assayed at 30 μM Fe(II); *B*, effect of *FET4* overexpression on high affinity uptake assayed at 1 μM Fe(II). The values represent the mean of two separate experiments each performed in duplicate. The *error bars* represent 1 S.D.

was detected in *fet3* cells overexpressing *FET4* (Fig. 3*B*). In wild type cells, *FET4* overexpression *reduced* high affinity uptake by approximately 50%. These data suggest that *FET4* overexpression increases an Fe(II) uptake activity other than the high affinity system.

FET4 overexpression might increase the Fe(II) uptake rate by increasing the activity of the low affinity system. Alternatively, a third, previously undetected, Fe(II) uptake system could be increased in activity by *FET4* overexpression. To address this question, we compared the kinetic properties of the Fe(II) uptake activity generated by *FET4* overexpression with those of the low affinity system measured in untransformed *fet3* mutant cells. *FET4* overexpressing *fet3* mutants exhibited a V_{max} of 2118 fmol/min/10^6 cells and an apparent K_m of 30 μM (Fig. 4). While the V_{max} of the low affinity system in untransformed *fet3* mutants was substantially lower than in the *FET4* overexpressing cells (291 fmol/min/10^6 cells), the apparent K_m values measured in these two strains were almost identical; the apparent K_m measured in the untransformed *fet3* mutant was 28 μM. An additional test was provided by our observation that

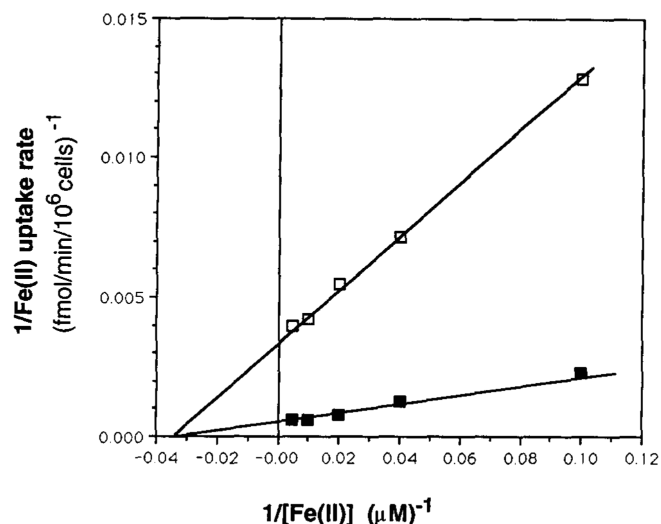
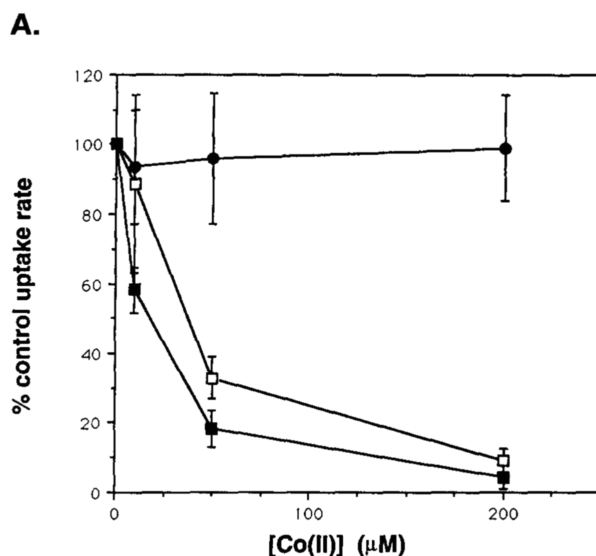


FIG. 4. **Kinetic analysis of Fe(II) uptake in *fet3* cells and *FET4* overexpressing *fet3* cells.** DEY1394 (*open squares*) and DEY1394 pCB1 transformants (*closed squares*) were grown in YPD and YPgal, respectively, and assayed for Fe(II) uptake rates over a range of Fe(II) concentrations. The data are shown on a Lineweaver-Burk reciprocal plot and represent the mean of two separate experiments, each performed in duplicate. The standard deviations for each experiment was <10% of the mean.



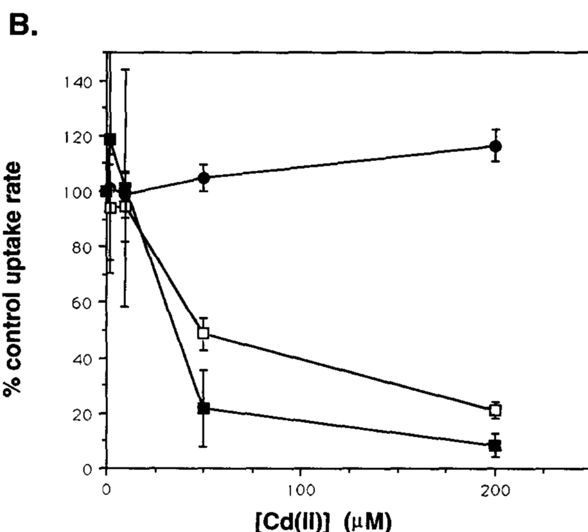


FIG. 5. **Inhibition of the uptake activity dependent on *FET4* overexpression by Co(II) and Cd(II).** High affinity activity was measured in YPD-grown wild type cells (DY1455, *closed circles*) at 1 μM Fe(II). Low affinity uptake was measured in YPD-grown *fet3* cells (DEY1394, *closed squares*) at 10 μM Fe(II). The uptake activity dependent on *FET4* overexpression was measured in YPgal-grown DEY1394 cells bearing pCB1 (*open squares*) at 10 μM Fe(II). These cells were assayed for Fe(II) uptake at the indicated concentrations of Co(II) (*A*) or Cd(II) (*B*). The values represent the mean of two separate experiments each performed in duplicate and plotted as the percent of the uptake rate measured in each strain's untreated control. The *error bars* represent ± 1 S.D.

Co(II) and Cd(II) are specific inhibitors of low affinity uptake. Both metals inhibited pCB1-dependent uptake to nearly the same degree that they inhibited the low affinity system at several concentrations of Co(II) (Fig. 5*A*) and Cd(II) (Fig. 5*B*). Neither metal affected high affinity uptake as indicated by similar assays performed on wild type cells at 1 μM Fe(II). These results demonstrate that *FET4* overexpression increases the activity of the low affinity system.

Sequence of the FET4 Gene—We sequenced the entire cDNA insert of pCB1 (Fig. 6). This fragment is 1,977 base pairs (bp) long with a single open reading frame encoding the *FET4* gene. This open reading frame is flanked by a 66-bp 5′-untranslated region and a 229-bp 3′-untranslated region. A 26-bp polyadenylate sequence was found at the 3′ end of the cDNA. The open reading frame is 1,656 bp long and encodes a 552-amino acid protein with a predicted molecular mass of 63 kDa. A detailed analysis of the FET4 amino acid sequence suggests that this protein is the Fe(II) transporter of the low affinity system. The *FET4* gene appears to encode an integral membrane protein. The protein is composed of 51.5% hydrophobic residues and, while lacking a recognizable signal sequence for targeting to the plasma membrane, hydropathy analysis indicates that FET4 contains several hydrophobic regions (data not shown). Six of these regions are 20 or more amino acids in length and may be transmembrane domains (Fig. 6). The pattern of hydrophobic regions observed in FET4 is common to many proteins that transport substrates across cellular membranes. However, *FET4* encodes a novel protein. The amino acid sequence of FET4 does not share significant similarity to any sequences, including the *E. coli feoB* Fe(II) transporter (24) and the yeast *CTR1* copper transporter (26), contained in the SWISS-PROT (Release 28, 2/94) and GenBank™ (Release 83, 6/94) data bases. Blot hybridization experiments performed at low stringency with the cDNA insert as probe failed to detect any other genes in the yeast genome similar in sequence to *FET4* (data not shown).

Disruption of the FET4 Gene—Our results demonstrate that overexpression of *FET4* increases low affinity activity. To determine if *FET4* is *required* for this system to function, we constructed a disruption mutation in the *FET4* gene. This allele, designated *fet4–1::LEU2*, was constructed by inserting the *LEU2* gene into a *Pst*I restriction site located in the center of the *FET4* open reading frame (Fig. 6). This insertion interrupts the FET4 amino acid sequence at residue 280. The *fet4–1::LEU2* allele retains little or no functional activity; overexpression of *fet4–1::LEU2* from pRS316-GAL1 did not increase Fe(II) uptake rate in a *fet3* mutant strain (data not shown). The disruption allele was transformed into a homozygous *fet3* diploid strain and a wild type haploid strain by gene transplacement (37). Transformants of both recipient strains that contained the correct substitution of the wild type *FET4* gene with *fet4–1::LEU2* were obtained and confirmed by blot hybridization analysis (data not shown). A *fet3*/*fet3 fet4*/+ diploid strain was sporulated; the asci were dissected, and each ascus yielded

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 -66 AGAAGGGAAAAGGTAGATATAGAAACCTAGTAAGACCCTTATTATTAAACATTCGTTAATCAAATT

   1 ATGGGTAAAATTGCAGAGTTTCTCGGGAAT CCAGGTGCTAGGCCTGACGTTCATCATAGA GCACCTACTGTTGACTGCAAACAGTACGAA
   1 M  G  K  I  A  E  F  L  G  N   P  G  A  R  P  D  V  H  H  R   A  P  T  V  D  C  K  Q  Y  E

  91 GAATTCGGTGATTCTAACGATTACAAAAAT GATGATGTGGTCAGAGTAGTCAGCCATAGC GATGAGAGTACTGATGACGAACTTTGTAAT
  31 E  F  G  D  S  N  D  Y  K  N   D  D  V  V  R  V  V  S  H  S   D  E  S  T  D  D  E  L  C  N

 181 GTGAATTTAACAGAAACAGGGGCAATCTTC ACGAGTAAAGGTTTTACCGGGTTAAGCAAA GGTTTCACAGATAAGACCCTGGATTTCCTG
  61 V  N  L  T  E  T  G  A  I  F   T  S  K  G  F  T  G  L  S  K   G  F  T  D  K  T  L  D  F  L

 271 GTACGAGTGGCCGGTTCGCAGGCGGTTTTT TTTATTGTTTGGATCATCCTCATAATTTGG GTGGTTATTGGTATTGTTTATAACGCACCT
  91 V  R  V  A  G  S  Q  A  V  F   F  I  V  W  I  I  L  I  I  W   V  V  I  G  I  V  Y  N  A  P

 361 TTCAATTGGCAAGTTGTTATGCAGGACGGA CAGTCCATTCAAAGTTATGTTTGGGACACA CTGTTGATGAGACAACAGTTGATGAGTACG
 121 F  N  W  Q  V  V  M  Q  D  G   Q  S  I  Q  S  Y  V  W  D  T   L  L  M  R  Q  Q  L  M  S  T

 451 CATGAACAAATTTTGATCTGCGGTAGATTG AAGTCGAGATTGGCTTCCTTCAAAAACTAT CTAACAAGAAGCACCCCAGAGGAAGAAAAA
 151 H  E  Q  I  L  I  C  G  R  L   K  S  R  L  A  S  F  K  N  Y   L  T  R  S  T  P  E  E  E  K

 541 GCAGACTGCACAGTTGAAGCTAATGAAGTC AGCTCTGTTGAAAATCATATAGACCCATCT GCCATTAACGGAGAACTGCCTGTGGAAAAT
 181 A  D  C  T  V  E  A  N  E  V   S  S  V  E  N  H  I  D  P  S   A  I  N  G  E  L  P  V  E  N

 631 TGGTACGACCGTTTATCTAATGTAGCAAGT AGGTATATGGGTTCAATTGCAGCAATGGTG ATATTTTGGATAGGTATTTTCGTTTGGATT
 211 W  Y  D  R  L  S  N  V  A  S   R  Y  M  G  S  I  A  A  M  V   I  F  W  I  G  I  F  V  W  I

 721 GGTTGCGGTGCTATCCCAAAAGATGCGGGC AACACTCCACCTTACACCGGAGAAACTACA GGTAGTAATCCAAGATTGAAAAAGTTCAGT
 241 G  C  G  A  I  P  K  D  A  G   N  T  P  P  Y  T  G  E  T  T   G  S  N  P  R  L  K  K  F  S
                                PstI
 811 GACGCTTGGCAGATGTATATTAACACTGCA GTTGCAATTTCCCTTCTAATTTGCACTACT TTCTTACAAAATATAAGGGCCAGACATGAT
 271 D  A  W  Q  M  Y  I  N  T  A   V  A  I  S  L  L  I  C  T  T   F  L  Q  N  I  R  A  R  H  D

 901 TATTTCACGGGGAGGTTTTTAGTTGATATC TTTGATATGGACGAGAAAATCGACTATCGT ATAAGAAAGCATTTTAACGATTTTGAAACA
 301 Y  F  T  G  R  F  L  V  D  I   F  D  M  D  E  K  I  D  Y  R   I  R  K  H  F  N  D  F  E  T

 991 CCTCACCCAGTTGTTACCATTGAGTCTAAA AAAAGGTCGACAGGGAGAAAGATGATTGAT TGGTATGCTGATATTATTGGTACTGGTATT
 331 P  H  P  V  V  T  I  E  S  K   K  R  S  T  G  R  K  M  I  D   W  Y  A  D  I  I  G  T  G  I

1081 GGTGTCCTGATCGGTGTGGCAGTGTTCGCT ACATGGATTGGTATTGGTTCGCCAATGAAG TGGGATGATAATTGGTGGTTGATTATCGGT
 361 G  V  L  I  G  V  A  V  F  A   T  W  I  G  I  G  S  P  M  K   W  D  D  N  W  W  L  I  I  G

1171 ACATACACAGGTTTAATTGGGTTTTTGGAC GGTTTCGTTTTGAGAGAAGTGTACTTCCGA ATCGTTCAACATGAAGAGAAAAATTATTCT
 391 T  Y  T  G  L  I  G  F  L  D   G  F  V  L  R  E  V  Y  F  R   I  V  Q  H  E  E  K  N  Y  S

1261 GATGTGGCTAAAGAAGACCTTGAATTATTC CAAGAATTAGGTATTGAATGTCCCGAAGAA CTTAGTGGTAAAGCTCCTGAAATTAACATT
 421 D  V  A  K  E  D  L  E  L  F   Q  E  L  G  I  E  C  P  E  E   L  S  G  K  A  P  E  I  N  I

1351 ATTGGTTACAGAACATCCCAATATATTAAT AGGATCTGCTCAACTCCATGGAGTGTTCTT GTATCCGTCATCATCATCATTGGTTTAATT
 451 I  G  Y  R  T  S  Q  Y  I  N   R  I  C  S  T  P  W  S  V  L   V  S  V  I  I  I  I  G  L  I

1441 TGTATTGCTTCCGGTTTGCGTTGGAGTACA ACAGGTCAATTGATTGCTAACACGCCAACT ATGATTATCGAAGAATTTTTCTTGCTAGTT
 481 C  I  A  S  G  L  R  W  S  T   T  G  Q  L  I  A  N  T  P  T   M  I  I  E  E  F  F  L  L  V

1531 TTGTTGCAAGCACATAATTGGGCCGATCGT CAAAGAAGAGTGGAGGTTACCGCTTTGTAC GCACGTAGGCGCATACTTCTATCATACGTA
 511 L  L  Q  A  H  N  W  A  D  R   Q  R  R  V  E  V  T  A  L  Y   A  R  R  R  I  L  L  S  Y  V

1621 GAAAAGCGTTTCCCAGAGGTTATGATGTTG GAAAAATAGTTTCATTGAACATTTTAGCAT TAGTGGGAAGAATTCCTCATGTTACTTATG
 541 E  K  R  F  P  E  V  M  M  L   E  K  *

1711 AGGGAGTTAATTTTCCTTAGGGATCTTAGG AATTTCTATACGGAGGTAGCGATCGACCTT AGAACTTTTATTTAGTTTGTACATATACCT
1801 CACCTGAGTTTTGCTTTTTTCTCTGGGAGC CTAAACCATTTAAAATGATATATAATAGAT AATAAATCCAGGATAAAATGTGGCTAAAAA
1891 AAAAAAAAAAAAAAAAAAAAA
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FIG. 6. **Nucleotide sequence of the *FET4* gene and the predicted amino acid sequence of its protein product.** The sequence of the cDNA insert of pCB1 is shown numbered beginning with the first nucleotide in the ATG initiation codon. Amino acids are numbered beginning with the initiator methionine residue. Putative membrane-spanning domains detected by hydropathy analysis (41) are *underlined*. The *Pst*I site into which the *LEU2* gene was inserted to construct the *fet4–1::LEU2* allele is located at nucleotide positions 836–841 and indicated by *boldface*.

four viable spores. These results indicate that the *FET4* gene is not essential for cell viability, even in a *fet3* mutant strain.

Fe(II) uptake assays were performed on haploid wild type, *fet3* mutant, *fet4* mutant, and *fet3 fet4* double mutant strains to determine how the *fet4* mutation affected the high and low affinity systems. As expected, the *fet3* and *fet3 fet4* mutant strains had little high affinity activity (Fig. 7*A*). Both wild type and *fet4* mutant cells possessed similar high affinity activities, indicating that *FET4* is not required for that system. In fact, the uptake rate in the *fet4* mutant assayed with 1 μM Fe(II) was higher than that observed in the wild type strain. The decrease in uptake rate observed from 1 to 5 μM for both wild type and *fet4* mutants was reproduced in several experiments and may be due to altered kinetic properties of the high affinity system with increasing Fe(II) concentrations. When the activity of the low affinity system was assayed, little increase in uptake rate was observed in the *fet4* and the *fet3 fet4* double mutant (Fig. 7*B*). Therefore, *FET4* is required for low affinity uptake.

The Low Affinity System Is a Biologically Relevant Source of Iron—Experiments in which *FET4* was overproduced or mutationally inactivated demonstrated the importance of this gene for low affinity uptake. The observation that *FET4* overexpressing *fet3* mutants grow better than the untransformed *fet3* strain on an iron-limiting medium suggests that this system can provide iron for cell growth under this artificial condition. To assess the role of the low affinity system as a source of Fe(II) when expressed at normal levels, we examined growth of wild type, *fet3*, *fet4*, and *fet3 fet4* cells under varying degrees of iron limitation. These conditions were achieved by adding increasing concentrations of BPS to iron-rich YPD medium. The *fet3 fet4* double mutant was hypersensitive to iron-limitation; growth was inhibited at 50 μM BPS, whereas both single mutants grew as well as the wild type strain on this concentration of BPS (data not shown). The hypersensitivity of the *fet3 fet4* double mutant to growth inhibition by BPS suggests that both high and low affinity systems provide iron to cells. The *fet3* single mutant showed intermediate sensitivity; growth of this strain was blocked at 200 μM BPS, while no difference in sensitivity was detected between the wild type strain and the *fet4* mutant at this concentration of chelator. This result was expected given that the high affinity system, the *FET3*-dependent pathway, would be of greater importance for iron-limited growth. Finally, neither *fet4* mutants nor wild type cells were capable of growth at the highest concentration of BPS used (500 μM).

To further examine the importance of the low affinity system for iron accumulation, we tested the effects of the *fet4–1::LEU2* mutation on the regulation of activities induced in response to iron-limiting conditions, the Fe(III) reductase (5, 38), and the high affinity system (6, 25). If both high and low affinity systems work together to maintain a certain intracellular iron content, loss of one uptake pathway may be compensated for by an increase in the activity of the other uptake system. Furthermore, the *fet4* allele, either singly or when combined with a *fet3* mutation, might cause an iron deficiency sufficient to induce Fe(III) reductase activity. We examined these activities in wild type, *fet3*, *fet4*, and *fet3 fet4* cells grown on an iron-rich medium (SD). SD medium was chosen for this experiment because wild

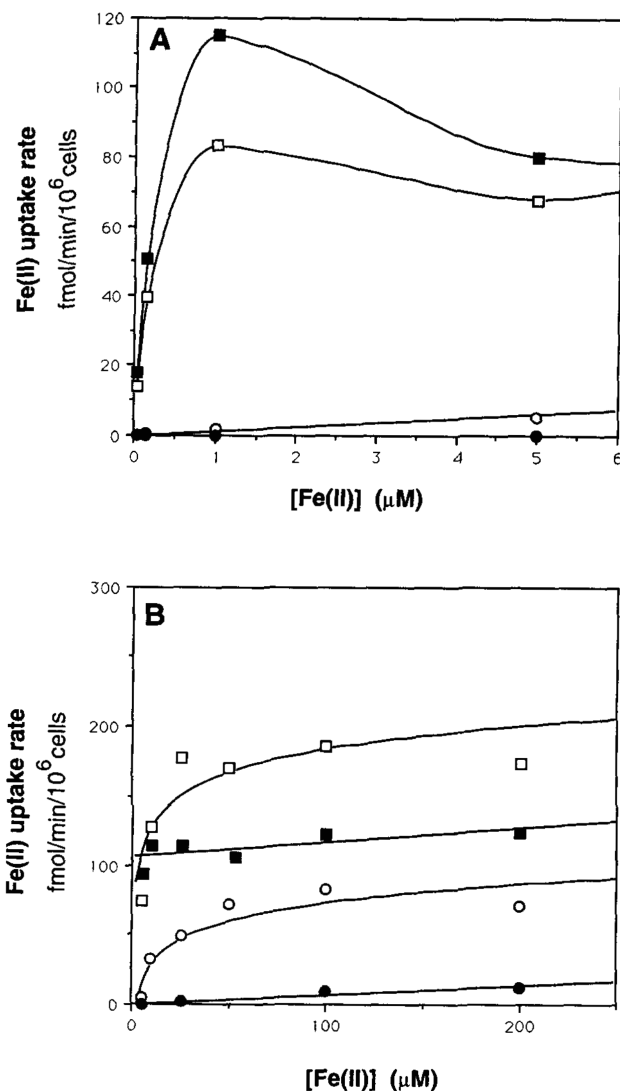

FIG. 7. **Effect of *fet3* and *fet4* mutations on high and low affinity uptake.** YPD-grown wild type (DY1455, *open squares*), *fet3* (DDY2, *open circles*), *fet4* (DDY33, *closed squares*), and *fet3 fet4* (DDY4, *closed circles*) cells were assayed for Fe(II) uptake at the indicated concentrations. *A*, effect of *fet3* and *fet4* mutations on high affinity uptake measured at 0.05–5 μM Fe(II). *B*, effect of *fet3* and *fet4* mutations on the low affinity uptake measured at 5–200 μM Fe(II). Fitting of the curves in *panel B* was performed using Cricket Graph Software Version 1.3, which fits the data to a logarithmic function using nonlinear regression based on the principle of least squares. The plotted values represent the mean of two separate experiments each performed in duplicate. The standard deviations for each experiment was <10% of the mean.

type cells growing on this medium appear iron replete; they have extremely low levels of both Fe(III) reductase and high affinity uptake activity (6). The rate of high affinity Fe(II) uptake in a *fet4* mutant was approximately 3-fold higher than in the wild type strain (Table IV). When Fe(III) reductase activity was measured in the wild type, *fet3* and *fet4* cells, each had extremely low levels of activity. However, the *fet3 fet4* double mutant's Fe(III) reduction rate was increased more than 4-fold. These results suggest that both high and low affinity uptake systems supply iron to growing yeast cells.

DISCUSSION

The *FET3* gene encodes a protein required for high affinity uptake in *S. cerevisiae*. Because of this requirement, mutations in *FET3* enabled a biochemical analysis of the low affinity system and the isolation of the *FET4* gene. We propose that

TABLE IV
Effect of fet3 and fet4 mutations on high affinity uptake and Fe(III) reduction

SD-grown DY1455 (wild type), DDY33 (*fet4*), DDY2 (*fet3*), and DDY4 (*fet3 fet4*) cells were assayed for Fe(II) uptake rate at 1 μM and Fe(III) reduction rate. Values shown are the means of two experiments each performed in duplicate.

Strain	High affinity Fe(II) uptake rate[a]	Fe(III) reduction rate[b]
Wild type	8.1 ± 1.5	0.00 ± 0.02
fet4	24.9 ± 4.0	0.01 ± 0.02
fet3	0.0 ± 0.3	0.04 ± 0.03
fet3 fet4	0.0 ± 0.2	0.19 ± 0.04

[a] fmol/min/10^6 cells (mean ± 1 S.D.).
[b] nmol/min/10^6 cells (mean ± 1 S.D.).

FET4 is the Fe(II) transporter of the low affinity system. Consistent with this hypothesis, the level of *FET4* expression correlated with low affinity uptake activity. Overexpression of *FET4* caused an increase in the activity of this system, whereas disruption of the *FET4* gene eliminated that activity. Furthermore, the predicted amino acid sequence of FET4 suggests that this protein plays a direct role in the transport of Fe(II). This sequence contains a high proportion of hydrophobic residues arrayed in six regions of sufficient length (20 amino acids or greater) to span a lipid bilayer membrane. This hydrophobicity suggests that FET4 is an integral membrane protein with multiple transmembrane domains. It is possible that FET4 is only one subunit of a heteromeric transporter complex, but this hypothesis seems unlikely given that overexpression of *FET4* alone was sufficient to increase Fe(II) uptake activity. However, if FET4 is a component of a multisubunit transporter, this result argues that FET4 would be a rate-limiting subunit.

FET4 is the first transmembrane Fe(II) transporter from a eukaryotic organism to be characterized at the molecular level. The only other Fe(II) transporter gene that has been isolated is *feoB* from *E. coli* (24). FeoB is a 773-amino acid protein with eight potential transmembrane domains. Aside from their shared hydrophobic character, FeoB and FET4 have dissimilar amino acid sequences. Regions of FeoB have similarity to the phosphate binding domain of eukaryotic and bacterial F-type ATPases and the nucleotide binding fold of other ATPases. This similarity suggested that ATP hydrolysis may drive Fe(II) uptake by FeoB (24). Similar sequences were not found in FET4, indicating that the yeast transporter and FeoB may function as Fe(II) transporters in different ways. A third gene involved in Fe(II) uptake that has been isolated is *FET3*. While the FET3 multi-copper oxidase is required for the high affinity system, it does not appear to encode a transporter protein *per se*. The FET3 protein has only a single potential transmembrane domain (25), an unlikely property for a transporter protein. We predict that the high affinity system has as yet unidentified protein components, perhaps complexed with FET3, that mediate the transport of Fe(II) across the plasma membrane.

Our results demonstrate that the high and low affinity systems are separate uptake pathways. Mutations in the *FET3* gene eliminated high affinity activity without greatly altering the low affinity system. Similarly, mutations in the *FET4* gene reduced low affinity uptake but not high affinity activity. We have also shown that the low affinity system is a biologically relevant source of iron for growing yeast cells. Previous biochemical and genetic studies on high affinity uptake demonstrated the importance of this system for the growth of iron-limited cells (6, 25). In contrast, the low affinity system has an apparent K_m approximately 300-times greater than that of the high affinity system. This low affinity called into question whether this system could serve as a mechanism for Fe(II) uptake under any but the most iron-rich conditions (5). Anal-

ysis of iron-limited growth of *fet3* and *fet4* mutants, as well as assays of Fe(III) reductase and Fe(II) uptake activity in high iron grown strains, has established that the low affinity system is an important source of iron under standard culture conditions. The high affinity system is induced under conditions of iron-deprivation. In *fet4* single mutants, high affinity activity was increased, suggesting that *fet4* mutant cells are more iron-limited than wild type cells. The low affinity system may also be induced by iron limitation. This was suggested by the fact that the V_{max} of the low affinity system is 2-fold higher in *fet3* mutant cells than in the wild type strain. Fe(III) reductase activity was induced in the *fet3 fet4* double mutant. We propose that the synergistic effect of *fet3* and *fet4* mutations on this property is due to an iron deficiency produced by the loss of both high and low affinity systems. Also consistent with this hypothesis, we observed that overexpression of *FET4* caused a decrease in the activity of the high affinity system. *FET4* overexpression and the resultant increase in low affinity activity may provide more iron to the cell via this pathway and cause the down-regulation of the high affinity system. An alternative explanation for these results is that the low and high affinity systems share a common subunit. Overexpression of *FET4* might then titrate this subunit away from the high affinity system, thus reducing the activity of this system. Conversely, disruption of the *FET4* gene might increase activity of the high affinity system simply because more of the limiting subunit is now available for its function.

Does the low affinity system play a role in the transport of transition metals other than Fe(II)? Fuhrmann and Rothstein (39) presented evidence for a divalent cation transporter in *S. cerevisiae* with an affinity series of Co(II), Zn(II) > Mn(II) > Ni(II). More recently, Norris and Kelly (40) demonstrated that Cd(II) was also a substrate for this system. Thus, it would appear from these studies that the major pathway for the accumulation of Mn(II) and Zn(II) was also capable of transporting Cd(II) and Co(II). Neither Zn(II) nor Mn(II) inhibited uptake of Fe(II) by the low affinity system, suggesting that this system, although perhaps capable of transporting Co(II) and Cd(II), is not the divalent cation transport system previously described.

Finally, our results suggest that *S. cerevisiae* may have iron uptake pathways in addition to the high and low affinity systems that have now been characterized. A strain lacking both of these systems, the *fet3 fet4* double mutant, grows well in spite of the almost undetectable level of iron uptake activity observed in this strain. Undoubtedly, these cells are obtaining iron. This uptake may represent residual activity of the high or low affinity systems, or a third system altogether. This activity may not be detectable in our uptake assays for a variety of reasons. For example, a transporter with very low affinity for Fe(II) may not be able to bind iron in the presence of the high concentration (20 mM) of citrate, an iron chelator, present in our assay medium. Alternatively, siderophore-mediated uptake would not be detectable in our experiments because the cells are washed free of the extracellular medium, and any siderophores present, prior to assay. Experiments designed to detect siderophore production in *S. cerevisiae* indicated that this organism does not produce these compounds (2). This previous research was performed with a wild type strain, and it is feasible that a latent siderophore-mediated system could be induced when both high and low affinity Fe(II) uptake systems are inactivated. Whatever the mechanism, it is now accessible to genetic and biochemical analysis. Just as *fet3* mutant strains made this study of the low affinity system possible, *fet3 fet4* double mutants will enable the analysis of additional iron-uptake pathways.

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