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MicroReview

Molecular mechanisms of iron uptake in fungi

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Summary

Fungi, like all free-living organisms, are in competition for limiting nutrients. In accumulating iron, fungi are faced also with a trace metal whose aqueous and redox chemistry make it both relatively bio-unavailable and strongly cytotoxic. Successful adaptation to this environmental context has provided fungi with an iron uptake strategy that has three features: it relies on redox cycling to enhance iron bio-availability and reduce iron cytotoxicity; it includes both high- and low-affinity pathways that are mechanistically distinct; and it is autoregulating so as to maintain intracellular iron homeostasis. Using *Saccharomyces cerevisiae* as a paradigm, this review summarizes current knowledge about the four pathways by which this yeast accumulates iron. These four pathways include: siderophore iron accumulation; high affinity iron uptake *via* an iron permease; and two lower affinity uptake pathways through relatively non-specific divalent metal ion transporters. All of these four pathways are directly or indirectly dependent on the activity of metalloreductase activity expressed extracellularly on the plasma membrane. A variety of experimental and genomics data indicate that this resourcefulness is shared by many, if not most, fungi. On the other hand, while the autoregulation of iron metabolism in Baker's yeast is well-understood, little is known about the apparent homeostatic mechanisms in these other yeasts and fungi. The integration of these multiple uptake mechanisms and their regulation into overall iron homeostasis in yeast concludes this brief review.

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Introduction

Iron is essential to the growth and proliferation of the vast majority of microorganisms. This essentiality derives from the role that iron in its biochemically accessible valence states plays in a wide variety of electron transfer processes. The few organisms that can substitute other transition metals in these diverse reactions, e.g. cobalt, manganese and copper, alone can thrive in the virtual absence of bio-available iron. This article briefly reviews our current knowledge and understanding about the mechanisms adopted by fungi to accumulate this essential redox cofactor.

Four features of iron chemistry and biochemistry have shaped the evolution and selection of those iron uptake mechanisms adopted. First is the redox chemistry of iron (Cotton *et al*., 1999; Lancashire, 2002). The reduction potential of the ferric/ferrous couple, $E^{\circ\prime}$ (Fe^{3+}/Fe^{2+}) at neutral pH is +0.77 V (Loach, 1968). This value makes this couple reducing in comparison to dioxygen reduction to water (+0.82 V). Simply put, under air iron is stable as Fe^{3+}; Fe^{2+} will spontaneously auto-oxidize. Second is the aqueous solution chemistry of ferric versus ferrous iron (Wilkins, 1991; Cotton *et al*., 1999; Lancashire, 2002). Fe^{3+} is essentially insoluble in water at neutral pH. In aqueous solution, $Fe^{3+}(H_2O)_n$ 'hydrolyses', that is, it loses protons to form a ferric hydroxide species that polymerizes into an amorphous gel. The initiating proton ionization occurs because Fe^{3+} is a strong Lewis acid. The relatively strong positive nuclear charge on Fe^{3+} promotes proton loss from the coordinated water molecules; the pK_a of bound water is ~ 3 (Lancashire, 2002). In contrast, Fe^{2+} is a fairly weak Lewis acid because the nuclear charge is suppressed by an additional electron. The waters bound to Fe^{2+} do not ionize readily and therefore aqua ferrous ion is relatively stable to hydrolysis – and soluble – at neutral pH. Third is the coordination chemistry of ferric versus ferrous iron. Fe^{3+} binds its ligands more tightly (due in part to the charge difference). This characteristic makes ferric iron complexes 'exchange inert'; that is, a ligand to Fe^{3+} in a complex can be replaced – exchanged with – an exogenous ligand only with difficulty. This is typically caused by a large kinetic barrier to the ligand exchange (Wilkins, 1991). Again, in contrast, ligand exchange in ferrous iron com-

plexes is much more facile. For example, H_2O exchange at Fe^{2+} is ~ 10^4 faster than at Fe^{3+} in otherwise similar ligand environments (Wilkins, 1991). Thus, even if the hydrolysis of $Fe^{3+}(H_2O)_n$ is suppressed, as it is at acidic pH values, Fe^{3+} still is relatively inaccessible to biologic ligands. And fourth is biologic competition – all the neighbours are out scavenging the available iron, too.

In this chemical and biologic context, success at obtaining sufficient iron to support competitive growth requires an iron accumulation mechanism that at the least addresses two problems: the strongly limited bioavailability of the dominant form of iron in the environment – Fe^{3+} – and the pervasive competition for this limited and growth-limiting nutrient. Furthermore, as outlined above, aerobic iron chemistry is so circumscribed. Consequently, the number of possible mechanisms effective in scavenging and accumulating iron will be similarly highly circumscribed: not only are most organisms competing for the same, limiting nutrient, but they will be constrained to use the same basic strategy(ies) to acquire it.

Obviously, the characteristics of an evolutionarily competitive strategy address the nature of aqueous iron chemistry as described above. First, this strategy must address the exchange-inert, insoluble nature of aqueous ferric iron in contrast to the more tractable properties of Fe^{2+}. This contrast suggests one feature of an effective strategy: labilize and solubilize Fe^{3+} by reducing it to Fe^{2+}. Indeed, essentially all eukaryotic cells express a functionally extracellular, plasma membrane metalloreductase that catalyses a first step in iron accumulation (Askwith and Kaplan, 1998; Dancis, 1998; Leong and Winkelmann, 1998; Nelson, 1999). Second, any strategy must address the problem of the local competition: grab the iron and hold on tight. Thus, the successful organism elaborates a 'high-affinity' system, one that binds iron with an association constant that can compete with the kinetic and equilibrium processes that lead to the precipitation of Fe^{3+}, with the auto-oxidation of Fe^{2+}, and with the sequestration of iron by the neighbours. Two such system types have evolved, one type based on extracellular chelation of ferric iron, the other type based on ferric iron reduction and subsequent 'free' iron permeation.

As essential as iron is for reproductive growth, it is equally cytotoxic. That is, ferrous iron, in particular, is a strong pro-oxidant. Fe^{2+} auto-oxidation produces superoxide radicals whereas the even more favourable oxidation by H_2O_2 produces the hydroxyl radical (Halliwell and Gutteridge, 1989). The latter is perhaps the most reactive and cytotoxic species encountered by aerobic and microaerobic cells under normal growth conditions. Thus, aerobic organisms have a dilemma: they need a scavenging system that is effective in accumulating iron yet can't afford to accumulate too much iron. The obvious solution is iron-dependent regulation of this scavenging system so that its activity is reduced in proportion to increases in cell iron. In fact, such regulation of high-affinity iron accumulation is seen in all microorganisms irrespective of the mechanism by which they accumulate this metal ion. But this solution imposes a second dilemma, namely, even when iron is highly available, and thus the high affinity system is 'off', cells still need iron. It is no surprise therefore that *Saccharomyces cerevisiae*, for example, produces both high and low affinity iron uptake systems, with the latter supplying iron to the cells in growth media that is replete with bioavailable iron (Dancis, 1998; Leong and Winkelmann, 1998; Nelson, 1999; Radisky and Kaplan, 1999). Indeed, baker's yeast expresses essentially every one of the four classes of iron uptake systems that have been characterized in eukaryotes.

The objective of this review is to summarize current information about these various iron sequestration strategies; about the functional properties of the protein components associated with each; and, briefly, about their integration into a rational iron homeostasis in fungi. Much of the primary data presented will be from *S. cerevisiae* as the most complete picture of fungal iron accumulation mechanisms has been obtained in this yeast. This is because of two features of the research in fungi in general: the comparative ease of doing classical and molecular genetic experiments in 'yeast' and the fact that its genome, alone among those of other fungal species, has been fully sequenced and annotated. Thus, in baker's yeast, we know that various iron permeases are encoded and produced as are a number of siderophore facilitators. Furthermore, the regulation of these iron uptake activities by environmental iron is fairly well understood. At least with respect to fungal iron accumulation, *S. cerevisiae* represents the quintessential paradigm. Table 1 lists proteins in *S. cerevisiae* involved in iron uptake mechanisms of all types and their functions in the various pathways of iron accumulation.

Uptake of siderophore iron

Among the subdivisions of the kingdom of fungi, iron uptake mechanisms in taxa of two of these subdivisions have been fairly well characterized: *Basidiomycota* and *Ascomycota*. The prime example from the former is *Ustilago maydis*, the corn smut (Ardon *et al*., 1997; 1998). Examples of *Ascomycota* include fungi from all three subclasses: for example, *Schizosaccharomyces pombe* (*Archaeascomycetes*); *Neurospora crassa* and *Aspergillus nidulans* (*Euascomycetes*); and *Saccharomyces cerevisiae* and *Candida albicans* (*Hemiascomycetes*). In all of these organisms with the exception of *S. pombe*, the presence of two uptake mechanism types has been established or inferred from proteomic analysis: one that depends on the extracellular reduction of ferric to ferrous

Table 1. The genomics and proteomics of fungal iron transport.

Uptake pathway	Protein component (substrate)	Organism/gene name (Archival designation)			
		S. cerevisiae (Systematic name)[a]	*C. albicans* (Stanford ORF)[b]	*S. pombe* (Proteome, Inc, PombePD™)[c]	*N. crassa* (Neurospora sequence databases)[d]
High affinity ($K_M < 2\ \mu M$)					
Siderophore-dependent	Facilitators (Fe^{3+}complex)	ARN1 *(YHL040C)* *TAF1* (YHL047C) *SIT1* (YEL065W) *ENB1* (YOL158C)	*CaSIT1* (orf6.6084)[e] (orf6.8902) (orf6.8417) (orf6. 1908)	None archived[f]	1C8/3.446 B15M5/3.356
Reductase/ferroxidase dependent	Reductases (Fe^{3+}, Cu^{2+} free or complexed)	FRE1 *(YLR214W)* FRE2 (*YKL220C*) FRE3 *(YOR381W)* FRE4 *(YNR060W)* FRE5 *(YOR384W)* FRE6 *(YLL051C)* FRE7 *(YOL152W)*	*CFL1* (orf6.5730) *CFL95* (orf6.6384) 6.2162 6.5606 6.1281 6.5676	frp1 (SPBC1683.09c) Homologous ORFs: (SPBC947.05c)	B15B3/3.88
	Ferroxidases (free Fe^{2+})	*FET3* (YMR058W) *FET5* (YFL041W)	*CaFET31* (orf6.5008)[g] *CaFET32* (orf6.5009) *CaFET33* (orf6.5010)	fio1 (SPAC1F7.08)	B23H20–030/3.186 3.237 3.321
	Permeases (ferroxidase-generated Fe^{3+})	*FTR1* (YER145C) *FTH1* (YBR207W)	*CaFTR1* (orf6.8119) *CaFTH1* (orf6.3061)		9A62/3.186
Low affinity ($K_M > 2\ \mu M$)					
Reductase/permease dependent	Permeases (free Fe^{2+}) (For Smf proteins: Mn^{2+})	*FET4* (YMR319C) *SMF1* (YOL122C) *SMF2* (YHR050W) *SMF3* (YLR034C)	None archived *CaSMF12* (orf6.3571) *CaSMF2* (orf6.7486) (orf6.460)	None archived pdt1 (SPAC27F1.08)	None archived 3.442 B11B22/3.514

a. *http://genome-www.stanford.edu/Saccharomyces/*. The systematic name is given in parentheses following the gene name.
b. *http://www-sequence.stanford.edu/group/candida/* and *http://genolist.pasteur.fr/CandidaDB/*. The latter site, maintained by the Galar Fungail Consortium, is linked directly to the SGD Database. The ORF number given by the Stanford Sequencing Project is in parentheses following the gene name used by the Consortium.
c. *http://www.proteome.com/databases/*. The Proteome Inc PombePD™ ORF designation is given in parentheses following the gene name.
d. *http://mips.gsf.de/proj/neurospora/*. Designations of ORFs archived in the German Sequencing project begin with a letter; those found in the Whitehead database are wholly numeric.
e. Except where designated by gene name, orthologues are listed in decreasing order of their identity/similarity to the query protein that, in all cases, is the index member of that protein class in *S. cerevisiae*.
f. 'None archived' indicates that no orthologous protein was identified in a given database by a tBLASTn search.
g. These are tandemly repeated ORFs. *CaFET31 has* the gene name *CaFET3*.

iron, another that depends on the internalization of an Fe^{3+}-siderophore complex. Homologous and orthologous loci encoding the proteins involved in these two iron uptake pathways in four fungal species are listed in Table 1.

Thus, in common with many prokaryotes, many if not most fungi express a siderophore-dependent iron uptake activity; in fact, data indicate that most species elaborate multiple such activities (Table 1). A common type is the ferrichrome-type siderophore uptake process although many fungal species produce other types of siderophores and facilitators for them, e.g. fusarinine, coprogen, rhodotorulic acid and rhizoferrin (Leong and Winkelmann, 1998). Generically, ferrichromes have a cyclic hexa- or heptapeptide structure, commonly Gly_3Orn_3, where Orn is ornithine (Albrecht-Gary and Crumbliss, 1998; Leong and Winkelmann, 1998). The ornithyl ε-amino groups are additionally modified by acetylation and hydroxylation (N^5-hydroxy-N^5-acyl) so that ferrichromes functionally are hydroxamates. In fact, a majority of siderophores contains hydroxamic acid functional groups (ferrichromes and the acyclic ferrioxamines; and fusarinines, coprogens and rhodotorulic acid), even if they also possess one of the other types of strong Fe^{3+} binding elements found in siderophores, i.e. catecholate and α-hydroxycarboxylic acid functionality. This review will focus on the uptake of the ferric siderophore complex, not on the biosynthesis and secretion of the siderophore itself.

Fungal siderophore iron uptake is mediated by plasma membrane permeases, all of which are members of the major facilitator superfamily (MFS) (Nelisson *et al*., 1997; Pao *et al*., 1998). These facilitators are composed of a single polypeptide chain and are all secondary transporters, that is, they are capable of transporting their respec-

tive solute molecules only down a (electro)chemical gradient established by some primary metabolic pump. Thus, they typically are co-transporters, with sym- or antiport transport of the primary solute driving uptake of the specific, secondary one. Biochemical, structural details of the fungal siderophore facilitators are lacking, but homology modeling indicates that that they contain 12 or 14 transmembrane domains. Nothing is known about the structure of these facilitators in the plasma membrane. One possible structure is a barrel composed of helical 'staves.' This concept is based on the structure of the FhuA ferrichrome transporter from *E. coli* that is a β-barrel composed of 21 antiparallel β-strands (Locher *et al*., 1998). A globular domain folds within the barrel to which the ferrichrome binds. It is this domain that confers siderophore specificity to FhuA and may drive uptake as indicated by structural studies of the FhuA homologue, FecA (Ferguson *et al*., 2002).

With respect to siderophores as a group, baker's yeast is the quintessential scavenger. Although producing no siderophores of its own, *S. cerevisiae* produces at least four distinct facilitators for their uptake (Table 1). These are: Arn1p (Yun *et al*., 2000a, b); Arn2p (Yun *et al*., 2000a, b), also known as Taf1p (Heymann *et al*., 1999); Arn3p (Yun *et al*., 2000a, b), better known as Sit1p (Lesuisse *et al*., 1998); and Arn4p (Yun *et al*., 2000a, b), also named Enb1p (Heymann *et al*., 2000a). The conflicting nomenclature has resulted from these loci being isolated more than once and in different labs. Two have been isolated as a consequence of complementing a defect in siderophore uptake: *TAF1* conferring triacylfusarinine uptake (Heymann *et al*., 1999), and *ENB1* conferring enterobactin (enterochelin) uptake (Heymann *et al*., 2000a). In as much as this specificity is most likely correct, these designations are appropriate. In contrast, the *ARN* notation resulted from the fact that the mRNAs for these facilitators were detected in a microarray as Aft1p-regulated (Yun *et al*., 2000a). Thus, *ARN* stands for *A*ft1p *R*eguloN. [Independently, Heymann and colleagues used 'ARN' to designate a specific class of ferrichromes possessing *A*nhydromevalonyl *R*esidues linked to N^{δ}-ornithine: *ARN* (Heymann *et al*., 2000b; see also below).] Aft1p is the primary, iron-regulated transcriptional activator of genes encoding iron uptake activities in *S. cerevisiae* (Yamaguchi-Iwai *et al*., 1996; Dancis, 1998). The *SIT* notation is more appropriate – *s*iderophore *i*ron *t*ransport – but suffers from a lack of specificity because 'siderophore' refers to all of these Fe^{3+} chelating agents, whereas Sit1p/Arn3p exhibits some selectivity for ferrioxamine B (FOB) (Lesuisse *et al*., 1998; 2001; Yun *et al*., 2000b). Nonetheless, this reviewer agrees with Lesuisse *et al*. (2001) who urged using gene designations that have been made on the basis of phenotype (*TAF1* and *ENB1*), or function (*SIT1*), and only lastly ones made on the basis of expression alone (*ARN1*). The designation *SIT* will be used when referring collectively to this group of genes irrespective of fungal species.

The primary information for the presence of these proteins in *S. cerevisiae* comes from either genetic complementation screens and/or by mining the yeast genome thus illustrating the experimental features of this fungus that have made it the leading model organism in studies on iron metabolism. Thus, *SIT1* (*ARN3*) was shown to encode a putative FOB facilitator by rescue, with a genomic library containing *SIT1*, of a mutant strain that lacked iron uptake activities of all types from failure to grow on FOB iron (Lesuisse *et al*., 1998). At the same time, the *SIT1* ORF – YEL065W (Table 1) – was recognized in the yeast genome as a likely MFS protein, albeit of unknown function (Nelisson *et al*., 1997; Pao *et al*., 1998). Isolation and characterization of the genomic clone that restored the mutant strain's ability to grow on FOB showed the clone to contain YEL065W; subsequent targeted disruption of this locus in the *SIT1* wild-type strain demonstrated that YEL065W was essential to iron accumulation from FOB.

This approach, in which the ability to grow, or not grow, on a single type of siderophore iron is matched to genotype, was used to suggest that *TAF1* (*ARN2*) encodes a facilitator specific for triacetylfusarinine (Heymann *et al*., 1999); that *ARN1* encodes a facilitator specific for a class of ferrichromes possessing anhydromevalonyl residues (Heymann *et al*., 2000b); and that *ENB1* (*ARN4*) encodes a facilitator specific for ferric enterobactin (Heymann *et al*., 2000a). The latter inference demonstrates the parasitic nature of *S. cerevisiae* when it comes to iron scavenging. Cyclic triester-based siderophores like enterobactin are not produced by fungi but are produced by many bacteria. One can assume that the role that Enb1p plays in yeast iron metabolism varies inversely with the quality of one's sterile technique. (Tit-for-tat: *Escherichia coli* doesn't produce ferrichromes, but does express FhuA on its outer membrane to scavenge this fungal siderophore!)

This precise assignment of the above siderophore specificity to these four facilitators has been questioned. Lesuisse and colleagues constructed otherwise isogenic strains of *Δarn1*, *Δtaf1*, *Δsit1*, and *Δenb1* genotype, respectively, evaluated iron accumulation from several iron sources and compared these values to uptake by the parental strain (Lesuisse *et al*., 2001). These comparisons are shown in Fig. 1. The data indicate that with respect to the four kinds of siderophore tested these facilitators are, more than anything, redundant. The strong exceptions to this broad conclusion are: Taf1p does confer a strong selectivity towards triacylfusarinine as a siderophore while Sit1p strongly supports the cells' uptake of iron from ferrioxamine B in comparison to a ferrichrome (ferrichrome or ferricrocin). These investigators did not directly test the proposed specificity of Enb1p towards enterobactin,

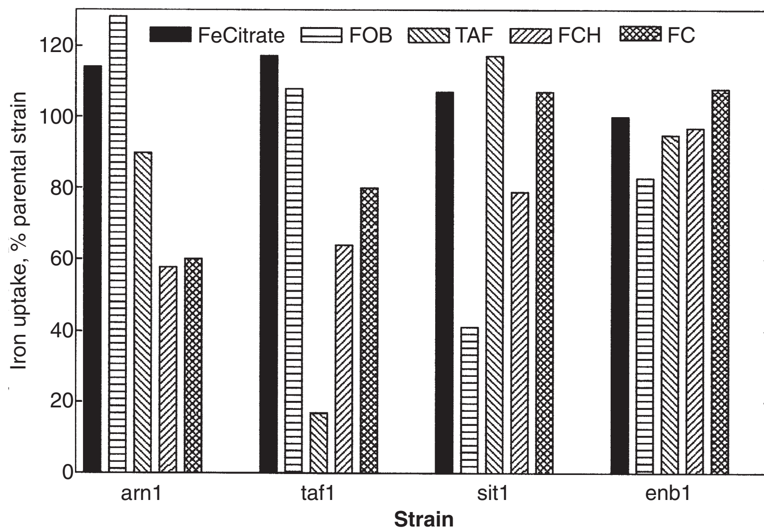

Fig. 1. Specificity of uptake of iron from ferric citrate and various ferric siderophore complexes by *S. cerevisiae*. ^{59}Fe uptake was measured in yeast strains carrying deletions in the siderophore permease loci as indicated; uptake values are presented as a percent of uptake by the parental wild type strain. The ferric complexes used were: $Fe(citrate)_3$; Ferrioxamine B (FOB); triacetylfusarinine C (TAF); ferrichrome (FCH); and ferricrocin (FC). The values in the figure are calculated from data presented in Lesuisse *et al*. (2001).

although their data show that this facilitator plays no role in the accumulation of iron from the other siderophores. Also, in comparing iron accumulation from these various sources in strains of different genetic background they noted significant variation in this pattern of facilitator 'specificity'. One explanation for this variation is that specificity is a multigenic property, that additional genetic loci contribute directly or indirectly to the ability of a given cell to accumulate iron from one versus another siderophore.

Yun and colleagues provide uptake data for ferrichrome, ferrioxamine and fusarinine iron in single and multiple 'arn' mutants (Yun *et al*., 2000b). They showed a 75% inhibition of iron uptake from FOB in a *Δsit1* strain but no inhibition in a *Δarn1* one. They also showed that the *arn1* mutant exhibited no defect in iron uptake from any of the siderophores. These results were similar to those of Lesuisse and colleagues. However, Yun and colleagues further demonstrated an essentially complete inhibition of ferrichrome uptake in the *Δarn1Δsit1* double mutant. Deletion of neither of the other two SIT loci, *TAF1* and *ENB1*, in the *Δsit1* background led to a similar complete loss of function. They did not test the double mutants for iron uptake from ferrioxamine, however, so their two sets of data cannot be directly compared. Although Yun *and* colleagues do not proffer an explanation for the apparent synergism between *Δsit1* and *Δarn1* in ferrichrome iron uptake, one explanation is simply that whereas Sit1p dominates in this uptake (at least 70% in wild-type cells), Arn1p does provide a fraction of the total. Furthermore, these results do contrast in general with those of Lesuisse and colleagues who concluded that if Sit1p had any specificity it was towards FOB not ferrichrome (Lesuisse *et al*., 2001). Lastly, Yun and colleagues also tested TAF uptake in various multiple mutants (Yun *et al*., 2000b). The 90% loss of TAF iron uptake in the *Δtaf1* background was not increased by deletion of additional *SIT* loci consistent with the inference that Taf1p alone among these Sit proteins was highly specific for fusarinine complexes.

One aspect of the work described by Yun and colleagues deserves comment and may relate to the inconsistencies noted. In many of the *arn* deletion strains used, the gene encoding a component of the other high affinity uptake system in yeast (and other fungi), *FET3*, was also deleted. Putting aside the reasons for using a *Δfet3* background in assigning function to a given Sit protein (see below), the apparent specificity of a particular facilitator *was* altered in this background. Thus, whereas Lesuisse *et al*. (2001) showed that deletion of *SIT1* had essentially no effect on ferrichrome utilization (Fig. 1), Yun *et al*. (2000b) showed that in the *Δfet3* background, deletion of *SIT1* resulted in the 65% loss of ferrichrome iron accumulation noted above. This result appears to confirm the inference made by Lesuisse and colleagues that siderophore uptake in *S. cerevisiae* is multigenic and serves as a caution in designing experiments and comparing results from different laboratories.

Reductase-dependent iron uptake

Fungi produce – or appear to encode in their genomes – one or more integral membrane reductases (Table 1). All are likely to be relatively non-specific. For example, the most abundant plasma membrane reductase in *S. cerevisiae*, the Fre1 protein, exhibits comparable activity towards Fe^{3+} and Cu^{2+}, and readily measurable activity towards one-electron acceptors like paraquat (methyl viologen), INT (iodophenylnitrophenylphenyltetrazolium chloride) and TTC (triphenyltetrazolium chloride) (Hassett and Kosman, 1995). A plasma membrane reductase like Fre1p is required for the cell accumulation of iron present

in the growth medium as Fe^{3+} in many different cell types, from fungi to those from plants and animals (Askwith and Kaplan, 1998; Dancis, 1998; Leong and Winkelmann, 1998; Nelson, 1999; Radisky and Kaplan, 1999).

As noted above, reduction of Fe^{3+} to Fe^{2+} does 'labilize' the metal ion, kinetically. The importance of this reduction-labilization to uptake is indicated by the fact that stabilizing the ferric iron so that it becomes resistant to reduction, that is, lowering its oxidation potential relative to that of the reductase, blocks iron uptake (Hassett and Kosman, 1995). Thus, citrate inhibits reduction of Fe^{3+} and uptake while EDTA completely inhibits both processes; E° for the Fe(III)citrate/Fe(II)citrate couple is ~ 0.0 V, while the E° for the Fe(III)EDTA^{-}/Fe(II)EDTA2^{-} one is –0.2 V. These values show that carboxylato complexes of Fe^{3+} are reducing relative to dihydroascorbic acid, for example (E° = + 0.06 V), and not much more oxidizing than is NAD^{+} (E° for the NAD^{+}/NADH couple is –0.32 V). The latter is used for comparison as it is likely that NAD(P)H is the reducing substrate in the reductase reaction (Lesuisse *et al*., 1996). At an even further extreme would be complexes of Fe^{3+} with essentially any of the siderophores which, at a minimum, exhibit a 10^{10}-fold greater affinity for ferric versus ferrous iron. The stability of the ferric-siderophore complex in comparison the ferrous one stabilizes the Fe(III) valence state; for example, E° values for ferric complexes of trishydroxamate siderophores are all in the range of –0.5 to –0.4 V, reducing even with respect to NADH (Albrecht-Gary and Crumbliss, 1998). In other words, although siderophore iron is taken into the fungal cell, it thermodynamically cannot readily do so *via* a reductase-dependent pathway as the ferric iron in the complex would be a poor substrate for the reductase.

Whereas reduction serves to make iron available for uptake, it also dictates the valence state of the iron that is substrate for a subsequent uptake pathway, i.e. Fe^{2+}. *Saccharomyces cerevisiae* again serves to illustrate all of those mechanisms that use this ferrous iron substrate for uptake. The mechanisms fall into two types: one that involves no further metabolism of the Fe^{2+}, that is, one in which the ferrous iron is transported directly into the cell; and a second mechanism that involves a reoxidation of the Fe^{2+} to Fe^{3+}, known generically as ferroxidation. Ferroxidation is ubiquitous in eukaryotes and is catalysed by a multicopper oxidase enzyme. These enzymes are known as ferroxidases (Frieden and Osaki, 1974).

Direct ferrous iron uptake

Ferrous iron is taken directly into cells through divalent metal ion transporters. Like the reductases, these are not specific to Fe^{2+} but can serve also as transporters for Mn^{2+}, Ni^{2+}, and Cu^{2+}, for example. There is a family of such transporters in *S. cerevisiae* encoded by *SMF1-3* (Pinner *et al*., 1997; Chen *et al*., 1999; Cohen *et al*., 2000). These transporters are orthologues of the Nramp proteins found in animal cells. For example, Nramp2, also known as DCT1, is the dominant plasma membrane divalent metal ion transporter found in all epithelial cells (Canonne-Hergaux *et al*., 1999). All archived fungal genomes contain these Nramp orthologues (Table 1).

Smf1p is somewhat different from the mammalian proteins in that it also supports the uptake of monovalent alkali metals (Li^{+}, K^{+} and Rb^{+}) (Chen *et al*., 1999). The transport of monovalent ions may be gratuitous. This is suggested by the fact that Fe^{2+} uptake through Smf1p is saturable (K_m = 2.2 μM) whereas sodium uptake is not. Also, iron but not sodium uptake through Smf1p is H^{+}-coupled, as is iron uptake through DCT1. The data indicate that this Fe^{2+}/H^{+} co-transport is stoichiometric, that is, one H^{+} for one Fe^{2+}. Thus, like the Sit proteins – or facilitators in general – the Smf proteins are secondary transporters in that they use a chemical gradient as driving force that is established by some other metabolic activity. In a free-living organism like a fungus, this gradient may be simply due to the pH of the growth medium, which, of course, can be modulated by metabolite utilization and/or secretion. Alternatively, the ATPase activity common to fungal plasma membranes may establish the necessary gradient. Neither model has been rigorously tested experimentally.

A second type of 'direct' ferrous iron transporter has been characterized in *S. cerevisiae*, but not in other fungi. This uptake is due to the protein encoded by *FET4* (Dix *et al*., 1994; 1997). Fet4p is a type 2 transmembrane protein that likely has six transmembrane, helical domains. Unlike all the other iron uptake transport processes, the Fet4p uptake is a low affinity one. That is, while the K_M values for the substrate for iron accumulation – ferric siderophore or Fe^{2+} – for the pathways summarized above vary from 0.1 to 2 μM, the K_M for Fe^{2+} uptake through Fet4p is 35 μM. On the other hand, like the Smf proteins, Fet4p has a relatively low substrate specificity. For example, it supports low affinity uptake of copper as well, K_M = 35 μM (Hassett *et al*., 2000). There is no homology between the Smf and Fet4 proteins; also, whereas iron transport through the former is proton-coupled, there is no evidence that the same is true of iron transport through the latter. Indeed, there appear to be no orthologues of Fet4p, based on a search of currently archived genome databases (Table 1).

Indirect ferrous iron uptake: ferroxidation/permeation

In baker's yeast, these latter two activities are provided by the Fet3 (Askwith *et al*., 1994; de Silva *et al*., 1997) and Ftr1 (Stearman *et al*., 1996) proteins respectively (Table 1). Fet3p is a type 1 membrane protein with a

single carboxy-terminal transmembrane domain. Fet3p is a multicopper oxidase (Hassett *et al.*, 1998) possessing the three distinct types of copper sites that have been characterized in copper proteins (Solomon *et al.*, 1996). Thus, Fet3p has one type 1, or 'blue' copper site; one type 2, or non-blue copper site; and one binuclear copper cluster, designated a type 3 site, in which the unpaired electrons on the two cupric ions in the cluster are spin-coupled. The organization of these sites is illustrated by the proposed structure of the Fet3 protein, Fig. 2A. As noted in the figure, oxidation of Fe^{2+} occurs at the type 1 copper site, whereas dioxygen is reduced to $2H_2O$ at the trinuclear cluster.

Post-translational processing of Fet3p includes insertion of the four copper atoms. This incorporation appears to occur in a *trans*- or post-Golgi compartment and is dependent on the Ccc2 protein, a P-type, copper ATPase (Yuan *et al.*, 1995; 1997). Targeting and processing also depends on the coproduction of the Ftr1 protein, the high affinity iron permease (Stearman *et al.*, 1996). That is,

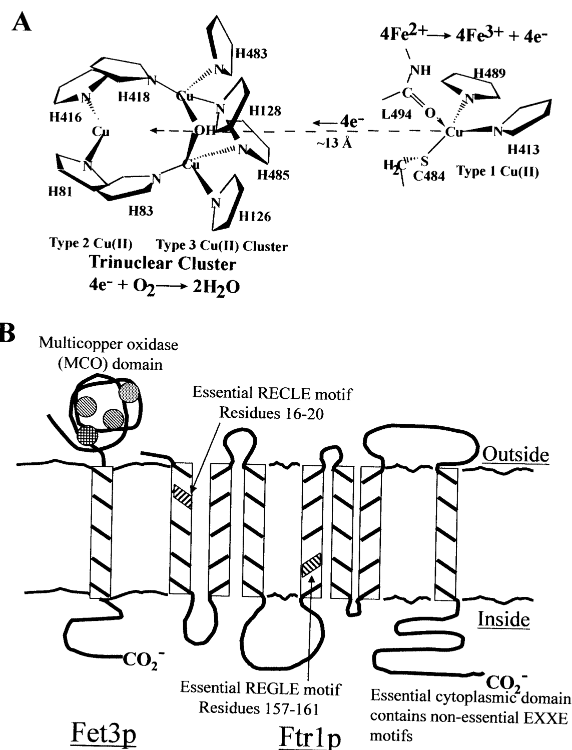

Fig. 2. The Fet3p ferroxidase and Fet3p/Ftr1p complex in *S. cerevisiae*.
A. A model of the relationship between the ferroxidase (type 1 copper) and oxygen reduction (trinuclear cluster) sites in Fet3p.
B. A model of the Fet3p/Ftr1p complex in the yeast plasma membrane. The topoplogy and orientation of Fet3p has been confirmed (de Silva *et al.*, 1995; Hassett *et al.*, 1998); the Ftr1p topology and orientation is based on sequence analysis. The MCO domain in Fet3p contains three copper sites: type 1 (shaded); type 2 (hatched); and type 3 binuclear cluster (stippled).

Fet3p produced in a cell of *Δftr1* genotype is retained in a cytoplasmic compartment in a copper-free free, inactive form. Correspondingly, Ftr1p produced in a cell of *Δfet3* genotype fails to reach the plasma membrane. These facts suggests a model in which the two proteins form a heterodimer or higher order structure, and it is this oligomer that is substrate for correct maturation and trafficking to the plasma membrane. The orientation and topology of Ftr1p is not known. Modeling suggests that it has either six or seven transmembrane domains. A cartoon depicting the latter model is shown in Fig. 2B. The complementary orientation of Fet3p is shown, also; this orientation has been confirmed (de Silva *et al.*, 1995; Hassett *et al.*, 1998).

Noted in the model of Ftr1p are two structural elements that are required for iron uptake. First, truncation of the cytoplasmic (in this model), C-terminal domain strongly reduces iron uptake without affecting the maturation and targeting of the Fet3 and Ftr1 proteins. (Stearman *et al.*, 1996) Within this domain are a series of EXXE motifs that potentially were responsible for the permeation activity supported by the C-terminus. However, substitution of one or all of the glutamic acid residues within these motifs with glutamine has no effect on iron uptake activity (Severance *et al.*, 2003). The second structural element type is represented by the REXLE motifs noted in the model; both elements, residues 16–20 and 157–161, respectively, are likely to be within transmembrane domains. Substitution of either (or both) E residue in either motif results in an Ftr1 protein that, although targeted to the plasma membrane (along with Fet3p) is inactive in iron uptake (Stearman *et al.*, 1996; Severance *et al.*, 2003). A glutamate residue in Fet3p essential to iron uptake has been identified, also: E185. Whereas the Fet3p(E185A) mutant retains ferroxidase activity, it does not support iron uptake (Severance *et al.*, 2003). Thus, this residue could be involved in the apparent direct channeling of Fet3p-produced Fe^{3+} to Ftr1p.

The mechanistic link between ferroxidation and iron permeation in which iron transport absolutely depends on the *coupling* of those two activities is not well understood. Indeed, even the biologic rationale, the selective advantage, of such a system is unclear. Put into context, this pathway, overall, is one of redox cycling environmental iron from ferric to ferrous to ferric prior to uptake. As discussed above, reductive mobilization is a clear advantage to an organism in its quest for iron; the advantage of subsequent reoxidation of the ferrous produced as a step coupled to permeation is not so obvious.

However, there is a clear kinetic advantage in that the K_M value for Fet3p/Ftr1p-mediated uptake is 0.2 μM (Dancis *et al.*, 1992) whereas, as noted, the K_M values for Smf1p and Fet4p uptake, both direct permeation pathways for reductase-produced Fe^{2+}, are 2 μM (Chen *et al.*,

1999) and 35 μM (Dix *et al*., 1994; 1997) respectively. On the other hand, the V_{max} values for Fet4p and Fet3p/Ftr1p uptake are comparable in cells producing only one or the other of these transport systems (Askwith *et al*., 1994; Dix *et al*., 1994; Hassett and Kosman, 1995; Stearman *et al*., 1996). This means that under conditions of iron limitation ([Fe] < 100 nM) the latter process will out-compete the former by a factor of ~ 200 (the ratio of their respective K_M values), a clear selective advantage to those organisms that possess this latter pathway. The kinetic constants for siderophore iron uptake are similar to those for Fet3p/Ftr1p accumulation (Yun *et al*., 2000b) indicating that this pathway provides a selective advantage under conditions of 'free' iron limitation as a result of extensive bacterial and fungal competition for environmental iron.

In addition, ferroxidation might actually protect the organism that possesses this pathway. The ferroxidases in mammals, ceruloplasmin in particular, appear to protect the organism from oxidative damage due to the pro-oxidant activity of Fe^{2+} (Qian and Ke, 2001). As noted in the Introduction, ferrous iron supports the production of the highly cytoxic hydroxyl radical. At the fungal membrane, one has the reductase-dependent generation of Fe^{2+}; ferroxidation could prevent the accumulation of this 'pro-toxin' while at the same time providing a substrate for uptake. This is a pathway that only aerobes can use as the ferroxidation reaction requires dioxygen. Of course, only in the presence of dioxygen does ferrous iron exhibit its cytotoxicity. In effect, with respect to iron accumulation, in adapting to geologic aerobiosis, facultative aerobes simply harnessed the same redox activity that makes ferrous iron toxic in coupling it enzymatically to iron uptake.

Regulation, functional overlap and iron homeostasis

An obvious question about iron uptake in fungi in general is whether *Saccharomyces cerevisiae* is a paradigm or an outlier. Do most or all fungal species elaborate multiple iron uptake pathways or has baker's yeast alone mastered all of them? The question itself may simply reflect the fact that it is only for *S. cerevisiae* that we have essentially all of the data, starting with a fully annotated genome. Although genomes of other fungi are not fully complete and/or annotated, they do provide evidence that *S. cerevisiae* is not unique (Table 1). For example, *Schizosaccharomyces pombe*, which produces a characterized reductase-ferroxidase uptake pathway (Askwith and Kaplan, 1997), is also likely to produce proteins with striking homology to Arn1p, Taf1p (Arn2p), and Sit1p (Arn3p). These are encoded by ORFs SPBC4F6, SPCC622 and SPAC1F8. Using Enb1p (Arn4p) as the query sequence identifies these three as well as four others, all of which, however, have a strongly reduced degree of homology. Thus, although not characterized in *S. pombe*, it would appear that a siderophore uptake pathway undoubtedly exists in this fungus. A similar search in the *Neurospora crassa* genome database uncovers a number of potential 'Sit' loci. Three of these – NCU07464.1, NCU06132.1 and NCU03789.1 – encode proteins that are ≥60% related to Arn1p. As noted above, the *N. crassa* genome also encodes ferroxidase and iron permease orthologues of Fet3 and Ftr1p, while the presence of both high affinity pathways – siderophore and ferroxidase-dependent – have been experimentally established in *Candida albicans* (Morrissey *et al*., 1996; Eck *et al*., 1999; Hammacott *et al*., 2000; Ramanan and Wang, 2000; Ardon *et al*., 2001; Knight *et al*., 2002). The evidence supports the conclusion that fungi in general are resourceful in their quest for iron, employing all of the mechanism types of iron accumulation that have been characterized in biology.

This apparent redundancy in metabolic function raises two questions: first, is there any mechanistic overlap between any two pathways, and, second, how is uptake through these multiple pathways integrated to provide for cellular iron homeostasis? For example, does any protein that functions in siderophore-dependent uptake also function in one of the other pathways? The one protein or activity that certainly might is the metalloreductase. Ferrireduction is definitely a prerequisite for uptake through the established reductase-dependent pathways, *via* an Smf-mediated process or a ferroxidase-dependent one. In addition, in *S. cerevisiae* siderophore iron uptake depends on the *FRE* gene products, too (Yun *et al*., 2001).

The *S. cerevisiae* genome contains seven *FRE* homologues (Martins *et al*., 1998; Georgatsou and Alexandraki, 1999). Deletion analysis indicates that only Fre1p and Fre2p function at the plasma membrane in support of reductase-dependent iron uptake whether *via* Fet4p, for example, or Fet3p/Ftr1p (Dancis *et al*., 1992; Georgatsou and Alexandraki, 1994; Hassett and Kosman, 1995). When assayed with either cupric or ferric ion as substrate, or by reductase-dependent copper or iron uptake, Fre1p accounts for >95% of the total reductase activity expressed on the plasma membrane of a wild-type yeast cell. Fre2p accounts for the rest (Georgatsou and Alexandraki, 1994; Hassett and Kosman, 1995). The function of the other Fre proteins is not known; one reasonable postulate is that they may participate in redox cycling of iron in or between intracellular compartments. Ferrireduction probably is a component of the labilization of ferric iron from internalized siderophores much as this chemistry is involved in the mobilization of Fe^{3+} from the transferrin/transferrin receptor complex in metazoan endosomes (Aisen *et al*., 1999).

Yun *et al*. (2001) investigated the role that Fre proteins might play in siderophore iron uptake. They tested the ability of four ferrisiderophore complexes to serve as

reductase substrate in whole cells with *FRE*$^+$ and various Δ*fre* genotypes, e.g. Δ*fre1*, Δ*fre2*, etc. Using a standard reductase assay employing a ferrous iron chelating agent that forms a strongly chromogenic complex with Fe^{2+} as indicator (ferrozine), Yun and colleagues showed apparent reductase activity towards FOB, TAF and rhodotorulic acid, but none towards ferrichrome. Deletion of *FRE1* or *FRE2* had no phenotype in this assay, but deletion of both resulted in loss of this apparent cell reductase activity. However, this assay is biased towards reduction because the ferrous iron chelator stabilizes Fe^{2+}, in effect, making the ferrisiderophore more oxidizing (making its E° less negative, see above). This thermodynamic 'pull' as a result of the indicating Fe^{2+} chelator is indicated by the fact that deletion of *FRE1*, from which >95% of the plasma membrane reductase activity derives, had no effect on the 'reduction' rates measured. Only by removing essentially *all* reducing power from the membrane, as in the Δ*fre1*Δ*fre2* strain, did Yun and colleagues find no reduction of any siderophore.

Given the cytotoxic potential of iron the second question about integration of these several iron uptake pathways is an important one, also. With two high-affinity and, at least in *S. cerevisiae*, two low affinity pathways of iron accumulation, a fungus would certainly accumulate more iron than is nutritionally essential and most likely more than could be metabolically handled. Thus, it is not surprising that the high affinity pathways in *S. cerevisiae* are stringently regulated. This regulation appears to be predominantly if not exclusively at the level of transcription and is due primarily to the Aft1 protein (Yamaguchi-Iwai *et al*., 1995; 1996; Dancis, 1998). Aft1p is a 78 kDa protein that contains a glutamine-rich, C-terminal region that could conform to a transactivation domain and an N-terminal region that could serve as nuclear localization one. Aft1p occupancy of a PyPuCACCCPu element in the 5′ non-coding regions of genes encoding proteins involved in ferroxidase-dependent high affinity iron uptake is observed in iron-deficient but not iron replete cells (Yamaguchi-Iwai *et al*., 1996). This element confers Aft1p-dependent activation of these genes under conditions of iron-depletion. Expression of these genes in a Δ*aft1*-containing background is essentially absent. Among loci containing this Aft1p *cis*-element are *FET3* and *FTR1*, and, of course, the so-called '*ARN*' loci (Yun *et al*., 2000a, b; Philpott *et al*., 2002). Thus, Aft1p is the direct regulator of the activity of both high affinity iron uptake activities in baker's yeast. On the other hand, the expression of *FET4* and the *SMF* loci is relatively independent of the iron status of the yeast cell, and of the Aft1 protein (Dix *et al*., 1994; Cohen *et al*., 2000). With a $K_M = 35\ \mu M$ for iron, Fet4p will not efficiently transport iron except at unusually high environmental iron levels. Except when over-produced, Smf proteins make an experimentally undetectable contribution to yeast cell iron uptake. In short, these low affinity pathways do not pose a serious 'threat' to yeast; indeed, if Fet4p is 'designed' to provide nutritional iron under conditions of iron excess, it can't be dependent on the iron-repressible Aft1 protein for its expression.

Thus, an iron homeostatic mechanism, one based on Aft1p, does exist in *S. cerevisiae*. Given the proliferation of orthologues of essentially all of the 'yeast' iron uptake proteins, one would naturally expect to find a complementary set of Aft1p orthologues in the same collection of fungal genomes. In fact, no such proteins can be found encoded in the *S. pombe*, *C. albicans*, or *N. crassa* genomes although all encode all of the iron uptake proteins described in this review and despite the fact that in all cases experimentally investigated, the level of expression of the respective genes is repressed by environmental iron. Loci have been isolated from *C. albicans* (*TUP1* and *IRO1*) (Garcia *et al*., 2001; Knight *et al*., 2002), and from *Aspergillus nidulans* (Haas *et al*., 1999; Oberegger *et al*., 2002) and *Neurospora crassa* (Zhou *et al*., 1998) (*sreA*) that appear to act as repressors of expression of genes encoding high affinity iron uptake activities, both ferroxidase- and siderophore-dependent ones. The regulatory roles these proteins play in their respective organisms are pleiotropic, however. For example, *TUP1* in *C. albicans* plays a role in repressing genes involved in morphologic change (Braun *et al*., 2000) whereas Tup1p and Ssn6p in *S. cerevisiae* form a repressor complex that functions in a wide variety of gene regulatory circuits (Smith and Johnson, 2000). Consequently, the effect that deletion of one of these loci has on the iron uptake activity of the mutant cell may be indirect and not involve the type of direct protein–DNA interaction that has been established for Aft1p-dependent regulation.

There is a second protein in baker's yeast that has transcriptional activity at the promoters of the 'iron regulon.' This is the Aft2 protein (Blaiseau *et al*., 2001; Rutherford *et al*., 2001). Deletion of *AFT2 has* no phenotype, but over-expression of the gene complements a deletion of *AFT1* indicating that Aft2p can directly or indirectly support the expression of the genes encoding high affinity iron uptake activities. Aft2p does have a transcriptional activation domain because in a fusion to the LexA DNA binding domain in a one-hybrid analysis, it does support transcription of the reporter gene (Rutherford *et al*., 2001). It does not, however, contain an easily recognizable transcriptional domain such as the glutamine-rich one in Aft1p. With respect to other fungi, an Aft2p orthologue is recognized in a BLAST search of the *C. albicans* genome (but, as noted, not an Aft1p one) but not in any of the others. In summary, iron homeostasis in fungi other than *S. cerevisiae* is maintained, but there is really no information about the underlying homeostatic mechanism(s) or the proteins involved.

A last question concerning the integration of these several iron uptake pathways focuses on the cellular trafficking of the iron once it crosses the plasma membrane, whether into the cytosol or cytosol-continuous compartment, or into a vesicular one. There are very limited data about the trafficking of iron in the fungal cell; again, most experimental data is for yeast. Biochemical and genetic evidence suggests that vacuolar compartments contribute to intracellular iron metabolism. In *S. cerevisiae*, newly arrived iron can be tracked to the vacuole (Raguzzi *et al*., 1988), and likely iron-handling proteins, e.g. ferroxidase (Fet5p) and permease (Fth1p) homologues are localized there (Urbanowski and Piper, 1999). Smf proteins also appear to be largely confined to the membranes of intracellular compartments; these proteins, however, may likely play a more dominant role in the metabolism of other divalent cations, most probably Mn^{2+}, not ferrous iron (Chen *et al*., 1999; Liu and Culotta, 1999; Cohen *et al*., 2000). Iron taken into the cell *via* a siderophore may enter the cytoplasm but then accumulate in an endosomal-like compartment (Yun *et al*., 2000a;Lesuisse *et al*., 2001; Hu *et al*., 2002; Kim *et al*., 2002). Subsequently, the siderophore-iron complex can be trafficked *via* retrograde vesicular transport. Following its release from the siderophore within this compartment, this iron can efflux directly into the cytosol. An alternative model is illustrated by the direct transfer of iron from the mammalian endosome into mitochondria; transfer of iron in this fashion could be to any cytosol discontinuous compartment. In any event, a putative endosomal metabolism of siderophore iron could involve reductase-dependent steps, as noted, and any of the ferrous iron transport pathways outlined in this review.

There is evidence for endosomal trafficking of siderophore iron, but somewhat contradictory evidence about its mechanism. *In situ* immunofluorescence does localize a ferrichrome derivative carrying a fluorescent tag to late endosomes/prevacuoles in yeast as indicated by perivacuolar imaging (Lesuisse *et al*., 2001), whereas epitope-tagged Sit1p (and Arn1p) cosediments with a protein marker of late endosomes when yeast membranes are fractionated by gradient sedimentation (Yun *et al*., 2000a). In *C. albicans*, GFP-CaArn1p was localized to the plasma membrane; upon exposure of cells to ferrichrome, some of this protein was internalized, apparently to discrete compartments, perhaps endosomes (Hu *et al*., 2002). A similar pattern of ferrichrome-induced plasma membrane to endosome cycling has been demonstrated in *S. cerevisiae* (Kim *et al*., 2002). On the other hand, siderophore iron uptake at the non-permissive temperature was not different than at the permissive one in a yeast strain carrying a temperature-sensitive allele of *ACT1* (Lesuisse *et al*., 2001). This strain is unable to carry out endocytosis at the non-permissive temperature consistant with the well-established role that actin polymerization/depolymerization plays in yeast, and probably mammalian, endocytosis (Wendland *et al*., 1998).

Can these contradictory observations concerning the role of vesicular trafficking in siderophore uptake be reconciled? For example, the immunofluorescence data can be an artifact and the fractionation results can be interpreted differently. In order to 'see' the fluorescence due to the tagged ferrichrome *in situ*, cells had to over-accumulate the siderophore; normal levels of accumulated siderophore were undectable by this technique (Lesuisse *et al*., 2001). It could be that siderophores accumulate in these prevacuolar vesicles only at elevated intracellular levels. With respect to the co-localization studies (Yun *et al*., 2000a), they do not exclude a model in which siderophore facilitators cycle normally between post-Golgi vesicles/late endosomal and plasma membranes. The fluorescent data on GFP-Arn1p are consistent with this model. Alternatively, these facilitators could localize stably to and function in both membranes. In any event, siderophore facilitators are, after all, members of a well-characterized family of *transporters*, not *receptors*. Thus, they most likely do *not* depend on vesicular trafficking to move their substrate siderophores from one compartment to another. Hopefully what is clear from this discussion is that *how* fungal siderophore facilitators work is not known.

Conclusion

At least within the highly limited slice of the fungal kingdom sampled here, except for receptor-mediated iron uptake all other iron uptake pathways are well-represented. In addition, this broad representation appears to be a recurring theme. Thus, a single model for iron uptake in this small collection of species can reasonably be proposed (Fig. 3). At [iron] $\leq 1\ \mu M$ in the growth environment, adequate iron stores can be maintained only by uptake *via* either the ferroxidase-permease or sidero-

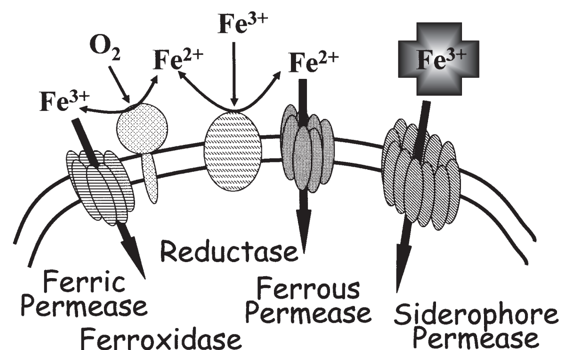

Fig. 3. Summary of iron uptake activities in the yeast plasma membrane.

phore permease pathways as both exhibit kinetic K_M values of 1–2 µM or less. The latter pathway, of course, requires that a siderophore (complexed to iron) is in the environment whether self-produced or not, while the former pathway is directly reductase-dependent. Metalloreductase activity appears more widely distributed among eucaria generally than is siderophore synthesis among fungi specifically suggesting that ferroxidation-permeation is the index pathway for high affinity iron uptake. This conclusion is also suggested by results that indicate a dependence of siderophore iron uptake on Fet3p production in *S. cerevisiae*; there is no inverse relationship, however. The molecular basis of this apparent interaction is not known but this epistatic relationship could be taken as further evidence for the relative essentiality of ferroxidase-dependent ferric iron uptake in comparison to siderophore permeation.

As far as low affinity iron uptake is concerned, there is far less information. The only data in fungi come from *S. cerevisiae* with the studies on Fet4p and the Smf proteins. Fet4p *can* support normal iron homeostasis in yeast when homologously produced in a yeast strain lacking high affinity iron uptake; that is, Fet4p produced at normal levels from the chromosomal *FET4* locus is sufficient to maintain normal cell growth at $[\text{iron}]_{\text{medium}} > 1$ µM. This is not true for the Smf proteins, the yeast orthologues to the mammalian Nramp transporters; rescue of iron uptake defects by Smf1p requires its overexpression. Whereas Nramp2/DCT1 clearly is essential to ferrous iron uptake in epithelial cells *in vivo*, the same is not true for any of the three yeast Smf proteins. As Nramp orthologues, they *do* transport Fe^{2+}, but there are no data that indicate these transporters play any role in iron homeostasis in a typical free-living fungus. A search of those other few archived fungal genomes for Fet4p and Smf protein orthologues indicates that the former could have a very limited distribution as none of these genomes encode a Fet4p-like protein. Possibly, low affinity iron uptake in baker's yeast is a specific adaptation to chronic high *iron* conditions similar to the gene duplication of the *CUP1* ORF found in CUP^R strains that is an adaptation to chronic high *copper* ones. On the other hand, all of these other genomes contain ORFs that conform to Nramp orthologues, perhaps only reminding us that manganese is as essential to the viable cell as is iron.

References

Aisen, P., Wessling-Resnick, M., and Leibold, E.A. (1999) Iron metabolism. *Curr Opin Chem Biol* **3:** 200–206.

Albrecht-Gary, A.-M., and Crumbliss, A.L. (1998) Coordination chemistry of siderophores: thermodynamics and kinetics of iron chelation and release. In *Iron Transport and Storage in Microorganisms, Plants, and Animals*, Sigel A., and Sigel H. (eds). New York, NY: Marcel Dekker, pp. 239–327.

Ardon, O., Weizman, H., Libman, J., Shanzer, A., Chen, Y., and Hadar, Y. (1997) Iron uptake in *Ustilago maydis*: studies with fluorescent ferrichrome analogues. *Microbiol* **143:** 3625–3631.

Ardon, O., Nudelman, R., Caris, C., Libman, J., Shanzer, A., Chen, Y., and Hadar, Y. (1998) Iron uptake in *Ustilago maydis*: tracking the iron path. *J Bacteriol* **180:** 2021–2026.

Ardon, O., Bussey, H., Philpott, C., Ward, D.M., Davis-Kaplan, S., Verroneau, S., *et al.* (2001) Identification of a *Candida albicans* ferrichrome transporter and its characterization by expression in *Saccharomyces cerevisiae*. *J Biol Chem* **276:** 43049–43055.

Askwith, C., and Kaplan, J. (1998) Iron and copper transport in yeast and its relevance to human disease. *Trends Biochem Sci* **23:** 135–138.

Askwith, C., Eide, D., Van Ho, A., Bernard, P.S., Li, L., Davis-Kaplan, S., *et al.* (1994) The *FET3* gene of *S. cerevisiae* encodes a multicopper oxidase required for ferrous iron uptake. *Cell* **76:** 403–410.

Askwith, C., and Kaplan, J. (1997) An oxidase-permease-based iron transport system in *Schizosaccharomyces pombe* and its expression in *Saccharomyces cerevisiae*. *J Biol Chem* **272:** 401–405.

Blaiseau, P.L., Lesuisse, E., and Camadro, J.M. (2001) Aft2p, a novel iron-regulated transcription activator that modulates, with Aft1p, intracellular iron use and resistance to oxidative stress in yeast. *J Biol Chem* **276:** 34221–34226.

Braun, B.R., Head, W.S., Wang, M.X., and Johnson, A.D. (2000) Identification and characterization of *TUP1*-regulated genes in *Candida albicans*. *Genetics* **156:** 31–44.

Canonne-Hergaux, F., Gruenheid, S., Ponka, P., and Gros, P. (1999) Cellular and subcellular localization of the Nramp2 iron transporter in the intestinal brush border and regulation by dietary iron. *Blood* **93:** 4406–4417.

Chen, X.Z., Peng, J.B., Cohen, A., Nelson, H., Nelson, N., and Hediger, M.A. (1999) Yeast SMF1 mediates H^+-coupled iron uptake with concomitant uncoupled cation currents. *J Biol Chem* **274:** 35089–35094.

Cohen, A., Nelson, H., and Nelson, N. (2000) The family of SMF metal ion transporters in yeast cells. *J Biol Chem* **275:** 33388–33394.

Cotton, F.A., Wilkinson, G., Murillo, C.A., and Bochmann, M. (1999) *Advanced Inorganic Chemistry*, 6th edn. New York, NY: John Wiley and Sons.

Dancis, A. (1998) Genetic analysis of iron uptake in the yeast *Saccharomyces cerevisiae*. *J Pediatrics* **132:** S24–S29.

Dancis, A., Roman, D.G., Anderson, G.J., Hinnebusch, A.G., and Klausner, R.D. (1992) Ferric reductase of *Saccharomyces cerevisiae*: molecular characterization, role in iron uptake and transcriptional control by iron. *Proc Natl Acad Sci USA* **89:** 3869–3873.

Dix, D.R., Bridgham, J.T., Broderius, M.A., Byersdorfer, C.A., and Eide, D.J. (1994) The *Fet4* gene encodes the low affinityFe (II) transport protein of *Saccharomyces cerevisiae*. *J Biol Chem* **269:** 26092–26099.

Dix, D., Bridgham, J., Broderius, M., and Eide, D. (1997) Characterization of the FET4 protein of yeast. Evidence for

a direct role in the transport of iron. *J Biol Chem* **272:** 11770–11777.

Eck, R., Hundt, S., Härtl, A., Roemer, E., and Künkel, W. (1999) A multicopper oxidase gene from *Candida albicans*: cloning, characterization and disruption. *Microbiol* **145:** 2415–2422.

Ferguson, A.D., Chakraborty, R., Smith, B.S., Esser, L., van der Helm, D., and Deisenhofer, J. (2002) Structural basis of gating by the outer membrane transporter FecA. *Science* **295:** 1715–1719.

Frieden, E., and Osaki, S. (1974) Ferroxidases and ferrireductases: their role in iron metabolism. *Adv Exper Med Biol* **48:** 235–265.

Garcia, M.G., O'Connor, J.E., Garcia, L.L., Martinez, S.I., Herrero, E., and del Castillo, A.L. (2001) Isolation of a *Candida albicans* gene, tightly linked to *URA*3, coding for a putative transcription factor that suppresses a *Saccharomyces cerevisiae aft*1 mutation. *Yeast* **18:** 301–311.

Georgatsou, E., and Alexandraki, D. (1994) Two distinctly regulated genes are required for ferric reduction, the first step of iron uptake in *Saccharomyces cerevisiae*. *Mol Cell Biol* **14:** 3065–3073.

Georgatsou, E., and Alexandraki, D. (1999) Regulated expression of the *Saccharomyces cerevisia*e Fre1p/Fre2p Fe/Cu reductase related genes. *Yeast* **15:** 573–584.

Haas, H., Zadra, I., Stoeffler, G., and Angermayr, K. (1999) The *Aspergillus nidulans* GATA factor SREA is involved in regulation of siderophore biosynthesis and control of iron uptake. *J Biol Chem* **274:** 4613–4619.

Halliwell, B., and Gutteridge, J.M.C. (1989) *Free Radicals in Biology and Medicine*, 2nd edn. Oxford: Clarendon Press.

Hammacott, J.E., Williams, P.H., and Cashmore, A.M. (2000) *Candida albicans CFL1* encodes a functional ferric reductase activity that can rescue a *Saccharomyces cerevisiae fre1* mutant. *Microbiol* **146:** 869–876.

Hassett, R., and Kosman, D.J. (1995) Evidence for Cu (II) reduction as a component of copper uptake by *Saccharomyces cerevisiae*. *J Biol Chem* **270:** 128–134.

Hassett, R.F., Yuan, D.S., and Kosman, D.J. (1998) Spectral and kinetic properties of the Fet3 protein from *Saccharomyces cerevisiae*, a multinuclear copper ferroxidase enzyme. *J Biol Chem* **273:** 23274–23282.

Hassett, R., Dix, D.R., Eide, D.J., and Kosman, D.J. (2000) The Fe (II) permease Fet4p functions as a low affinity copper transporter and supports normal copper trafficking in *Saccharomyces cerevisiae*. *Biochem J* **351:** 477–484.

Heymann, P., Ernst, J.F., and Winkelmann, G. (1999) Identification of a fungal triacetylfusarinine C siderophore transport gene (*TAF*1) in *Saccharomyces cerevisia*e as a member of the major facilitator superfamily. *Biometals* **12:** 301–306.

Heymann, P., Ernst, J.F., and Winkelmann, G. (2000a) A gene of the major facilitator superfamily encodes a transporter for enterobactin (Enb1p) in *Saccharomyces cerevisiae*. *Biometals* **13:** 65–72.

Heymann, P., Ernst, J.F., and Winkelmann, G. (2000b) Identification and substrate specificity of a ferrichrome-type siderophore transporter (Arn1p) in *Saccharomyces cerevisia*e. *FEMS Microbiol Lett* **186:** 221–227.

Hu, C.-J., Bai, C. Zheng, X.-D. Wang, Y.-M., and Wang, Y. (2002) Characterization and functional analysis of the siderophore-iron transporter CaArn1p in *Candida albicans*. *J Biol Chem* **277:** 30598–30605.

Kim, Y., Yun, C.-W., and Philpott, C.C. (2002) Ferrichrome induces endosome to plasma membrane cycling of the ferrichrome transporter, Arn1p. *Saccharomyces cerevisiae*. *EMBO J* **21:** 3632–3642.

Knight, S.A., Lesuisse, E., Stearman, R., Klausner, R.D., and Dancis, A. (2002) Reductive iron uptake by *Candida albicans*: role of copper, iron and the *TUP*1 regulator. *Microbiol* **148:** 29–40.

Lancashire, R.J. (2002) Chemistry of iron. http://www.chem.uwimona.edu.jm: 1104/courses/iron.html.

Leong, S.A., and Winkelmann, G. (1998) Molecular biology of iron transport in fungi. In *Iron Transport and Storage in Microorganisms, Plants, and Animals*. Sigel A., and Sigel H. (eds). New York, NY: Marcel Dekker, pp. 147–186.

Lesuisse, E., Casteras-Simon, M., and Labbe, P. (1996) Evidence for the *Saccharomyces cerevisiae* ferrireductase system being a multicomponent electron transport chain. *J Biol Chem* **271:** 13578–13583.

Lesuisse, E., Simon-Casteras, M., and Labbe, P. (1998) Siderophore-mediated iron uptake in *Saccharomyces cerevisiae*: the SIT1 gene encodes a ferrioxamine B permease that belongs to the major facilitator superfamily. *Microbiol* **144:** 3455–3462.

Lesuisse, E., Blaiseau, P.-L., Dancis, A., and Camadro, J.-M. (2001) Siderophore uptake and use by the yeast *Saccharomyces cerevisiae*. *Microbiol* **147:** 289–298.

Liu, X.F., and Culotta, V.C. (1999) Post-translation control of Nramp metal transport in yeast: Role of metal ions and the *BSD2* gene. *J Biol Chem* **274:** 4863–4868.

Loach, P.A. (1968) Oxidation-reduction potentials, absorbance bands and molar absorbance of compounds used in biochemical studies. In *Handbook of Biochemistry. Selected Data for Molecular Biology*. Sober H. A. (ed.). Cleveland, OH: Chemical Rubber, pp J27–J48.

Locher, K.P., Rees, B., Koebnik, R., Mitschler, A., Moulinier, L., Rosenbusch, J.P., and Moras, D. (1998) Transmembrane signaling across the ligand-gated FhuA receptor: crystal structures of free and ferrichrome-bound states reveal allosteric changes. *Cell* **95:** 771–778.

Martins, L.J., Jensen, L.T., Simon, J.R., Keller, G.L., and Winge, D.R. (1998) Metalloregulation of *FRE1* and *FRE2* homologs in *Saccharomyces cerevisiae*. *J Biol Chem* **273:** 23716–23721.

Morrissey, J.A., Williams, P.H., and Cashmore, A.M. (1996) *Candida albicans* has a cell-associated ferric-reductase activity which is regulated in response to levels of iron and copper. *Microbiol* **142:** 485–492.

Nelisson, B., De Wachter, R., and Goffeau, A. (1997) Classification of all putative permeases and other membrane plurispanners of the major facilitator superfamily encoded by the complete genome of *Saccharomyces cerevisiae*. *FEMS Microbiol Rev* **21:** 113–134.

Nelson, N. (1999) Metal ion transporters and homeostasis. *EMBO J* **18:** 4361–4371.

Oberegger, H., Zadra, I., Schoeser, M., Abt, B., Parson, W., and Haas, H. (2002) Identification of members of the *Aspergillus nidulans* SREA regulon: genes involved in siderophore biosynthesis and utilization. *Biochem Soc Trans* **30:** 781–783.

Pao, S.S., Paulsen, I.T., and Saier, M.H., Jr (1998) Major facilitator superfamily. *Microbiol Mol Biol Rev* **62:** 1–34.

Philpott, C.C., Protchenko, O., Kim, Y.W., Boretsky, Y., and Shakoury-Elizeh, M. (2002) The response to iron deprivation in *Saccharomyces cerevisiae*: expression of siderophore-based systems of iron uptake. *Biochem Soc Trans* **30:** 698–702.

Pinner, E., Gruenheid, S., Raymond, M., and Gros, P. (1997) Functional complementation of the yeast divalent cation transporter family *SMF* by Nramp2, a member of the mammalian natural resistance-associate macrophage protein family. *J Biol Chem* **272:** 28933–28938.

Qian, Z.M., and Ke, Y. (2001) Rethinking the role of ceruloplasmin in brain iron metabolism. *Brain Res Rev* **35:** 287–294.

Radisky, D., and Kaplan, J. (1999) Regulation of transition metal transport across the yeast plasma membrane. *J Biol Chem* **274:** 4481–4484.

Raguzzi, F., Lesuisee, E., and Crichton, R.R. (1988) Iron storage in *Saccharomyces cerevisiae*. *FEBS Lett* **231:** 253–258.

Ramanan, N., and Wang, Y. (2000) A high-affinity iron permease essential for *Candida albicans* virulence. *Science* **288:** 1062–1064.

Rutherford, J.C., Jaron, S., Ray, E., Brown, P.O., and Winge, D.R. (2001) A second iron-regulatory system in yeast independent of Aft1p. *Proc Natl Acad Sci USA* **98:** 14322–14327.

Severance, S.W., Wang, T.-P., and Kosman, D.J. (2003) Targeted suppression of the Ferroxidase and iron-trafficking activities of the multicopper oxidase, Fet3p, from *Saccharomyces cerevisiae*. *J Biol Chem* **278:** in press.

de Silva, D.M., Askwith, C.C., Eide, D., and Kaplan, J. (1995) The *FET3* gene product required for high affinity iron transport in yeast is a cell surface ferroxidase. *J Biol Chem* **270:** 1098–1101.

de Silva, D., Davis-Kaplan, S., Fergestad, J. and Kaplan, J. (1997) Purification and characterization of Fet3 protein, a yeast homologue of ceruloplasmin. *J Biol Chem* **272:** 14208–14213.

Smith, R.L., and Johnson, A.D. (2000) Turning genes off by Ssn6-Tup1: a conserved system of transcriptional repression in eukaryotes. *Trends Biochem Sci* **25:** 325–330.

Solomon, E.I., Sundaram, U.M., and Machonkin, T.E. (1996) Multicopper oxidases and oxygenases. *Chem Rev* **96:** 2563–2605.

Stearman, R., Yuan, D.S., Yamaguchi-Iwai, Y., Klausner, R.D., and Dancis, A. (1996) A permease-oxidase complex involved in high-affinity iron uptake in yeast. *Science* **271:** 1552–1557.

Urbanowski, J.L., and Piper, R.C. (1999) The iron transporter Fth1p forms a complex with the Fet5 iron oxidase and resides on the vacuolar membrane. *J Biol Chem* **274:** 38061–38070.

Wendland, B., Emr, S.D., and Riezman, H. (1998) Protein traffic in the yeast endocytic and vacuolar protein sorting pathways. *Curr Opin Cell Biol* **10:** 513–522.

Wilkins, R.G. (1991) *Kinetics and Mechanism of Reactions of Transition Metal Complexes*, 2nd edn. New York, NY: VCH Publishers.

Yamaguchi-Iwai, Y., Dancis, A., and Klausner, R.D. (1995) AFT1: a mediator of iron regulated transcriptional control in *Saccharomyces cerevisiae*. *EMBO J* **14:** 1231–1239.

Yamaguchi-Iwai, Y., Stearman, R., Dancis, A., and Klausner, R.D. (1996) Iron-regulated DNA binding by the AFT1 protein controls the iron regulon in yeast. *EMBO J* **15:** 3377–3384.

Yuan, D.S., Stearman, R., Dancis, A., Dunn, T., Beeler, T., and Klausner, R.D. (1995) The Menkes/Wilson disease gene homologue in yeast provides copper to a ceruloplasmin-like oxidase required for iron uptake. *Proc Natl Acad Sci USA* **92:** 2632–2636.

Yuan, D.S., Dancis, A., and Klausner, R.D. (1997) Restriction of copper export in *Saccharomyces cerevisiae* to a late Golgi or post-Golgi compartment in the secretory pathway. *J Biol Chem* **272:** 25787–25793.

Yun, C.-W., Ferea, T., Rashford, J., Ardon, O., Brown, P.O., Botstein, D., *et al.* (2000a) Desferrioxamine-mediated iron uptake in *Saccharomyces cerevisiae*. Evidence for two pathways of iron uptake. *J Biol Chem* **275:** 10709–10715.

Yun, C.-W., Tiedeman, J., Moore, R.E., and Philpott, C.C. (2000b) Siderophore-iron uptake in *Saccharomyces cerevisiae*. Identification of ferrichrome and fusarinine transporters. *J Biol Chem* **275:** 16354–16359.

Yun, C.-W., Bauler, M., Moore, R.E., Klebba, P.E., and Philpott, C.C. (2001) The role of the *FRE* family of plasma membrane reductases in the uptake of siderophore-iron in Saccharomyces cerevisiae. *J Biol Chem* **276:** 10218–10223.

Zhou, L.-W., Haas, H., and Marzluf, G.A. (1998) Isolation and characterization of a new gene, sre, which encodes a GATA-type regulatory protein that controls iron transport in *Neurospora crassa*. *Mol Gen Genet* **259:** 532–540.