BioMetals **13:** 65–72, 2000.

A gene of the major facilitator superfamily encodes a transporter for enterobactin (Enb1p) in *Saccharomyces cerevisiae*

Petra Heymann[1], Joachim F. Ernst[2] & Günther Winkelmann[1,*]
[1]*Department of Microbiology & Biotechnology, University of Tübingen, Tübingen, Germany* [2] *Institute of Microbiology, University of Düsseldorf, Düsseldorf, Germany* [*]*Author for correspondence: (Fax: (+49) 7071 2973094; E-mail: Winkelmann @ uni-tuebingen.de)*

Received 16 November 1999; Accepted 3 December 1999

Key words: major facilitator superfamily, iron transport, siderophores, enterobactin, *Saccharomyces cerevisiae*

Abstract

While in fungi iron transport via hydroxamate siderophores has been amply proven, iron transport via enterobactin is largely unknown. Enterobactin is a catecholate-type siderophore produced by several enterobacterial genera grown in severe iron deprivation. By using the *KanMX* disruption module in vector pUG6 in a *fet3*Δ background of *Saccharomyces cerevisiae* we were able to disrupt the gene *YOL158c Sce* of the major facilitator super family (MFS) which has been previously described as a gene encoding a membrane transporter of unknown function. Contrary to the parental strain, the disruptant was unable to utilize ferric enterobactin in growth promotion tests and in transport assays using ^{55}Fe-enterobactin. All other siderophore transport properties remained unaffected. The results are evidence that in *S. cerevisiae* the *YOL158c Sce* gene of the major facilitator super family, now designated *ENB1*, encodes a transporter protein (Enb1p), which specifically recognizes and transports enterobactin.

Introduction

Enterobactin (Figure 1) is a well known catecholate-type siderophore of enterobacterial genera, designed to transport iron into the bacterial cells. It has been detected in *Escherichia coli* (O'Brian & Gibson 1970), *Salmonella typhimurium* (Pollack & Neilands 1970), the *Erwinia-Enterobacter-Pantoea* group (Berner *et al.* 1991) and the non-enteric bacterium *Aeromonas hydrophila* (Telford *et al.* 1994). Also, its regulation by the *fur* gene has been early documented (Ernst *et al.* 1978). Since catecholate groups represent the most efficient iron binding bidentates, enterobactin is regarded as the ferric-specific siderophore with the highest stability constant ($K > 10^{49}$) ever found in nature (Albrecht-Gary & Crumbliss 1998). The structure of enterobactin consists of three 2,3-dihydroxybenzoylserine moieties linked via a triester ring. Besides enterobactin, a variety of other catecholate containing siderophores have been detected in bacteria. Thus, vibriobactin, parabactin, azotochelin, amonabactin, serratiochelin and corynebactin have been shown to represent catecholate-containing siderophores (see recent review in: Drechsel and Winkelmann (1997). Also linear trimer, dimer and monomer degradation or precursor products of enterobactin can be found in bacterial culture fluids some of which may serve as siderophores as well (Hantke 1990).

Ferric enterobactin transport in enterobacteria requires an outer membrane receptor (FepA), which has recently been crystallized and whose structure has been solved by x-ray diffraction (Buchanan *et al.* 1999). FepA has a 22-stranded ß-barrel structure functioning as a gated channel. An analogous FhuA outer membrane receptor for fungal ferrichromes has also been isolated and crystallized from *E. coli*, resulting in a similar barrel structure (Ferguson *et al*. 1998). An N-terminal domain folds into the barrel, plugging the barrel pore, which is assumed to open and close upon interaction with the TonB protein, to allow entrance of the siderophore to the periplasmic binding proteins (e.g., FhuD, FepB), which subsequently deliver the siderophore to the cytoplasmic membrane proteins

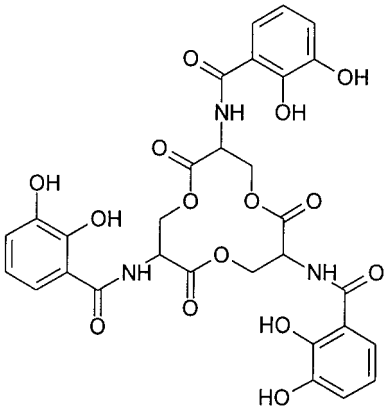

Fig. 1. Structural formula of enterobactin

(FhuB,C or FepDG,C) for final transfer to the interior of the cell (Mademidis *et al*. 1997).

Due to the highly negative reduction potential of enterobactin (−750 mV vNHE) an internal reductive mechanism of iron release has been excluded and an esterase attack prior to iron reduction has been suggested. In fungi the role of catecholate compounds in iron transport is unclear. Although various phenolic and catechole-containing secondary metabolites have been described to be synthesized in fungi, especially in basidiomycetes, where pigment formation is very prominent (Winkelmann & Drechsel 1997). While there is evidence of an involvement in biodegradation of wood, its role in iron transport has not been demonstrated (Goodell *et al.* 1997). Since enterobactin is regarded as a typical siderophore of enterobacteria genera, its utilization in fungi has so far not been studied in detail.

Genome sequencing data of *S. cerevisiae* have opened the possibility to investigate transporters of the so-called major facilitator superfamily (MFS), which besides the ATP-binding cassette (ABC) superfamily are essential for the transport of nutrients in microbial systems. Of the currently known MFS-genes in *S. cerevisiae* six UMF genes have been described, for which a function was so far unknown (Nelissen *et al.* 1997; Pao *et al.* 1998). Of the six UMF genes two have recently been identified: *YEL065w Sce* has been found to encode a transporter (Sit1p) for the bacterial ferrioxamine B (Lesuisse *et al.* 1998) and *YHL047c Sce* has been assigned a function in the transport of the fungal siderophore ferric triacetylfusarinine C (Taf1p) (Heymann *et al.* 1999) so that the UMF gene family can now be called **S**iderophore-**I**ron **T**ransporter (SIT) family according to Lesuisse (Lesuisse *et al.* 1998).

The present investigation shows that after disruption of the *YOL158c Sce* gene in a Δ*fet3*-background of *S. cerevisiae,* iron uptake via enterobactin was abolished, while all other siderophore uptake systems remained functioning. Although *S. cerevisiae* is unable to synthesize enterobactin, the disruption experiments clearly confirmed that the (MFS)-transporter gene *YOL158c*, now termed *ENB1*, is capable of transporting ferric enterobactin into the fungal cells enabling growth under iron-restricted conditions. Thus, the present investigation reports for the first time on the existence of an enterobactin-specific catecholate siderophore transporter in fungi.

Materials and methods

Siderophores and HPLC separation

Enterobactin, triacetylfusarinine C, ferrichromes and ferrioxamines were from the stock of our laboratory. Enterobactin was isolated from *Escherichia coli* AN311 (*fepB*$^{-}$strain) as described earlier (Winkelmann *et al.* 1994). Deferration of hydroxamate siderophores was according to Wiebe and Winkelmann (1975) using 8-hydroxyquinoline. The purity of siderophores was checked by HPLC using a reversed phase column (Nucleosil C18, 5 μm, 4 × 250 mm, Grom, Herrenberg, Germany) and an acetonitrile/water gradient (6–40%) with 0.1% trifluoroacetic acid (TFA) added to both solvents. HPLC separation was run on a HPLC (LC-10AT pumps, equipped with gradient controler and automatic sampler, Shimadzu, Duisburg, Germany). Detector wavelength was set at 435 nm or 220 nm which allowed to detect hydroxamate siderophores, desferrisiderophores and impurities.

Strains and culture conditions

Yeast strains were maintained on YPD (1% yeast extract, 2% peptone, 2% glucose) agar and grown overnight in liquid YPD medium at 30 °C under aeration on a rotary shaker. For disruption experiments the strain *S. cerevisiae* DEY1394 Δ*fet3*(*MATα*, *fet3::HIS3 ade6 can1 trp1 leu 2 his3 ura3*) was used which was kindly provided by David Eide. This strain (Δ*fet3*-strain) is defect in the high affinity reductive iron transport system (Askwith *et al*. 1996) which allows to distinguish between reductive iron uptake and intact siderophore iron uptake.

Gene disruption

Disruption of the genomic MFS-encoding sequence (*YOL158c Sce*, Accession No. GB CAA99180.1) was accomplished by using the *kanMX* disruption module present on vector pUG6 (Wach *et al.* 1994). Amplification of the *kanMX* module by PCR was performed by using primers containing 3′ sequences homologous to the *kanMX* module (underlined) and 5′ sequences homologous to the *YOL158c Sce* gene (italics): YOL158c-L, 5′-*ATG CTG GAA ACT GAT CAC TCT AGG AAT GAC AAT TTA GAC G*CA GCT GAA GCT TCG TAC GC 3′; YOL158c-R, 5′-*TCA ATA TCC AAT TAC ACG ACG GAG CCA TGA TTG TTG TTT G*GC ATA GGC CAC TAG TGG ATC TG-3′. Thus the primers contained genomic and disruption module sequences. A 1.5 kb PCR-fragment was obtained and checked by agarose gel electrophoresis.

Competent cells of *S. cerevisiae* DEY1394 Δ*fet*3 were transformed with the 1.5 kb PCR fragment and positive clones were selected on YPD/G418 plates containing 200 μg/ml geneticin (G418, Calbiochem, Germany) (Wach *et al.* 1994). To verify correct integration of the disruption module into the *YOL158c Sce* gene control PCRs were performed with G418-resistant clones using the following primer pairs: YOL158c-A: 5′-TAG TCC TGA GTC AAC TGC-3′; YOL158c-D: 5′-ATC GCT AGT ACT CTC CAG-3′, and KanB: 5′-GGA TGT ATG GGC TAA ATG-3′ and KanC: 5′-CCT CGA CAT CAT CTG CCC-3′. Consistent with correct integration of the disruption module a 1874 bp fragment was obtained with primers YOL158c-A and YOL158c-D, a 467 bp fragment with primers YOL158-A and KanB and 233 bp fragment with primers YOL158c-D and KanC.

Growth promotion assays

SD-soft agar plates (0.67% yeast nitrogen base (Difco, Detroit), 2% glucose, 0.5% agar) were prepared containing 500 μM bathophenanthroline disulfonic acid (BPDS) and an inoculum of overnight-grown yeast cells. Siderophores (10 μM) were pipetted on sterile filter disks (10 μl/disk), dried in a microwave oven and placed on the agar plates. Growth zones were read after 24 and 48 h incubation at 30 °C. The same SD-medium (without agar) was used for concentration dependent growth assays in order to tests the growth response when iron-free enterobactin or ferric enterobactin was added to the medium.

Radioactive iron uptake

Strains were grown overnight in chemically defined medium (SD medium: 0.67% yeast nitrogen base (Difco, Detroit), 2% glucose, 0.5% agar). Transport kinetics were performed in SD medium containing 500 μM BPDS and 4 μM ^{55}Fe-enterobactin. Enterobactin was labeled with $^{55}FeCl_3$ (Amersham, UK, ^{55}Fe in 0.1 M HCl) in buffered solution (1 M Tris HCl, pH 7.3). For time-dependent uptake studies cells (1 ml) were harvested at intervals by filtering through nitrocellulose filters and washed with 10 ml cold saline. Siderophore-bound iron taken up by the cells was measured by liquid scintillation counting. Radioactive iron taken up was calculated as pmoles per mg dry weight.

Results

Construction of a YOL158c Sce disruptant

Disruption of the MFS-encoding *YOL158c Sce* gene was performed using the *kanMX* disruption module (pUG6) which was amplified by PCR using primers homologous to the *kanMX* sequences and MFS-encoding sequences. The PCR amplified fragment (1.5 kb) was then used to transform a *S. cerevisiae* strain possessing a deletion in the multi-copper oxidase (Fet3) of the high affinity iron uptake system (Fet3/Ftr1) (Askwith *et al.* 1996). Iron transport via this transport system may interfere with siderophore iron uptake when exogenous reduction of siderophore iron is involved, yielding ferrous iron which in turn may enter via the Fet3/Ftr1 system. The low-affinity Fet4 system is only active at high ionic iron concentrations which is not likely to occur in enterobactin or hydroxamate siderophore transport assays or growth promotion tests. Thus, the rational for choosing a Δ*fet3* back-ground for disruption was to prevent uptake by other routes. We have shown earlier that gene disruption using the *kanMX* disruption module can be successfully applied to the unknown major facilitator gene *YHL047c Sce* which we have assigned a function in ferric triacetylfusarinine C transport (Taf1p) in *S. cerevisiae* (Heymann *et al.* 1999).

Growth promotion tests

When the parental fet3-strain (DEY1394 $\Delta fet3$) and the disruptant (DEY1394 $\Delta fet3\Delta enb1$ = PHY3) were compared in growth promotion tests (agar plates)

Table 1. Growth promotion tests using hydroxamate and catecholate siderophores in the parental stain *S. cerevisiae* DEY 1394 (Δ*fet3*) and in the disrupted strain PHY3 (Δ*fet3* Δ*enb1*).

Siderophores	DEY1394 (Δ*fet3*)	PHY3 (Δ*fet3* Δ*enb1*)
Ferrichrysin	+	+
Ferricrocin	+	+
Ferrirubin	+	+
Ferrirhodin	+	+
Ferrichrome	+	+
Ferrichrome A	+	+
Ferrioxamine B	+	+
Ferrioxamine E	+	+
Coprogen	+	+
Triacetyfusarinine C	+	+
Enterobactin	+	−
Azotochelin	−	−
Amonabactins (P+T)	−	−
Dihydroxybenzoic acid	−	−

Growth was tested on agar plates using filter disks containing 10 μl of siderophores (10 μM). Enterobactin (iron-free) was added as a concentration of 100 μM.

containing various fungal and bacterial siderophores, growth was observed in the presence of ferrichromes (ferrichrysin), fusigens (triacetylfusarinine C), and with the bacterial ferrioxamines (B and E) and also with enterobactin in the parental strain, whereas the Δenb1 disruptant (PHY3) completely failed to utilize enterobactin. The results of growth promotion tests with various siderophores are summarized in Table 1, showing that the parental strain and disruptant behave identical, with regard to hydroxamate iron uptake but differ in utilization of enterobactin.

As ferric enterobactin is very sensitive to oxidative degradation, it is common to use the iron-free form in growth promotion tests. Due to the high formation constant of enterobactin (K= 10^{49}), loosely bound iron present in the growth medium will be complexed after addition to the growth medium. While the hydroxamate siderophores were added as the ferric complex at 10 μM (10 μl/disk) concentration, the concentration of iron-free enterobactin used in growth promotion test was 100 μM, enabling the formation of sufficient ferric enterobactin in growth promotion tests.

In order to determine the actual concentration range of enterobactin required for growth of *S. cerevisiae* in liquid medium, iron-free and ferric enterobactin were added in increasing concentrations (0–100 μM) to inoculated SD medium. Although a

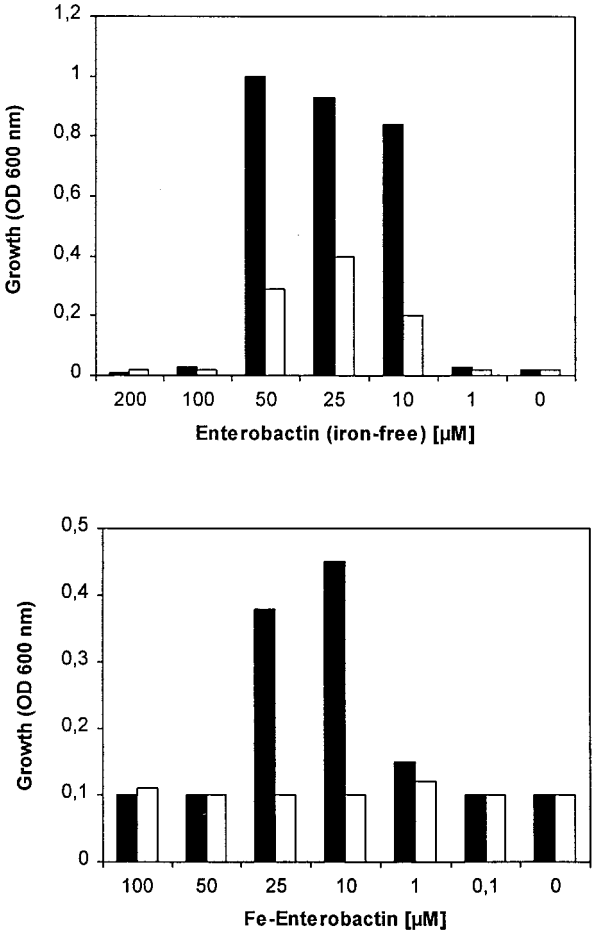

Fig. 2. Time-dependent uptake of ^{55}Fe-enterobactin (4 μM) by the parental strain, *S. cerevisiae* DEY1394 (Δ*fet*3) (●) and the disruptant PHY3 (Δ*fet*3 Δ*enb*1) (■). In addition uptake of ferric enterobactin in the presence of sodium azide (5 mM, 5 min preincubation) is shown (parental strain ○, disruptant □). Cells were grown overnight in SD-medium, sedimented and resuspended in SD medium, containing 500 μM BPDS and labeled ferric enterobactin. Aliquots of 1 ml cell suspension were taken at intervals, filtered, washed and the radioactivity was counted as described in Materials and methods. Uptake of ^{55}Fe-enterobactin was calculated as pmoles per mg dry weight.

fet3-strain was used, the SD medium contained in addition BPDS (500 μM) to prevent any residual ferrous iron uptake. As shown in Figure 2 (upper), maximal growth was observed in the concentration range of 10–50 μM enterobactin (iron-free). Since the actual amount of residual iron in the medium forming the ferric enterobactin complex-formation was unknown, additional tests with added ferric enterobactin were performed. As shown in Figure 2 (lower), maximal growth was observed at about 10 μM ferric

enterobactin. In both assays growth promotion of the Δenb1 disruption strain (PHY3) was significantly reduced. Interestingly, higher concentration of iron-free enterobactin ($> 50\ \mu$M) completely inhibited growth of *S. cerevisiae* cells, suggesting that larger amounts of enterobactin, because of its strong metal-binding formation constant or because of other properties, have an adverse effect on the fungal metabolism.

Enterobactin uptake studies

Figure 3 shows time-dependent uptake of ^{55}Fe-labeled enterobactin by parental strain and the Δ*enb1* disruptant of *S. cerevisiae* during incubation in SD-medium. Samples were taken at intervals and the radioactivity was counted as described in Material and methods. While the parental stain showed a rapid increase of uptake followed by a saturation phase after 5 min, the disruptant showed a low but constant level of radioactivity, possibly due to lipophilic adsorption of the ferric enterobactin to fungal membranes. Uptake of labeled enterobactin in the presence of sodium azide (5 mM) remained low, confirming an active transport mechanism for ferric enterobactin in *S. cerevisiae* (parental strain). Although the actual values of uptake varied within several experiments, the tendency of high uptake in the parental strain and low uptake in the disruptant was obvious and compared well with growth promotion tests in liquid or agar media. However, the absolute amount of iron incorporated during the transport experiments in fluid media also depended on the pH. When larger amounts of cells were used, rapid acidification (pH < 5) of the incubation medium occured which in turn decreased the stability of the enterobactin iron complex. Therefore, ferric enterobactin transport in *S. cerevisiae* seems to be optimal at neutral pH and decreases when the cells are actively acidifying the culture fluid.

Uptake of other catecholate siderophores. As enterobactin is a catecholate-type siderophore, we wanted to know whether other catecholate siderophores are transported as well. However, neither azotochelin from *Azotobacter vinelandii* (Duhme *et al*. 1997) nor the amonabactins (mixture of T and P) from *Aeromonas hydrophila* (Telford *et al*. 1994) or simple 2,3-dihydroxybenzoic acid supported growth of *S. cerevisiae* strains (data not shown), suggesting a high specificity of the Enb1p transporter protein for the three-dimensional structure of ferric enterobactin. In all transport assays with ferric enterobactin significant uptake was observed in the parental strain which was

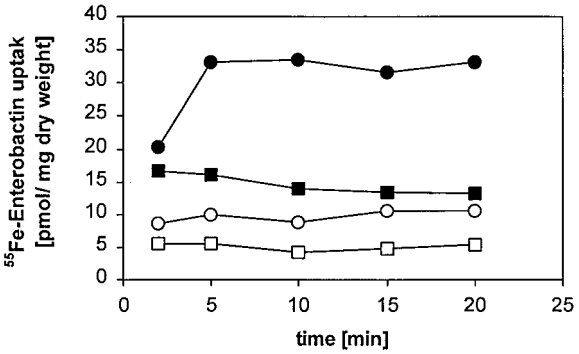

Fig. 3. Concentration dependent growth of *S. cerevisiae*, DEY1394 (Δ*fet*3), parental strain, (black) and disruptant PHY3 (Δ*fet*3 Δ*enb*1) (white) in the presence of (iron-free) enterobactin (upper) or ferric enterobactin (lower). Cells were incubated in YPD medium containing increasing amounts of (ferric or iron-free) enterobactin. Growth was monitored by measuring the optical density at 600 nm after 24 h of growth.

absent in the Δ*enb1* disruptant, confirming disruption of an enterobactin transporter gene. Thus the unknown major facilitator gene *YOL158c* in *S. cerevisiae* encodes a transport protein (Enb1p), which specifically recognizes the ferric enterobactin complex.

Discussion

As shown in the present paper, enterobactin is used as a heterologous siderophore for *S. cerevisiae*. Since unspecific iron uptake by various siderophores is sometimes observed in microorganisms, a final proof for specific uptake can only be made by a genetic approach. The fact that enterobactin failed to stimulate the growth of the disruption strain (PHY3), while other hydroxamates still functioned, is a direct proof that ferric enterobactin is recognized by a specific and separate transport protein (receptor) independent of hydroxamate transporters. This contrasts to a common transport system which we have proposed for the more closely related ferrichromes and coprogens in *Neurospora crassa* (Huschka *et al*. 1985).

In order to prove that disruption of the *YOL158c* gene affected a transporter protein, short-time transport experiments were performed using ^{55}Fe-labeled enterobactin. While growth promotion requires hours (12–24 h), short-time transport experiments showed translocation of radiolabeled molecules within minutes (1–20 min). The results with ^{55}Fe-enterobactin clearly indicated that ferric enterobactin was transported into the parental strain, but not into the disruptant. Although Yol158c Sce, based on

computer-aided protein sequence analysis, had been previously classified as a permease of unknown function (Pao *et al*. 1998), an experimental proof of its function and substrate specificity was still lacking and has now been reported for the first time in the present investigation.

Lesuisse and coworkers (1998) were the first to show that a ferrioxamine B transporter gene (*SIT1*) is a member of the unknown major facilitator superfamily genes (*YEL065w Sce*) which led us to conclude that further genes of the SIT family are involved in siderophore transport. We have shown that another homolog (*YHL047c*) of this family, encodes a specific transporter (*TAF1*) for the siderophore ferric triacetylfusarinine (Heymann *et al*. 1999). The *YOL158c* gene having been assigned here encoding a ferric enterobactin transport protein (Enb1p) and the *YEL065w* gene, encoding the Sit1p transporter for ferrioxamine B, are the most divergent members of the SIT family (Pao *et al*. 1998), reflecting the structural diversity of catecholate and hydroxamate compounds. Earlier kinetic studies have suggested that the transport of ferric enterobactin is based on simple diffusion (Lesuisse *et al*. 1998), whereas our inhibition studies using sodium azide indicated that the transport mechanism of ferric enterobactin in *S. cerevisiae* is energy-dependent. This makes sense, as actively respiring cells need more iron for growth and biosynthesis of cytochromes and iron-enzymes than fermenting cells, suggesting that siderophore-iron transport also occurs only under high repiratory chain activity and energy consumption. Siderophore transport in fungi has been shown earlier to depend on a functioning membrane potential (Huschka *et al*. 1983) which results from a proton extruding ATPase. High concentrations of protons generated by the fungal plasma membrane ATPase in turn allow a proton-coupled transport of siderophores to the cell interior (inside negative, 200 mV) which has been discussed as the most probable mechanism of siderophore transport in fungi (Huschka *et al*. 1983). Although these results were based on hydroxamate transport in *N. crassa*, energy-driven siderophore transport is now a generally accepted concept in microbial physiology, which allows active acquisition of siderophore-solubilized ferric iron. As enterobactin is regarded as a typical bacterial siderophore, its function in a fungus remains obscure. Moreover, the superior stability with iron and its negative redox potential (−750 mV vNHE) (Albrecht-Gary & Crumbliss 1998) requires specific iron release mechanisms. An esterase mechanism has been shown to be active in enterobacteria (Brickman & MacIntosh 1992) and an esterase has also been shown to be involved in ornithine ester degradation in fusarinines (Emery 1976).

It is well known that *Escherichia coli* and other Gram-negative bacteria express outer membrane receptors for both bacterial and fungal siderophores, such as ferrichromes or coprogens (Braun & Hantke 1997). The reverse is also true as exemplified by the Sit1p and Enb1p transporters. It may be anticipated that bacteria and microbial fungi compete in a common environment by using siderophores of both groups. The advantage is obvious, because a low-iron environment can be made suitable for a variety of other organisms and competitors profit much from other siderophores,which they do not need to biosynthesize. While *ENB1* and *SIT1* represent genes encoding transporters for bacterial siderophores, we have previously shown that also the UMF gene *YHL047c* of *S. cerevisiae* encodes a transporter (Taf1p) specific for the fungal siderophores ferric triacetylfusarinine C and fusigen. The fusarinines are typical fungal siderophores produced by *Aspergillus* strains and other ascomycetous fungi. The paralog *YHL040c* (close evolutionary relative to *YHL047c*) has been assigned a function in ferrirubin (ferrirhodin and ferrichrome A) transport, named *ARN1* (Heymann *et al*. 2000), which led us to assume that the iron-surrounding *N*-acyl residues in these molecules might be recognized by both transport proteins. This correlates well with earlier studies on the importance of iron surrounding *N*-acyl residues of ferrichromes in siderophore transport by *N. crassa* (Huschka *et al*. 1986). The presence of an enterobacterial siderophore transporter in *Saccharomyces*–an organism lacking any siderophore biosynthesis–underlines the perfect adaption to environmental iron sources which allows to utilize a broad collection of bacterial and fungal siderophores.

Acknowledgements

We thank David Eide and Andrew Dancis for providing yeast strains, Anne-Kathrin Duhme for a sample of azotochelin and Rowe Byers for a sample of amonabactins. We also thank Nadja Fahrbach and Michaela Gerads for technical assistance and Claudia Timpel for helpful advice in disruption and transformation techniques.

References

Albrecht-Gary A, Crumbliss AL. 1998 Coordination chemistry of siderophores: Thermodynamics and kinetics of iron chelation and release. In: Sigel A, Sigel H, eds. *Metal Ions in Biological Systems*, Vol 35. *Iron Transport and Storage in Microorganisms, Plants and Animals*. New York: Marcel Dekker; 239–327.

Askwith CC, de Silva D, Kaplan J. 1996 Molecular biology of iron acquisition in *Saccharomyces cerevisiae*. *Molec Microbiol* **20**, 27–34.

Berner I, Greiner M, Metzger J, Jung G, Winkelmann G. 1991 Identification of enterobactin and linear dihydroxybenzoylserine compounds by HPLC and ion spray mass spectrometry (LC/MS and MS/MS). *BioMetals* **4**, 113–118.

Berner I, and Winkelmann G. 1990 Ferrioxamine transport mutants and the identification of the ferrioxamine receptor protein (FoxA) in *Erwinia herbicola* (*Enterobacter agglomerans*). *BioMetals* **2**, 197-202.

Braun V, Hantke K. 1997 Receptor-mediated bacterial iron transport. In: Winkelmann G, Carrano CJ, eds. *Transition Metals in Microbial Metabolism*. Amsterdam: Harwood Academic Publisher; 67–145.

Brickman TJ, McIntosh MA. 1992 Overexpression and purification of ferric enterobactin esterase from *Escherichia coli*. Demonstration of enzymatic hydrolysis of enterobactin and its iron complex. *J Biol Chem* **267**, 12350–12355.

Buchanan SK, Smith BS, Venkatramani L, Xia D, Esser L, Palnitkar M, Chakraborty R, van der Helm D, Deisenhofer J. 1999 Crystal structure of the outer membrane active transporter FepA from *Escherichia coli*. *Nature Struct Biol* **6**, 56–63.

Dancis A, Klausner RD, Hinnebusch AG, Barriocanal JG. 1990 Genetic evidence that ferric reductase is required for iron uptake in *Saccharomyces cerevisiae*. *Mol Cell Biol* **10**, 2294–2301.

Drechsel H, and Winkelmann G. 1997 Iron chelation and siderophores, In: Winkelmann G, Carrano CJ, eds. *Transition Metals in Microbial Metabolism*. Amsterdam: Harwood Academic Publishers; 1–49.

Duhme A, RC Hider, Khodr HH. 1997 Synthesis and iron-binding properties of protochelin, the tris(catecholamide) siderophore of *Azotobacter vinelandii*. *Chem Ber /Recueil* **130**, 969–973.

Emery T. 1976 Fungal ornithine esterases: relationship to iron transport. *Biochemistry* **15**, 2723–2728.

Ernst JF, Bennet L, Rothfield LI. 1978 Constitutive expression of the iron-enterochelin and ferrichrome uptake systems in a mutant strain of *Salmonella typhimurium*. *J Bacteriol* **135**, 928–934.

Ferguson AD, Hofmann E, Coulton JW, Diederichs K, Welte W. 1998 Siderophore-mediated iron transport: Crystal structure of FhA with bound lipopolysaccharide. *Science* **282**, 2215–2220.

Goodell B, Jellison J, Liu J, Daniel G, Paszcczynski A, Fekete F, Krisnamurthu S, Jun L, Xu G. 1997 Low molecular weight chelators and phenolic compounds isolated from wood decay fungi and their role in the fungal biodegradation of wood. *J Biotechnol* **53**, 133–162.

Goffeau A, Park J, Paulsen IT, Jonniaux J-L, Dinh T, Mordant P, Saier MH. 1997 Multidrug-resistant transport proteins in yeast: complete inventory and phylogenetic characterization of yeast open reading frames within the major facilitator superfamily. *Yeast* **13**, 43–54.

Hantke K. 1990 Dihydroxybenzoylserine–a siderophore for *E. coli*. *FEMS Microbiol Lett* **67**, 5–8.

Heymann P, Ernst JF, Winkelmann G. 1999 Identification of a fungal triacetylfusarinine C siderophore transport gene (TAF1) in *Saccharomyces cerevisiae* as a member of the major facilitator superfamily. *BioMetals* **12** (in press, please include).

Heymann P, Ernst JF, Winkelmann G. 2000 Identification and substrate specificity of a ferrichrome-type siderophore transporter (ARN1p) in *Saccharomyces cerevisiae* (submitted).

Howard DH. 1999 Acquisition, transport and storage of iron by pathogenic fungi. *Clin Microbiol Rev* **12**, 394–404.

Huschka H, Winkelmann G. 1989 Iron limitation and its effect on membrane proteins and siderophore transport in *Neurospora crassa*. *BioMetals* **2**, 108–113.

Huschka H, Müller G, Winkelmann G. 1983 The membrane potential is the driving force for siderophore iron transport in fungi. *FEMS Microbiol Lett* **20**, 125–129.

Huschka H, Naegeli HU, Leuenberger-Ryf H, Keller-Schierlein W, Winkelmann G. 1985. Evidence for a common siderophore transport system but different siderophore receptors in *Neurospora crassa*. *J Bacteriol* **162**, 715-721.

Huschka H, Jalal MAF, van der Helm D, Winkelmann G. 1986 Molecular recognition of siderophores in fungi: Role of iron-surrounding N-acyl residues and the peptide backbone during membrane transport in *Neurospora crassa*. *J Bacteriol* **167**, 1020–1024.

Lesuisse E, Labbe P. 1994 Reductive iron assimilation in Saccharomyces cerevisiae. In: Winkelmann G, Winge DR, eds. *Metal Ions in Fungi*. New York: Marcel Dekker; 149–178.

Lesuisse E, Simon-Casteras M, 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. *Microbiology* **144**, 3455–3462.

Leong SA, Winkelmann G. 1998 Molecular biology of iron transport in fungi. In: Sigel A, Sigel H, eds. *Metal Ions in Biological Systems*, Vol. 35, New York: Marcel Dekker; 147–186.

Mademidis A, Killmann H, Kraas W, Flechsler I, Jung G, Braun V. 1997 ATP-dependent ferric hydroxamate transport system in *Escherichia coli*: periplasmic FhuD interacts with a periplasmic and with a transmembrane/cytoplasmic region of the integral membrane protein FhuB, as revealed by competitive peptide mapping. *Mol Microbiol* **26**, 1109–1123.

Nelissen B, De Wachter R, 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 Lett* **21**, 113–134.

Neilands J B, Konopka K, Schwyn B, Coy M, Francis RT, Paw BH, Bagg A. 1987 Comparative Biochemistry of microbial iron assimilation. In: Winkelmann G, van der Helm D, Neilands JB, eds. *Iron Transport in Microbes, Plants and Animals*, Weinheim: VCH-Wiley; 3–33.

O'Brian IG, Gibson F. 1970 The structure of enterochelin and related 2,3-dihydroxy-N-benzoylserine conjugates from *Escherichia coli*. *Biochim Biophys Acta* **215**, 393–402

Pao SS, Paulsen IT, Saier Jr MH. 1998 Major facilitator superfamily. *Microbiol Molec Biol Rev* **62**, 1–34.

Pollack JR, Neilands JB. 1970 Enterobactin, an iron transport compound from *Salmonella typhimurium. Biochem Biophys Res Commun* **5**, 989–992.

Telford JR, Leary JA, Tunstad LMG, Byers BR, Raymond KN. 1994 Amonabactin: Characterization of a series of siderophores from *Aeromonas hydrophila*. *J Am Chem Soc* **116**, 4499–4500.

Van der Helm D, Winkelmann G. 1994 Hydroxamates and polycarboxylates as iron transport agents (siderophores) in fungi. In: Winkelmann G, Winge DR, eds. *Metal Ions in Fungi.* New York: Marcel Dekker; 39–98.

Wach A, Brachat A, Pohlmann R, Philippsen P. 1994 New heterologous modules for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae. Yeast* **10**, 1793–1808.

Wiebe C, Winkelmann G. 1975 Kinetic studies on the specificity of chelate-iron uptake in *Aspergillus*. *J Bacteriol* **123**, 837–842.

Winkelmann G. 1979 Evidence for stereospecific uptake of iron chelates in fungi. *FEBS Lett* **97**, 43–46.

Winkelmann G. (ed.) 1991 Handbook of Microbial Iron Chelates Boca Raton FL: CRC Press.

Winkelmann G. 1993 Kinetics, energetics, and mechanisms of siderophore iron transport in fungi. In: Barton LL, Hemming BC, eds. *Iron Chelation in Plants and Soil Organisms*, San Diego: Academic Press; 219–239.

Winkelmann G, Braun V. 1981 Stereoselective recognition of ferrichrome by fungi and bacteria. *FEMS Microbiol Lett* **11**, 237–241.

Winkelmann G, Huschka H 1984. A study on the mechanism of siderophore transport–a proton symport. *J Plant Nutr* **7**, 479–487.

Winkelmann G., and D.R. Winge. (eds.) 1994 *Metal Ions in Fungi.* New York: Marcel Dekker.

Winkelmann G, Cansier A, Beck W, Jung G. 1994 HPLC separation of enterobactin and linear 2,3-dihydroxybenzoylserine derivatives: a study on mutants of *Escherichia coli* defective in regulation (fur), esterase (fes) and transport (fepA). *BioMetals* **7**, 149–154.

Winkelmann G, Drechsel H. 1997 Microbial siderophores. In: Kleinkauf H, von Döhren H, eds, *Biotechnology*, 2nd ed., Vol. 7, Weinheim VCH-Wiley; 199–264.