1 Molecular Genetics of Iron Uptake and Homeostasis in Fungi

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I. Introduction

Most prokaryotes and all eukaryotes require iron for their growth. This transition metal has two readily available ionization states, ferrous and ferric iron, and thus is involved in a great variety of enzymatic processes including electron transfer in respiration, redox reactions carried out by numerous oxygenases and hydrogenases, and DNA-synthesis. While iron is one of the most abundant metals on earth, in aerobic environments it is present mostly in very insoluble compounds such as oxyhydroxide polymers. Consequently, the concentration of ferric iron in solution at neutral pH is probably not greater than 10^{-18} M (Neilands 1995). On the other hand, an excess of iron within cells can be deleterious, because of the potential to catalyze the generation of cell damaging reactive oxygen species. Therefore, microbes have developed various highly regulated systems for iron uptake and storage. In the last decade, great advances have been made in our understanding of iron transport and intracellular distribution at the molecular level, especially in the baker's yeast *Saccharomyces cerevisiae*. This yeast certainly provides a useful paradigm of iron metabolism for other organisms. Due to a remarkable conservation of certain mechanisms involved in securing metal homeostasis between *Saccharomyces* and humans, studies of homologs of human disease genes in this yeast have shed light on the pathophysiology of several disorders (Askwith and Kaplan 1998). However, an important difference exists between this best-studied eukaryotic model microorganism and most other fungi – *S. cerevisiae* lacks the ability to synthesize siderophores (low molecular iron specific chelators, see below), although it can utilize siderophores produced by other species (Neilands et al. 1987).

Various reviews concerned with different aspects of regulation of iron metabolism in fungi have been published recently (Askwith et al. 1996; Leong and Winkelmann 1998; Howard 1999; de Luca and Wood 2000; Eide 2000; Winkelmann 2001). The present review focuses primarily on the comparison of molecular genetics of iron metabolism of various fungi. For a simplified overview, various aspects of iron metabolism of *S. cerevisiae* described in this review are summarized in Fig. 1. In order to investigate evolutionary conservation of various mechanisms involved in iron uptake and regulation, homologs of characterized genes have been searched in the following genomic databasis: http://www.sanger.ac.uk/Projects/A_fumigatus/ for *Aspergillus fumigatus,* http://

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The Mycota III
Biochemistry and Molecular Biology, 2nd Edition
R. Brambl and G.A. Marzluf (Eds.)
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www.genome.wi.mit.edu/annotation/fungi/neurospora/ for *Neurospora crassa*, and http://www.sequence.stanford.edu/group/candida/ for *C. albicans*, respectively. A compilation of genes involved in iron uptake and regulation, including function and occurrence of homologs present in various fungal species, is found in Table 1.

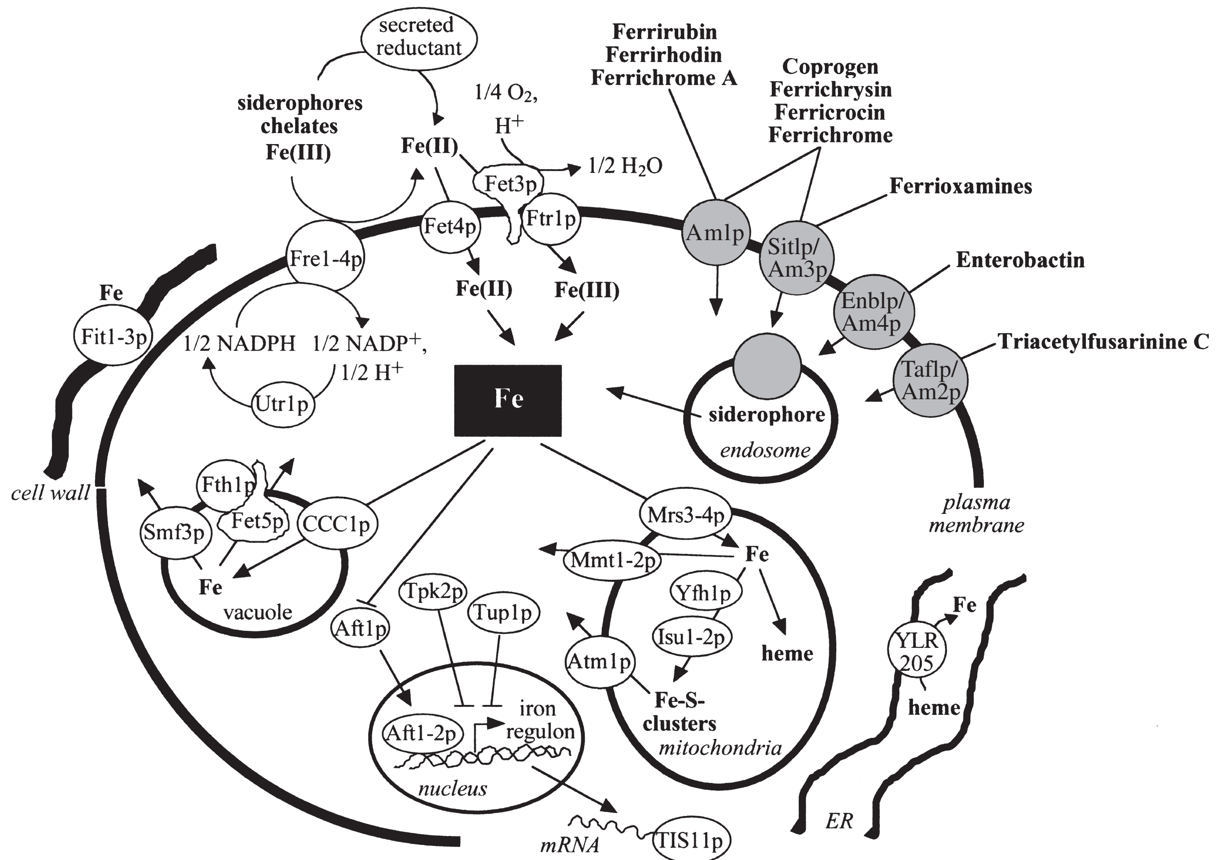

Fig. 1. Iron metabolism in *S. cerevisiae*

Table 1. Fungal orthologous genes involved in iron uptake and regulation

Function/similarity	Organism	Gene[a]	Reference
Structural genes			
High-affinity ferric iron oxidase	*S. cerevisiae*	*FET3*, *FET5*	Askwith et al. (1994); Dancis et al. (1994); Urbanowski and Piper (1999)
	S. pombe	*fio1*	Askwith and Kaplan (1997)
	C. albicans	*CaFET3*, *CaFET99*	Eck et al. (1999); Knight et al. (2002)
	P. pastoris	*FET3*	Paronetto et al. (2001)
	A. adeninivorans	*AFET3*	Wartmann et al. (2002)
	N. crassa	+	H. Haas (unpubl.)
	A. fumigatus	+	H. Haas (unpubl.)
High-affinity ferrous iron transporter	*S. cerevisiae*	*FTR1*, *FTH1*	Stearman et al. (1996); Urbanowski and Piper (1999)
	S. pombe	*fip1*	Askwith and Kaplan (1997)
	C. albicans	*CaFTR1*, *CaFTR2*	Ramanan and Wang (2000)
	N. crassa	+	H. Haas (unpubl.)
	A. fumigatus	+	H. Haas (unpubl.)

Table 1. *Continued*

Function/similarity	Organism	Gene[a]	Reference
Metallo reductases	*S. cerevisiae*	*FRE1–7*	Dancis et al. (1992); Georgatsou and Alexandraki (1994, 1999); Martins et al. (1998)
	S. pombe	*frp1*	Askwith and Kaplan (1997)
	C. albicans	*CFL1, CaCFL95/RBT2*	Braun et al. (2000); Hammacott et al. (2000); Knight et al. (2002)
	A. nidulans	*freA*	Oberegger et al. (2002a)
	N. crassa	+	H. Haas (unpubl.)
	A. fumigatus	+	H. Haas (unpubl.)
Low affinity ferrous iron transporter	*S. cerevisiae*	*FET4*	Dix et al. (1994)
	S. pombe	AL392143	GenBank
	C. albicans	–	
	N. crassa	+	H. Haas (unpubl.)
	A. fumigatus	+	H. Haas (unpubl.)
Siderophore transporter	*S. cerevisiae*	*ARN1, ENB1/ARN4, SIT1/ARN3, TAF1/ARN2*	Heymann et al. (1999, 2000a, b); Lesuisse et al. (1998); Yun et al. (2000b)
	S. pombe	*AL031534*, *AL033127*, *Z81312*	GenBank
	C. albicans	*CaARN1/CaSITA*	Ardon et al. (2001); Heymann et al. (2002); Hu et al. (2002)
	A. nidulans	*mirA*, *mirB*, *mirC*	Oberegger et al. (2001); Haas et al. (2002)
	A. fumigatus	+	H. Haas (unpubl.)
	N. crassa	+	H. Haas (unpubl.)
Ornithine N^5-oxygenase	*U. maydis*	*sid1*	Mei et al. (1993)
	S. cerevisiae	–	
	S. pombe	AL138854	GenBank
	C. albicans	–	
	A. nidulans	*sidA*	Oberegger et al. (2002b)
	A. fumigatus	+	H. Haas (unpubl.)
	N. crassa	+	H. Haas (unpubl.)
	A. pullulans	U85909	GenBank
Ferrichrome nonribosomal peptide synthetase	*U. maydis*	*sid2*	Yuan et al. (2001)
	S. cerevisiae	–	
	S. pombe	AL138854	GenBank
	C. albicans	–	
	A. nidulans	*sidC*	Oberegger et al. (2002b)
	A. fumigatus	+	H. Haas (unpubl.)
	N. crassa	+	H. Haas (unpubl.)
	A. pullulans	U85909	GenBank
Regulator encoding genes			
Iron-responsive transcriptional repressor	*U. maydis*	*urbs1*	Voisard et al. (1993)
	S. cerevisiae	–	
	S. pombe	*GAF2/FEP1*	Hoe et al. (1996); Pelletier et al. (2002)
	C. albicans	*SFU1*	GenBank
	A. nidulans	*sreA*	Haas et al. (1999)
	A. fumigatus	+	H. Haas (unpubl.)
	N. crassa	*sre*	Zhou et al. (1998)
	P. chrysogenum	*sreP*	Haas et al. (1997)
	B. cinerea	*bir1*, AJ309051	GenBank
Iron responsive transcriptional activator	*S. cerevisiae*	*AFT1, AFT2*	Yamaguchi-Iwai et al. (1995); Blaiseau et al. (2001); Rutherford et al. (2001)
	C. albicans	+	H. Haas (unpubl.)
	A. fumigatus	–	
	N. crassa	–	
Iron-responsive transcriptional activator	*C. albicans*	*IRO1*	Garcia et al. (2001)
	S. cerevisiae	–	
	A. fumigatus	–	
	N. crassa	–	

[a] +, Searches in the genome databases – http://www.sanger.ac.uk/Projects/A_fumigatus/ for *A. fumigatus*, http://www.genome.wi.mit.edu/annotation/fungi/neurospora/ for *N. crassa*, and http://www.sequence.stanford.edu/group/candida/ for *C. albicans*, respectively – have revealed sequences with significant homology; –, searches in genome databases did not identify homologs.

II. Acquisition of Iron

As most species lack an excretory route for iron, the primary control point for iron homeostasis appears to be regulation of metal uptake across the plasma membrane. *S. cerevisiae* uses a variety of iron acquisition strategies, including separate high-affinity and multiple low-affinity uptake systems. This might also hold for other fungi and the explanation for such a diversity is probably that alternative mechanisms provide the organism with the ability to deal with a variety of environmental challenges. High-affinity systems are important under iron-limited conditions, whereas low-affinity systems play an important role when iron is more abundant. Furthermore, pathogenic fungi have potentially developed additional systems specialized to utilize host iron sources.

A. High-Affinity Iron Uptake

Because iron is most commonly found as virtually insoluble ferric hydroxides, a general feature of high-affinity uptake systems is the necessity to solubilize ferric iron, whereby two major strategies have evolved in microorganisms: copper-dependent reductive iron uptake and copper-independent siderophore transport. The latter system is often termed “nonreductive iron assimilation”. However, it is important to note that nonreductive iron assimilation also contains a reductive step which occurs in contrast to reductive iron assimilation intracellularly subsequent to the uptake of iron. Various fungi utilize both strategies and siderophore uptake is also found in fungi unable to synthesize siderophores. Furthermore, siderophore-bound iron can in many cases be utilized by the reductive iron assimilatory pathway.

1. Reductive Iron Assimilation

Reductive iron assimilation begins with solubilization of iron by extracellular reduction of ferric iron to ferrous iron which is subsequently taken up.

a) Extracellular Reduction of Iron

Ferric iron is reduced to ferrous iron at the plasma membrane through transmembrane electron transfer mediated by the iron-regulated paralogous metalloreductases Fre1p, Fre2p, Fre3p, and Fre4p (Dancis et al. 1992; Georgatsou and Alexandraki 1994; Yun et al. 2001). Fre1p and Fre2p have additionally been shown to facilitate copper uptake (Hassett and Kosman 1995; Georgatsou et al. 1997) – therefore the term metalloreductases is more appropriate than ferrireductases. The involvement of Fre1p in copper metabolism is underscored by the fact that expression of the encoding gene is in addition to Aft1p-mediated iron regulation (see Sect. IV.A), controlled by the copper-responsive transcription factor Mac1p (Georgatsou and Alexandraki 1999). The Fre-proteins share significant sequence similarity with the $gp91^{phox}$ subunit of cytochrome b_{558}, the human phagocyte respiratory burst oxidase, by containing the motifs implicated in binding the essential cofactors flavin adenine dinucleotide (FAD) and nicotinamide adenine dinucleotide phosphate (NADPH). Furthermore, four critical histidine residues that are required to coordinate a bis-heme structure between transmembrane domains are conserved in the Fre1–7p and $gp91^{phox}$ (Rotrosen et al. 1992; Finegold et al. 1996). In support, heme-deficient mutants are defective in ferric iron reduction as well as accumulation, but in contrast are still able to utilize ferrous iron (Lesuisse and Labbe 1989; Amillet et al. 1996). Consistent with the relationship of $gp91^{phox}$ and Fre-proteins, crude preparations of yeast ferric reductase require NADPH and FAD for activity (Lesuisse et al. 1990). Moreover, yeast plasma membranes have a *b*-type cytochrome absorbance spectrum which correlates with the level of ferric reductase activity (Lesuisse et al. 1996; Shatwell et al. 1996). Lesuisse et al. (1996) suggested that Fre1p-dependent reduction of iron involves at least two components, Fre1p and a NADPH dehydrogenase. Furthermore, ferric reductase activity requires the product of *UTR1* (Anderson et al. 1994; Lesuisse et al. 1996), which has recently been shown to encode a cytosolic NAD-kinase necessary for supply of the cofactor NADP (Kawai et al. 2001). In addition to the membrane-bound ferric reductase system, excreted compounds may contribute to reduction of ferric iron in *S. cerevisiae* (Lesuisse et al. 1992; Georgatsou and Alexandraki 1994; de Luca and Wood 2000).

Substrates for the reductive iron assimilatory system include iron salts, low-affinity iron chelates as ferric citrate, and siderophores like ferrioxamine B, ferrichrome, triacetylfusarinine C, enterobactin and rhodotorulic acid. Fre1p appears to constitute the major activity for reduction of iron salts and

low-affinity iron chelates because *FRE1*-deletion mutants are unable to grow on iron-limited media (Dancis et al. 1990, 1992). Uptake of siderophore-bound iron by this system requires the iron first to be dissociated from the siderophore through reduction. Fre1p, Fre2p, Fre3p and Fre4p are all involved in facilitation of this reduction process, but they are not functionally interchangeable (Yun et al. 2001). Fre1p and Fre2p constitute the major siderophore reductase activity. Fre3p and Fre4p cannot facilitate growth on low concentrations of the catecholate enterobactin, but Fre3p can specifically facilitate reduction and uptake of iron bound to the trihydroxamate siderophores ferrioxamine B, ferrichrome, and triacetylfusarinine C and to the dihydroxamate rhodotorulic acid. Fre4p by itself can facilitate growth only on high concentrations of rhodotorulic acid. The differences of the metalloreductases in the specificities and affinities for their substrates may be based on differences in the structure of the iron coordination site in the siderophores and/or in the reduction potentials of the siderophores. Noteworthy, reduction of siderophore-bound iron at the cell surface is a low-affinity mechanism – at low extracellular concentrations (1 μM), uptake of siderophore-bound iron is essentially nonreductive (see Sect. II.A.2) and only when the siderophore concentration is raised, the reductive mechanism becomes quantitatively more important as the nonreduc-tive high-affinity system approaches saturation (Lesuisse and Labbe 1989).

In addition to *FRE1–4*, the *S. cerevisiae* genome contains five additional paralogues, *FRE5–7*, YLR047c and YGL160w. Suggestions for the functions of the gene products come from expression analysis studies (Martins et al. 1998; Georgatsou and Alexandraki 1999). Similar to *FRE1–4*, transcription of *FRE5–6* is AFT1p-dependent upregulated by iron starvation, suggesting a function in iron metabolism. The expression of *FRE7* is inducible by copper deprivation and therefore appears to be important for copper metabolism. The expression of two further paralogous genes, YLR047c and YGL160w, is affected by neither iron nor copper limitation. The peptide sequences predict a membrane localization for all of the encoded putative metalloreductases. Some of these enzymes might be localized in membranes other than the plasma membrane, such as those of the vacuole and mitochondria, and may be involved in intracellular reduction of metals.

Evidence for membrane-bound reductive iron assimilatory systems has been obtained from studies of a broad array of fungi, including *Schizosaccharomyces*, *Candida*, *Pichia*, *Hyphopichia*, *Kluyveromyces*, *Endomyces*, *Yarrowia*, *Cryptococcus*, *Ustilago*, *Histoplasma*, *Arxula*, and *Rhodotorula* (Ecker and Emery 1983; Lesuisse et al. 1995; Morrissey et al. 1996; Askwith and Kaplan 1997; Fedorovich et al. 1999; Nyhus and Jacobson 1999; Timmerman and Woods 1999). Homologs to *S. cerevisiae* metalloreductase-encoding genes have been detected in various fungi. A *S. pombe* strain mutated in the *FRE* homolog *frp1* is deficient in ferric reductase activity and deficient in ferric iron uptake, while ferrous iron uptake is not impaired (Roman et al. 1993). The *C. albicans* genome contains multiple *FRE* homologs and the products of two genes, *CaCFL1* and *CaCFL95*, are able to restore the growth of a *S. cerevisiae* mutant lacking Fre1p (Hammacott et al. 2000; Knight et al. 2002); *CaCFL95* is identical to *RBT2* which was previously identified as a target of the general repressor Tup1p (Braun et al. 2000). The genomes of the filamentous fungi *Aspergillus nidulans*, *A. fumigatus* and *N. crassa* contain multiple *FRE*-homologous sequences and transcription of one of the *A. nidulans* homologs, *freA*, is induced by iron depletion (Oberegger et al. 2002a). Interestingly, *Arabidopsis thaliana* FRO2, the major ferric-chelate reductase in the roots of this plant, displays significant homology to the fungal metalloreductases (Robinson et al. 1999), whereas the mammalian b-type cytochrome ferric reductase Dcytb, expressed in the duodenal mucosa and probably involved in dietary ferric iron absorption, lacks sequence homology to the fungal enzymes (McKie et al. 2001). In addition to cell surface ferric reducing agent(s) and extracellular low-molecular weight nonproteinaceous ferric reductant(s), *Histoplasma capsulatum* produces an extracellular glutathione-dependent ferric reductase potentially involved in iron acquisition (Timmerman and Woods 2001).

The reduced iron is subsequently taken up by low-affinity iron uptake systems active in iron-replete cells or the siderophore-independent high-affinity ferrous iron uptake system, which is expressed in iron-limited cells.

b) High-Affinity Ferrous Iron Uptake

In *S. cerevisiae*, the high-affinity uptake system for ferrous iron has an apparent K_m of approximately