Siderophore uptake and use by the yeast *Saccharomyces cerevisiae*

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The non-reductive uptake of several siderophores (ferrioxamine B, ferrichrome, triacetylfusarinine C and ferricrocin) by various strains of *Saccharomyces cerevisiae* was studied. Several aspects of siderophore transport were examined, including specificity of transport, regulation of transport and intracellular localization of the ferri-siderophores. Ferrioxamine B was taken up preferentially via the products of the *SIT1* gene and triacetylfusarinine C by the *TAF1* gene product, but the specificity was not absolute. Ferrichrome and ferricrocin uptake was not dependent on a single major facilitator superfamily (MFS) gene product. The apparent specificity of transport was strongly dependent on the genetic background of the cells. Non-reductive uptake of siderophores was induced under more stringent conditions (of iron deprivation) than was the reductive uptake of ferric citrate. Regulation of transport depended on the transcriptional factors Aft1 and Tup1/Ssn6. Cells disrupted for the *TUP1* or *SSN6* genes were constitutively derepressed for the uptake of ferrichrome, ferricrocin or ferrioxamine B, but not for the uptake of triacetylfusarinine C. Cells bearing the *AFT1*^{up} mutation accumulated large amounts of ferric siderophores. Intracellular decomplexation of the siderophores occurred when transcription of the *AFT1*^{up} gene was repressed. Ferrioxamine B and ferrichrome seemed to accumulate in an endosomal compartment, as shown by biochemical studies and by confocal microscopy study of cells loaded with a fluorescent derivative of ferrichrome. Endocytosis was, however, not involved in the non-reductive uptake of siderophores.

Keywords: iron, siderophore, yeast, *Saccharomyces cerevisiae*

INTRODUCTION

The yeast *Saccharomyces cerevisiae* can use two different high-affinity mechanisms (reductive and non-reductive) to take up iron from the extracellular medium. The reductive mechanism (Lesuisse *et al.*, 1987; Dancis *et al.*, 1990) involves the labilization of extracellular ferric chelates by reduction at the surface of the cells via the inducible plasma membrane reductases Fre1p and Fre2p (Dancis *et al.*, 1992; Georgatsou & Alexandraki, 1994). A permease–oxidase complex (Ftr1p and Fet3p) is then involved in translocating iron through the plasma membrane (Askwith *et al.*, 1994; Stearman *et al.*, 1996). The non-reductive

mechanism requires that a ferric complex enters the cells prior to any reduction step. In 1989, we described a non-reductive mechanism for ferrioxamine B (FOB) uptake by *S. cerevisiae* (Lesuisse & Labbe, 1989). This siderophore can enter cells via an FOB receptor present at the cell surface. We showed that the siderophore transport system of *S. cerevisiae* is specific, saturable, energy-dependent and has a high affinity for FOB (Lesuisse & Labbe, 1989). We called this an 'opportunistic strategy of iron uptake' because *S. cerevisiae* can use siderophores excreted by other micro-organisms although it does not produce any itself. The presence in a fungus of two different uptake systems, one reductive and another non-reductive, was first demonstrated by Ecker & Emery (1983) for *Ustilago sphaerogena*.

The existence of two mechanisms for iron uptake makes the specific study of non-reductive uptake somewhat complicated. Uptake kinetics do not fit well with simple

Abbreviations: BPS, bathophenanthrolinedisulfonate; FC, ferricrocin; FCH, ferrichrome; FOB, ferrioxamine B, mesylate form (Desferal); MFS, major facilitator superfamily; TAF, triacetylfusarinine C.

Michaelis–Menten representations (Lesuisse & Labbe, 1989). Reduction of siderophores at the cell surface is a low-affinity and low-specificity mechanism. At low extracellular concentrations (around 1 μM), uptake of the siderophore is essentially non-reductive. When the siderophore concentration is raised, the reductive mechanism becomes quantitatively more important as the non-reductive, high-affinity system approaches saturation (Lesuisse & Labbe, 1989). Genetic studies are also not simple: a mutation that affects some step of non-reductive iron assimilation in *S. cerevisiae* will be difficult to detect because iron can still enter the cells by the reductive mechanism. Emery (1986) used gallium analogues of siderophores, which cannot be reduced, to distinguish between reductive and non-reductive uptake of siderophores by *U. sphaerogena*. We used mutants of *S. cerevisiae* in which the *FET3* and *FET4* genes, encoding components of the high-affinity and low-affinity reductive uptake systems, respectively (Askwith *et al.*, 1994; Dix *et al.*, 1994), had been deleted. Such mutants are unable to grow on minimum medium (containing about 1 μM iron) unless a siderophore (or large amounts of a ferric salt) is added to the growth medium. This property allowed us to select mutants unable to take up FOB, and to identify a gene, *SIT1* (Siderophore Iron Transport), whose product is probably responsible for the translocation of FOB through the plasmalemma (Lesuisse *et al.*, 1998). Sit1p belongs to the major facilitator superfamily (MFS) and has several homologues that may be expected to participate in the transport of other siderophores. This was recently demonstrated in three short studies (Heymann *et al.*, 1999, 2000a, b) in which the authors attributed the product of the YHL047c ORF to triacetylfulvarinine C (TAF) transport, the product of the YHL040c ORF to the transport of a specific class of ferrichromes (FCHs), possessing anhydromevalonyl residues linked to *N*(δ)-ornithine, and the product of the YOL158c ORF to the transport of enterobactin. They postulated that the product of any single gene of the *SIT* family was responsible for recognizing and transporting a single siderophore. This is not consistent with our own observations (present work) or with other recently published results (Yun *et al.*, 2000b). The present report re-examines some aspects of siderophore transport by *S. cerevisiae*, including the specificity of transport, energy requirements, the regulation of transport and the intracellular use of iron. To respect priority of names and to avoid unnecessary confusion, the following nomenclature is used throughout the paper: YEL065w is referred to as *SIT1* (Lesuisse *et al.*, 1998), YHL047c as *TAF1*, YHL040c as *ARN1* and YOL158c as *ENB1* (Heymann *et al.*, 1999, 2000a, b).

METHODS

Strains, media and iron compounds. The various strains of *S. cerevisiae* used in this study are described in Table 1. Unless otherwise stated, cells were grown at 30 °C (in 50 ml Falcon tubes in a gyroschaker) in minimal YNB-glucose (without copper and iron) medium (Bio101) plus the required amino

acids, 1 μM copper sulphate and various iron sources (siderophores or ferric citrate). For iron-deficient cultures, 100 μM BPS (bathophenanthrolinedisulfonate) was added to YNB-glucose medium without copper and iron. Desferri-compounds were obtained as described by Wiebe & Winkelmann (1975). When required, they were labelled with ^{55}Fe [50 mCi mg^{-1} (1.85 GBq mg^{-1})]. Throughout this work, the name '(des)ferrioxamine B' refers to the commercially available mesylate derivative, Desferal (Novartis). The fluorescent derivative of FCH used was the glycine-based analogue B9 described by Berner *et al.* (1991).

Iron uptake assays. Iron uptake was measured in micro-titration plates. Cells were grown overnight at 30 °C in various media (as specified above) to stationary phase. In the morning, cells were diluted 10-fold in the same medium and cultured for 6 h at 30 °C. Cells were then washed three times with distilled water and suspended in 50 mM citrate (trisodium) buffer (pH 6.5) containing 5% glucose to give an OD_{600} of 3. The cell suspension was distributed into the wells of a micro-titration plate (50 μl cells per well) at 0 °C. Iron was added (as a ^{55}Fe chelate) to give a final concentration of 1–5 μM and the plate was incubated for 15–60 min at 30 °C. The cells were collected with a cell harvester (Brandel) and washed on a filter.

Cell fractionation. Cell fractions were purified (Raguzzi *et al.*, 1988) after protoplast lysis (moderate osmotic shock) by centrifugation on a discontinuous Ficoll gradient.

Other. Low-temperature spectra (–191 °C) of whole cells were prepared as described by Lesuisse & Labbe (1989). The optical path length was 1 mm and the reference was a single sheet of wet filter paper. Spectra were corrected for baseline shift. SDS-PAGE and subsequent transfer to nitrocellulose were done using standard procedures. The proteins on nitrocellulose were probed with monoclonal antibodies or with an antiserum specific for Hem15p that was purified in our laboratory (Camadro & Labbe, 1988). Primary antibodies were detected with a horseradish-peroxidase-conjugated anti-mouse IgG and ECL chemiluminescence (Amersham). Fluorescence confocal microscopy was performed as described by Ardon *et al.* (1998). Cells bearing the *AFT1^{up}* mutation were grown overnight on a minimum medium containing 10 μM of fluorescent FCH analogue and then examined for intracellular fluorescence. New mutants affected in siderophore transport were generated by insertion mutagenesis (Tn3) as described by Burns *et al.* (1994). Conditional expression of *AFT1^{up}* was done by cloning the *AFT1^{up}* ORF into the centromeric plasmid pRS316-Gal.

RESULTS

The specificity of Sit1 and homologues for various siderophores is not absolute

We previously showed (Lesuisse *et al.*, 1998) that FOB and ferricrocin (FC) are taken up by *S. cerevisiae* via an active transport system, and that Sit1p is responsible for the uptake of FOB. It was then predictable that gene products homologous to Sit1p should be involved in the uptake of other siderophores. We subsequently identified FCH and TAF as siderophores transported by an active, energy-dependent mechanism by *S. cerevisiae*. This has also been reported recently by others (Yun *et al.*, 2000b; Heymann *et al.*, 1999). We measured the rate of uptake of FOB, FC, FCH, TAF and ferric citrate by a

Table 1. Description of *S. cerevisiae* strains used

Strain name	Genotype
DEY1455	<i>MATα ade2 can1 his3 leu2 trp1 ura3 gal</i> (wild-type)
DEY1433	<i>MATα ade2 his3 leu2 trp1 ura3 fet3::HIS3 fet4::LEU2</i>
DDY4	<i>MATα ade6 can1 his3 leu2 trp1 ura3 fet3-2::HIS3 fet4-1::LEU2</i>
DEY1455 Δ <i>sit1</i>	<i>MATα ade2 can1 his3 leu2 trp1 ura3 gal sit1::KanMX4</i>
DEY1433 Δ <i>sit1</i>	<i>MATα ade2 his3 leu2 trp1 ura3 fet3::HIS3 fet4::LEU2 sit1::KanMX4</i>
CM3260	<i>MATα trp1 leu2 gcn4 his3 ura3</i> (wild-type)
CM3260 Δ <i>sit1</i>	<i>MATα trp1 leu2 gcn4 his3 ura3 sit1::KanMX4</i>
M2p	<i>MATα trp1 leu2 gcn4 his3 ura3 AFT1^{up}</i>
CM3260 Δ <i>aft1</i>	<i>MATα trp1 leu2 gcn4 his3 ura3 aft1::TRP1</i>
BY4742	<i>MATα lys2 leu2 his3 ura3</i> (wild-type)
BY4742 Δ <i>sit1</i>	<i>MATα lys2 leu2 his3 ura3 sit1::KanMX4</i>
BY4742 Δ <i>taf1</i>	<i>MATα lys2 leu2 his3 ura3 taf1::KanMX4</i>
BY4742 Δ <i>arn1</i>	<i>MATα lys2 leu2 his3 ura3 arn1::KanMX4</i>
BY4742 Δ <i>enb1</i>	<i>MATα lys2 leu2 his3 ura3 enb1::KanMX4</i>
NY13	<i>MATα act1 ura3 gal2</i>
FY105	<i>MATα leu2 his3 ura3</i> (wild-type)
BSHFT5	<i>MATα leu2 his3 ura3 tup1::HIS3</i>
PHF105	<i>MATα leu2 his3 ura3 ssn6::HIS3</i>

Fig. 1. Iron uptake by various strains of *S. cerevisiae* from different iron sources. Strains of the BY4742 background disrupted for the *ARN1*, *TAF1*, *SIT1* or *ENB1* ORFs were first cultured overnight in iron-deficient medium (without iron, but with 1 μ M copper). Cultures were then diluted 10-fold in the same medium + 100 μ M BPS and cultured for 6 h before iron uptake was measured as described in Methods. Iron sources tested (each 1 μ M) were: ferric citrate (■), FOB (□), TAF (▨), FCH (▩), and FC (▩). Values are means $\pm$ SE from 15 independent experiments.

wild-type strain and isogenic mutants disrupted for either the *SIT1* gene, the *TAF1* gene, the *ARN1* gene or the *ENB1* gene to evaluate the contribution of Sit1p and homologues to the transport of these various siderophores (Fig. 1). These data indicate that TAF transport

mainly depends on *TAF1*, as recently shown by Heymann *et al.* (1999). FCH transport was affected in strains in which the *ARN1*, *TAF1* or *SIT1* genes were disrupted (Fig. 1). This confirms the recent results of Yun *et al.* (2000b) showing that FCH uptake does not depend on a single MFS gene. Our data also indicate that the same is true for the FCH-type siderophore FC. Transport of that siderophore was affected in strains whose *ARN1* or *TAF1* genes were disrupted (Fig. 1). Curiously, disruption of *ARN1* seemed to facilitate the transport of FOB (Fig. 1). Members of the Sit family are thus not completely specific for a single siderophore. *SIT1* and *TAF1* seem to encode the most specific transporters, for FOB and TAF, respectively. The relative non-specificity of the transporters was also indicated by the following observation. Constitutive overexpression of the *SIT1* ORF in various strains led to an increase in the rate of uptake of FOB, but also of FC and FCH, and to a lesser extent of TAF (Table 2).

Analysis of new mutants also indicated that each Sitp member is not completely specific for a single siderophore. Cells lacking the *YFH1* gene are sensitive to iron (Babcock *et al.*, 1997), especially when it is presented as FOB, FC or FCH (see below). We used this property to select new mutants able to grow on plates enriched with 10 μ M FCH. One of these mutants could not take up FOB, FCH or FC. The transport of these three siderophores by the mutant cells was restored when they were transformed with *SIT1* on a multicopy plasmid (data not shown). Other mutants obtained in this way are being analysed. We previously showed that FC uptake is completely unaffected when the *SIT1* gene is disrupted in the strain DEY1433 (Lesuisse *et al.*, 1998). We disrupted the same gene in strains of various genetic backgrounds and observed that the effect of *SIT1* disruption on siderophore transport differed greatly

Table 2. Iron uptake from different sources by various strains of *S. cerevisiae*

Strains of the BY4742 background disrupted for *ARN1*, *TAF1* or *SIT1* ORFs were transformed with a plasmid bearing the *SIT1* ORF under a strong constitutive promoter (pVT100U-*SIT1*). Transformed and untransformed cells were first cultured overnight in iron-deficient medium (without iron but with 1 μ M copper), diluted 10-fold in the same medium + 100 μ M BPS and cultured for 6 h. Iron uptake from ferric citrate, FOB, TAF, FC or FCH (each 2 μ M) was then measured as described in Methods. Values are means $\pm$ SE from three independent experiments.

Strain	Iron uptake rate [pmol min ⁻¹ (OD ₆₀₀ unit) ⁻¹]				
	Fe(III) citrate	FOB	TAF	FC	FCH
Wild-type	1.2 $\pm$ 0.30	1.3 $\pm$ 0.20	0.9 $\pm$ 0.05	2.9 $\pm$ 0.40	4.2 $\pm$ 0.30
Δ arn1	1.1 $\pm$ 0.20	1.5 $\pm$ 0.15	0.8 $\pm$ 0.03	2.1 $\pm$ 0.30	2.9 $\pm$ 0.30
Δ taf1	1.3 $\pm$ 0.20	1.7 $\pm$ 0.10	0.1 $\pm$ 0.02	3.1 $\pm$ 0.35	3.6 $\pm$ 0.20
Δ sit1	0.9 $\pm$ 0.15	0.3 $\pm$ 0.04	1.2 $\pm$ 0.01	2.8 $\pm$ 0.20	2.7 $\pm$ 0.40
Δ arn1- <i>SIT1</i>	1.2 $\pm$ 0.10	4.0 $\pm$ 0.30	1.8 $\pm$ 0.15	4.1 $\pm$ 0.30	5.2 $\pm$ 0.60
Δ taf1- <i>SIT1</i>	1.0 $\pm$ 0.20	5.8 $\pm$ 0.40	0.3 $\pm$ 0.01	4.6 $\pm$ 0.50	5.7 $\pm$ 0.50
Δ sit1- <i>SIT1</i>	1.3 $\pm$ 0.15	5.2 $\pm$ 0.30	1.4 $\pm$ 0.10	4.5 $\pm$ 0.40	5.2 $\pm$ 0.30

Table 3. Iron uptake from different sources by various strains of *S. cerevisiae*

Strains of different genetic backgrounds were tested for iron uptake before and after disruption of the *SIT1* gene. Cells were cultured overnight in iron-deficient medium (without iron but with 1 μ M copper), diluted 10-fold in the same medium + 100 μ M BPS and cultured for 6 h. Iron uptake from ferric citrate, FOB, TAF, FC or FCH (each 2 μ M) was then measured as described in Methods. Values are means $\pm$ SE from three independent experiments.

Strain	Iron uptake rate [pmol min ⁻¹ (OD ₆₀₀ unit) ⁻¹]				
	Fe(III) citrate	FOB	TAF	FC	FCH
CM3260	0.9 $\pm$ 0.07	1.5 $\pm$ 0.16	0.4 $\pm$ 0.05	3.5 $\pm$ 0.40	4.3 $\pm$ 0.35
CM3260 Δ sit1	1.2 $\pm$ 0.10	0.3 $\pm$ 0.01	0.3 $\pm$ 0.02	1.3 $\pm$ 0.10	1.8 $\pm$ 0.20
DEY1433	0.07 $\pm$ 0.01	2.4 $\pm$ 0.15	0.7 $\pm$ 0.09	5.5 $\pm$ 0.50	5.9 $\pm$ 0.65
DEY1433 Δ sit1	0.05 $\pm$ 0.01	0.2 $\pm$ 0.03	0.6 $\pm$ 0.05	5.9 $\pm$ 0.50	5.6 $\pm$ 0.60
BY4742	1.3 $\pm$ 0.07	1.4 $\pm$ 0.10	1.0 $\pm$ 0.16	3.2 $\pm$ 0.40	4.0 $\pm$ 0.60
BY4742 Δ sit1	1.2 $\pm$ 0.15	0.3 $\pm$ 0.02	1.3 $\pm$ 0.11	3.1 $\pm$ 0.35	2.5 $\pm$ 0.20

from one strain to another (Table 3). Disruption of *SIT1* led to a specific defect in FOB uptake in cells of the DEY1433 background, but it led to a decrease in FOB, FC and FCH uptake in cells of the CM3260 background (Table 3). *SIT1* disruption affected FOB and FC uptake by cells of the BY4742 background, but not that of FCH (Fig. 1, Table 2). As cells of the CM3260 background are mutated in the *GCN4* gene, we tested whether the expression of *GCN4* interfered with siderophore transport. We found that overexpression of *GCN4* on a multicopy plasmid in CM3260 and CM3260 Δ sit1 cells did not significantly change the rate of iron uptake from FOB, FC, FCH or TAF (data not shown).

As previously shown for FOB and FC (Lesuisse *et al.*, 1998), there was competition between FOB and FCH, and between FCH and FC, in the DEY1433 strain (data not shown). TAF did not inhibit the uptake of FOB, FC or FCH (data not shown). Our kinetic parameters for

the uptake of TAF and FCH were very similar to those published by Yun *et al.* (2000b) (data not shown).

Differences in the regulation of siderophore transport and reductive iron uptake

In a previous study (Lesuisse *et al.*, 1998), we presented evidence that the transport of siderophore is regulated in an Aft1p-independent manner. Our conclusion arose from analysis of a mutant strain obtained by EMS mutagenesis. This strain was identified as an *aft1* mutant by complementation with *AFT1* on a centromeric plasmid (data not shown). However, a recent study (Yun *et al.*, 2000b) showed unambiguously that our conclusion was wrong: the *SIT1* gene and homologues are actually regulated by Aft1p. We are currently trying to understand how a point mutation in *AFT1* could lead to inactivation of the reductive pathway of iron uptake

Table 4. Rates of iron uptake from ferric citrate or FOB by *S. cerevisiae* cells precultured and cultured under various conditions

Cells of the strain CM3260 were cultured overnight in a minimum medium (without iron or copper) supplemented with 1 μ M copper ('-Fe'), or 1 μ M copper plus 5 μ M ferric citrate ('+Fe'). Cells were then washed three times with distilled water, diluted 10-fold in the same medium with or without iron [as 5 μ M ferric citrate ('+Fe^a') or 5 μ M FOB ('+Fe^b')] and cultured for 6 h. Where indicated ('+BPS'), the culture medium contained 100 μ M BPS. Iron uptake by the cells was then measured as described in Methods with either 2 μ M ferric citrate or 2 μ M FOB. Representative data from one of three independent experiments are shown.

Preculture conditions	Culture conditions	Iron uptake rate [pmol min ⁻¹ (OD ₆₀₀ unit) ⁻¹]	
		Reductive uptake of Fe(III) citrate	Non-reductive uptake of FOB
+Fe	+Fe ^a	0.5	0.2
+Fe	+Fe ^b	0.5	0.8
+Fe	-Fe	1.2	1.5
+Fe	-Fe+BPS	6.8	3.0
-Fe	+Fe ^a	2.7	0.3
-Fe	+Fe ^b	3.5	2.5
-Fe	-Fe	4.5	5.0
-Fe	-Fe+BPS	2.1	6.2

without affecting the non-reductive uptake of siderophores. The specific aspects of Aft regulation of siderophore transport will be discussed in another paper (P.-L. Blaiseau, E. Lesuisse & J.-M. Camadro, unpublished). We confirm here that deletion of *AFT1* completely abolished the transport of FOB, FC, FCH and TAF by the cells (data not shown). But the reductive and non-reductive pathways of iron uptake in *S. cerevisiae* were not regulated identically. First, full induction of both siderophore uptake and ferrous iron uptake did not occur under the same growth conditions. We previously reported (Lesuisse *et al.*, 1998) that the induction of ferrous transport under iron deprivation was much higher than the induction of siderophore transport under the same conditions of growth in an iron-deficient medium. This was true when cells that had been cultured in a complete medium were washed and transferred to an iron-deficient medium for 6 h before measuring iron uptake from Fe²⁺ or FOB. The cell ferrireductase activity was then maximum and the rate of ferrous iron uptake was 10- to 30-fold higher than in cells grown in an iron-rich medium. Under the same conditions, the rate of FOB uptake was only increased fivefold (Lesuisse *et al.*, 1998). However, when cells were cultured in iron-deficient medium only, there was a much greater increase in siderophore uptake. Thus, the induction of non-reductive uptake of siderophore seems to require more stringent conditions than reductive uptake (Table 4); the assumption that siderophore uptake is non-reductive is justified by our use of low (1 μ M) siderophore concentrations (see Introduction). Table 4 shows that a long period of iron deprivation (culture and preculture) was needed for the full induction of FOB transport, while reductive uptake

of ferric citrate was maximum after only 6 h of iron deprivation. We previously observed that cell ferrireductase activity seemed to depend more on the flux of iron entering the cells in exponential-growth phase than on the absolute iron content of the cells (Lesuisse & Labbe, 1989). This could account for the high rate of reductive iron uptake by iron-replete cells subjected to iron-deprivation for short periods. Table 4 also shows that iron in the growth medium was more efficient at repressing reductive and non-reductive iron uptake when presented as ferric citrate than as FOB. This suggests that the intracellular processing of the two forms of iron is different.

The Ssn6-Tup1 repressor forms one of the largest and most important gene-regulatory circuits in yeast. In previous unpublished experiments, we observed an effect of *TUP1* deletion on cell ferrireductase activity. We checked if *TUP1/SSN6* expression could influence reductive and/or non-reductive uptake of iron. The relative dependence of the two pathways on these transcription factors indicated differences in the regulation of reductive and non-reductive iron uptake pathways in *S. cerevisiae*. The reductive uptake of ferric citrate was decreased in strains with disrupted *TUP1* or *SSN6* genes (Fig. 2). The opposite was true for the non-reductive uptake of siderophores, especially for that of the FCH-type siderophores (Fig. 2). The reductive uptake of ferric citrate by Δ *tup1* or Δ *ssn6* strains was decreased under iron-sufficient conditions and unchanged by iron deprivation (compared to the wild-type grown under iron-sufficient conditions). Under the same conditions, the non-reductive uptake of FC and FCH was constitutively activated (Fig. 2). TAF uptake did not

Fig. 2. Effect of disrupting *TUP1* and *SSN6* genes on reductive uptake of ferric citrate and non-reductive uptake of siderophores in *S. cerevisiae*. Wild-type, $\Delta tup1$ and $\Delta ssn6$ cells were cultured overnight in iron-deficient medium ('-Fe'; without iron but with 1 μ M copper), or in iron-rich medium ('+Fe', same medium plus 10 μ M ferric citrate). In the morning, cells were diluted 10-fold in the same medium and cultured for 6 h before iron uptake (1 μ M) was measured as described in Methods. ■, Wild-type + Fe; □, wild-type - Fe; ▨, $\Delta tup1$ + Fe; ▩, $\Delta tup1$ - Fe; ▤, $\Delta ssn6$ + Fe; ▥, $\Delta ssn6$ - Fe. Representative data from one of six independent experiments are shown.

respond the same way as FC/FCH uptake to *TUP1*/*SSN6* disruption. The uptake of TAF was completely repressed in $\Delta tup1$ or $\Delta ssn6$ mutant cells grown in iron-rich medium, and was significantly decreased (compared to the wild-type) in the same mutants grown in iron-deficient medium (Fig. 2).

It has been suggested that members of the Sit family function as H^+ /drug antiports (Goffeau *et al.*, 1997). We therefore compared the effect of extracellular pH on the non-reductive uptake of siderophores (FCH and FC) and the reductive uptake of ferric citrate (Fig. 3). The effect of pH on reductive and non-reductive uptakes was different. Reductive uptake was much more efficient at low pH, while non-reductive uptake was maximal at pH 6–7 (Fig. 3). This result is not conclusive, but it is compatible with the hypothesis that Sit family proteins act as siderophore_{in}/ H^+ _{out} antiports.

Intracellular fate of iron

The gain-of-function *AFT1*^{up} mutation causes cells to be constitutively activated for the reductive transport of iron (Yamaguchi-Iwai *et al.*, 1995). It had the same effect on the non-reductive uptake of FC, FCH, FOB and TAF (data not shown). *AFT1*^{up} mutants seemed to accumulate siderophores inside the cells as the undissociated

Fig. 3. Effect of pH on the reductive uptake rate of ferric citrate and on the non-reductive uptake rate of FC in *S. cerevisiae*. Cells of the strain CM3260 were cultured overnight in iron-deficient medium (without iron but with 1 μ M copper), diluted 10-fold in the same medium + 100 μ M BPS and cultured for 6 h. Iron uptake was measured as described in Methods with either 2 μ M ferric citrate (●) or 2 μ M FCH (□) at various pHs (buffered by MES/MOPS, each 0.1 M). Representative data from one of three independent experiments are shown.

Fig. 4. Accumulation of FOB inside *AFT1*^{up} mutant cells of *S. cerevisiae*. *AFT1*^{up} (a) and wild-type (b) cells were plated on minimum agar medium containing 10 μ M FOB and grown for 3 d at 30 °C.

ferric complex (Fig. 4). *AFT1*^{up} cells grown on a FOB-containing plate had the colour of the siderophore itself. The same observation was previously made with haemless mutants (Lesuisse & Labbe, 1989). There are two possible explanations for these observations. One is that the siderophore itself acts as an iron-storage form, as shown for the fungus *Neurospora crassa* (Matzanke *et al.*, 1987). The cells would then accumulate large amounts of the ferric siderophore as a direct conse-

Fig. 5. Low-temperature spectra of *S. cerevisiae* cells loaded with FOB and induced (A) or repressed (B) for the expression of the *AFT1^{up}* gene. $\Delta aft1$ cells were transformed with a multicopy plasmid bearing the *AFT1^{up}* ORF under the *GAL4* promoter. Cells were grown for 24 h in a YNB-galactose medium containing 100 μ M FOB, washed with distilled water and transferred to an iron-deficient medium (without iron and copper) with either galactose or glucose as the carbon source and grown for 24 h. Cells were then washed and low-temperature spectra were recorded.

quence of the deregulation of transport. The other is that *AFT1^{up}* cells are defective at some step in the intracellular decomplexation of siderophores. A haem component could be involved in this dissociation step, which would explain why the same result was obtained with haem-less mutants (Lesuisse & Labbe, 1989). We transformed a $\Delta aft1$ strain with a plasmid bearing the *AFT1^{up}* ORF under the inducible promoter of *GAL4*. The cells were grown on a galactose medium containing a large excess of FOB (100 μ M), harvested, washed with water and placed in an iron-deficient medium with galactose or glucose as the carbon source for 24 h. We then recorded the low-temperature spectra of the washed cells (Fig. 5). Cells placed in the glucose medium (*AFT1^{up}* expression repressed) showed a spectrum similar to that of the wild-type, indicating that most of the intracellular FOB was dissociated. In contrast, cells placed in galactose medium still showed a high absorbance peak in the range 420–440 nm, indicating that the FOB remained undissociated inside the cells (Fig. 5). The total iron content of the cells were identical (not shown). This suggests that Aft1p is involved in the intracellular decomplexation of siderophores. Another observation supports the hypothesis that ferric siderophores are normally not accumulated inside the cells: in cells deleted for the *YFH1* gene, reductive iron uptake is constitutively increased, iron accumulates into the mitochondria and is toxic (Babcock *et al.*, 1997). The non-reductive uptake of FOB or FCH (but interestingly not of TAF) is also constitutively increased in $\Delta yfh1$

Fig. 6. Relative toxicity of ferric citrate and of FCH for a $\Delta yfh1$ mutant of *S. cerevisiae*. Wild-type (WT) and $\Delta yfh1$ mutant (M) cells were sequentially fivefold-diluted and then plated on YNB-glucose agar medium containing either 5 μ M ferric citrate (1) or 5 μ M FCH (2).

mutants (data not shown). If FOB/FCH were mainly accumulated inside the cells as the undissociated form, one would expect these siderophores to be less toxic than ferric citrate. But the opposite was true (Fig. 6), which could be due to the fact that the uptake rate for FCH was higher than that for ferric citrate. We used this property to select new mutants whose siderophore uptake/accumulation was affected. Aft1p is known as a transcriptional activator, not as a repressor (Yamaguchi-Iwai *et al.*, 1995). Thus, we needed to know how overexpression of *AFT1^{up}* could lead to a defect in siderophore use by the cells. One explanation is that Aft1p activates a gene whose product is involved in the intracellular storage of siderophores in a compartment where decomplexation of the molecule cannot occur. We investigated the intracellular storage of siderophores using fluorescent derivatives of FCH, as previously described for *Ustilago maydis* (Ardon *et al.*, 1998). Wild-type cells took up very little of the fluorescent probes, so that we could not see any signal by confocal fluorescence microscopy (data not shown). But an *AFT1^{up}* strain took up fluorescent material, which was seen in peri-vacuolar structures (Fig. 7). Fractionation on a Ficoll gradient showed that most of the ^{55}Fe -labelled FOB taken up into *AFT1^{up}* cells was in the same fraction as the late endosome marker Pep12 (Fig. 8). Unexpectedly, much less iron was associated with the vacuolar fraction, while a significant peak of iron co-migrated with the mitochondrial fraction (Fig. 8). Yun *et al.* (2000a) recently showed that Sit1p was mostly associated with an internal compartment (probably late endosome), unlike the Fet3p–Ftr1p complex (components of the reductive uptake system), which was mostly associated with the plasma membrane fraction. Therefore, we tested the possibility that the uptake of siderophore

Fig. 7. Confocal micrographs of *S. cerevisiae* M2p. Cells were grown for 24 h with 10 μ M of a B9 fluorescent analogue of FCH (Ardon *et al.*, 1998). Fluorescence is concentrated in vesicles within the cells. Superimposition of the pictures recorded by phase-contrast microscopy (a) and by confocal fluorescence microscopy (b) shows that the fluorescent material lies around the vacuole (c). Bars, 5 μ m (bar in a also applies to b).

Fig. 8. Intracellular distribution of iron in an *AFT1^{up}* strain of *S. cerevisiae* loaded with ^{55}Fe -labelled FOB. Cells of the strain M2p were grown overnight in YNB-raffinose medium containing 100 μ M FOB, lysed and the cell lysate separated on a discontinuous Ficoll gradient. The total amount of iron in each fraction (numbered 1 to 8 from top to bottom) was measured. Samples of the same fractions were used for Western blotting experiments, in which markers of mitochondria (ferrochelatase, A), of the vacuole (vacuolar ATPase, B) and of the late endosome (pep12p, C) were revealed by specific antibodies.

depended on endocytosis. We tested the capacity of a thermosensitive *act1* strain, unable to carry out endocytosis at 37 °C (Kubler & Riezman, 1993), to reductively take up iron from ferric citrate, or non-reductively from FOB (each 1 μ M), after growing in various media (complete medium, minimum iron-deficient medium, minimum iron-rich medium) and incubation at 28 or 37 °C in the presence of ^{55}Fe . The reductive uptake of ferric citrate by the *act1* strain was somewhat lower than that of the wild-type, but its non-reductive uptake of FOB seemed to be unaffected. Data from a typical experiment were as follows: cells of the wild-type strain and of the *act1* mutant were cultured for 6 h at 28 °C in iron-deficient medium and then shifted to 37 °C for 30 min before iron uptake was measured (at 37 °C). The uptake rate of ferric citrate was $9.4 \pm 1.1 \text{ pmol min}^{-1} (\text{OD}_{600} \text{ unit})^{-1}$ for the wild-type and $6.9 \pm 0.8 \text{ pmol min}^{-1} (\text{OD}_{600} \text{ unit})^{-1}$ for the *act1* mutant. These values were, respectively, 2.0 ± 0.15 and $2.8 \pm 0.2 \text{ pmol min}^{-1} (\text{OD}_{600} \text{ unit})^{-1}$ for the uptake of FOB (mean $\pm$ SE from three experiments). The transport of TAF, FC and FCH was similarly unaffected in the *act1* mutant at the non-permissive temperature (data not shown). We conclude that siderophore uptake does not require Act1-mediated endocytosis.

DISCUSSION

S. cerevisiae has proven a very good model for studying reductive iron uptake in eukaryotes (reviewed by Askwith & Kaplan, 1998) and it will probably help us to better understand non-reductive iron uptake as well, even though it does not itself produce any siderophores. We previously showed that disruption of *SIT1* blocked FOB uptake by *S. cerevisiae* (strain DEY1433) without

affecting the transport of FC (Lesuisse *et al.*, 1998). However, there is competition for the transport of the two siderophores. This suggested that the uptake of a given siderophore is very specific and that some step common to several siderophores are upstream or downstream of the transport system itself (Lesuisse *et al.*, 1998). Here again, we found that there is competition between FOB, FC and FCH. Like Yun *et al.* (2000b), we found that siderophore transport by *S. cerevisiae* is not strictly specific. While FOB and TAF are each taken up via a single transporter (Sit1p and Taf1, respectively), several transporters (especially Sit1p and Arn1p) contribute to the uptake of FCH and FC. The apparent specificity is also strongly strain-dependent. Thus, it is not clear whether or not a single protein is responsible for both recognition and transport of siderophores by *S. cerevisiae*. We are examining this question with *in vitro* binding experiments on isolated plasma membranes. The fact that the uptake of several siderophores (FOB, FC, FCH) is competitive implies that other proteins are involved in their transport. We are currently trying to identify these proteins by analysing new mutant strains affected in siderophore uptake/use. The present study suggests that the siderophore FOB is associated with an endosomal compartment when it accumulates inside the cells. Another study (Yun *et al.*, 2000a) showed that Sit1p is also associated with the late endosome compartment. We used *AFT1^{up}* mutant cells, which accumulate large amounts of siderophores. The intracellular processing of siderophores is probably blocked in *AFT1^{up}* cells. This would explain why iron was released from FOB within the cells when the *AFT1^{up}* gene on a plasmid was turned off in a Δ *aft1* strain loaded with FOB. Yun *et al.* (2000a) proposed that Sit1p is involved downstream of the uptake system for FOB, which itself could involve endocytosis. However, this possibility can be ruled out since a mutant completely blocked for endocytosis showed no defect in siderophore uptake. In addition, involvement of Sit1p downstream of the uptake system would not explain why disruption of *SIT1* completely abolished FOB uptake in some strains. Sit1p is more likely to act as a transporter at the cell surface and be rapidly recycled by internalization. Nothing is known about the intracellular processing of siderophores in *S. cerevisiae*. Preliminary studies suggest that a haemoprotein is involved in the reductive decomplexation of siderophores inside the cells (Lesuisse & Labbe, 1989). This could involve one of the Fre1p/Fre2p homologues. Many other aspects of siderophore metabolism in *S. cerevisiae* remain to be clarified, including the energetics of transport, the relationship between recognition and transport, and its regulation. These questions are under investigation in our laboratory.

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