Characterization of the *Aspergillus nidulans* transporters for the siderophores enterobactin and triacetylfusarinine C

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The filamentous ascomycete *Aspergillus nidulans* produces three major siderophores: fusigen, triacetylfusarinine C, and ferricrocin. Biosynthesis and uptake of iron from these siderophores, as well as from various heterologous siderophores, is repressed by iron and this regulation is mediated in part by the transcriptional repressor SREA. Recently we have characterized a putative siderophore-transporter-encoding gene (*mirA*). Here we present the characterization of two further SREA- and iron-regulated paralogues (*mirB* and *mirC*), including the chromosomal localization and the complete exon/intron structure. Expression of *mirA* and *mirB* in a *Saccharomyces cerevisiae* strain, which lacks high affinity iron transport systems, showed that MIRA transports specifically the heterologous siderophore enterobactin and that MIRB transports exclusively the native siderophore triacetylfusarinine C. Construction and analysis of an *A. nidulans mirA* deletion mutant confirmed the substrate specificity of MIRA. Phylogenetic analysis of the available sequences suggests that the split of the species *A. nidulans* and *S. cerevisiae* predates the divergence of the paralogous *Aspergillus* siderophore transporters.

Key words: fungi, iron uptake, major facilitator, phylogenetic analysis.

INTRODUCTION

Most prokaryotes and all eukaryotes require iron for their growth. Iron is most commonly found as virtually insoluble ferric hydroxide and therefore micro-organisms require high affinity iron-uptake systems. Under iron starvation most fungi excrete low molecular mass ferric iron-specific chelators, termed siderophores, to mobilize extracellular iron with high affinity and specificity. Subsequently cells recover the solubilized iron from the ferri–siderophore complexes [1–3]. Remarkably many micro-organisms have developed transport systems for heterologous siderophores produced by other species. The acquisition of iron is also recognized as one of the key steps in the infection process by any pathogen since this metal is tightly sequestered by high-affinity iron-binding proteins in mammalian hosts [4]. Therefore, siderophores have often been suggested to function as virulence factors. Furthermore, siderophore biosynthesis and uptake represent possible targets for an antifungal chemotherapy because the underlying biochemical pathways are absent in human cells [5].

The best-studied eukaryotic model micro-organism, *Saccharomyces cerevisiae*, lacks the ability to synthesize siderophores although it can utilize siderophores produced by other species [6]. This yeast employs two distinct high-affinity iron-uptake systems. The first mechanism, termed 'reductive iron assimilation', requires the action of surface metalloreductases to reduce ferric ions to ferrous ions [7–9] which are subsequently transported into the cell by the permease–oxidase complex Ftr1p/Fet3p [10,11]. This system allows the uptake of both siderophore-bound and -unbound iron [12]. The second iron-uptake system, called 'non-reductive iron assimilation', is specialized for the uptake of siderophore-bound iron and depends on four members of the unknown major facilitator (UMF) family of the major facilitator superfamily [12–17]. Two of these transporters display a high substrate specificity: Enb1p/Arn4p transports enterobactin (EB) and Taf1p/Arn2p transports *N,N′,N″*-triacetylfusarinine C (TAF). Other transporters are less specific: Sit1p/Arn3p transports ferrioxamine and ferrichrome (FCH), and Arn1p transports a wide range of hydroxamate siderophores. Expression of genes encoding siderophore transporters is repressed by iron and this regulation is mediated by the paralogous transcriptional activators Aft1p and Aft2p [12,18,19]. Repression of expression of these genes involves the negative acting Tpk2p protein kinase A and the general transcriptional repressors Tup1p and Ssn6 [20,21].

The yeast *Candida albicans* is also able to take up iron reductively and non-reductively, and has an independent haem uptake system [22,23]. The reductive transport involves orthologues to *Saccharomyces cerevisiae* Fre1p, Fet3p and Ftr1p and it has been shown that CaFTR1-deficient *C. albicans* mutants are unable to establish systemic infection in mice [24–26]. Some reports have described the production of siderophores by *C.*

Abbreviations used: BPS, bathophenanthroline disulphonate; EB, enterobactin; EDDHA, ethylenediamine-*N,N′*-bis-(*o*-hydroxyphenyl)acetic acid; EST, expressed sequence tag; FC, ferricrocin; FCH, ferrichrome; TAF, *N,N′,N″*-triacetylfusarinine C; UMF, unknown major facilitator.

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The nucleotide sequence data reported will appear in DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under the accession numbers AY131330 and AY135152 for *mirB* and *mirC* respectively.

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albicans [27,28], but the nature of such siderophores has not been elucidated. Interestingly, the *C. albicans* genome at 5 × coverage seems to lack genes necessary for synthesis of hydroxamate siderophores (H. Haas, unpublished work). Nevertheless, *C. albicans* possesses a single siderophore transporter, CaArn1p/CaSit1p, which is orthologous to the *S. cerevisiae* siderophore permeases [23,29,30]. CaArn1p/CaSit1p displays a broad substrate specificity by transporting various FCH-type siderophores including ferricrocin (FC), FCH, ferrichrysin, ferrirubin, TAF and coprogen. Remarkably, CaArn1p/CaSit1p is required in a specific process of infection, namely epithelial invasion and penetration, while it is not essential for systemic infection by *C. albicans* [23,31].

Various species of *Aspergillus*, filamentous ascomycetes, are important pathogens of immunocompromised hosts, causing pneumonia and invasive disseminated disease with high mortality [32]. From a medical point of view, therefore, it is important to understand the iron acquisition mechanisms of these fungi. In contrast to *Aspergillus fumigatus*, for example, *A. nidulans* is a much rarer cause of human disease but represents a model ascomycete [33]. *A. nidulans* produces the intracellular siderophore FC, which is involved in iron storage, and two major extracellular siderophores, fusigen and its acetylated form TAF [34,35]. In *A. nidulans*, siderophore biosynthesis and uptake are negatively regulated by iron and derepressed in strains lacking the GATA transcription factor SREA [34,35]. Differential mRNA display led to the identification of putative SREA target genes, including *mirA* which encodes an orthologue of the *S. cerevisiae* and *C. albicans* siderophore permeases [34,36]. Subsequently, expression analysis of gene fragments found in an *A. nidulans* expressed sequence tag (EST) database suggested the existence of two further iron- and SREA-regulated *mirA* paralogues, termed *mirB* and *mirC*.

So far, siderophore transporters have only been functionally characterized from yeasts which probably utilize exclusively siderophores produced by other species. In this study we present the molecular and functional characterization of siderophore-transporter-encoding genes of the siderophore-producing filamentous fungus *A. nidulans*.

EXPERIMENTAL

Strains, vectors, growth media and general molecular techniques

Genotypes and sources of the *A. nidulans* and *S. cerevisiae* strains, as well as descriptions of the plasmids used, are summarized in Table 1. Plasmids were propagated in *Escherichia coli* DH5α (Life Technologies, Vienna, Austria). The *A. nidulans* strains WG355, WGTRAN, SRK01 and MIKO3 are designated in the text as *argB*⁻, *wt*, Δ*sreA* and Δ*mirA*, respectively. Construction of WGTRAN and MIKO3 is described below.

Generally, *A. nidulans* strains were grown according to Pontecorvo et al. [37] at 37 °C in minimal medium containing 1 % glucose as the carbon source and 20 mM glutamine as the nitrogen source. Growth media for iron-replete conditions contained 10 μM $FeSO_4$; for iron-depleted conditions, iron was omitted. The iron status of the cells was confirmed by analysis of extracellular siderophore production using the chrome azurol S liquid assay [38]. Biotin (20 μg/l) or/and L-arginine (200 mg/l) was added to the media as required. *S. cerevisae* strains were grown in YPD medium [1 % (w/v) yeast extract/2 % (w/v) peptone/2 % (w/v) glucose].

Standard molecular techniques were performed as described by Sambrook et al. [39]. *A. nidulans* DNA was isolated as described previously [40]; for RNA isolation TRI Reagent™ (Sigma) was used. Transformation of *S. cerevisiae* was carried out by the lithium acetate method [57].

Table 1 Strains and plasmids used

Strain or plasmid	Genotype or description	Reference
A. nidulans strains		
WG355 (*argB*⁻)	argB2; bgA0; biA1	[56]
WGTRAN (*wt*)	as WG355, but argB2::pTRAN, argB⁺	this work
MIKO3 (Δ*mirA*)	as WG355, but ΔmirA::argB⁺	this work
SRKO1 (Δ*sreA*)	as WG355, but ΔsreA::argB⁺	[35]
S. cerevisiae strain		
PHY14	MATα, ade6, can1, his3, leu2, trp1, ura3, fet3::HIS3, Δarn1::loxP, Δarn2::loxP, Δsit1::loxP, Δenb1::loxP-kanMX-loxP[23]	
A. nidulans plasmids		
pBluescript®II	3.0 kb cloning vector (pBKS)	Stratagene
pILJ16	2.9-kb genomic *Sal*I–*Pst*I *argB* DNA fragment in pUC8	[41]
pMIRA	5.9-kb genomic *Eco*RI–*Hin*dIII *mirA* fragment in pBKS	this work
pMIRAKO	as pMIRA, but replacement of the *mirA*-containing 3.5-kb *Cla*I–*Bgl*II fragment by the 2.9-kb *Pst*I–*Bam*HI *argB* fragment of pILJ16	this work
pTRAN3-1A	reporter vector carrying a defective *argB* copy	[43]
S. cerevisiae plasmids		
Ycp22Gal	*S. cerevisiae* GAL1/10-promoter expression vector	[23]
Ycp22Gal/mirA	1.8-kb *Bam*HI–*Hin*dIII *mirA* cDNA fragment in Ycp22Gal	this work
Ycp22Gal/mirB	1.8-kb *Bam*HI–*Hin*dIII *mirB* cDNA fragment in Ycp22Gal	this work
Ycp22Gal/mirC	1.8-kb *Xba*I–*Sph*I *mirC* cDNA fragment in Ycp22Gal	this work

Disruption of *mirA* and generation of WGTRAN

For disruption of *mirA* in *A. nidulans*, the 3.5 kb *Cla*I–*Bgl*II fragment of the *mirA*-containing 5.9 kb *Eco*RI–*Hin*dIII fragment was replaced by the 2.9 kb *Pst*I–*Bam*HI *argB*-encoding fragment from pILJ16 [41] subsequent to blunt-ending of the *Pst*I and *Cla*I restriction sites with T4 polymerase. The resulting plasmid was digested with *Eco*RI/*Pst*I and the 6.2 kb fragment was gel-purified prior to transformation of *A. nidulans* strain WG355. Transformation of *A. nidulans* was carried out according to Tilburn et al. [42]. Screening of disruption mutants was performed by PCR using the primers: oHar20, 5′-GCTGGAATGATAA-CTACC-3′; oHar8, 5′-TAGAGACAGACAGTGAGG-3′; and oArgBnew, 5′-ATGTCACGAATGCGGAGT-3′. In order to obtain homokaryotic transformants, colonies from single homokaryotic spores were picked and genomic integration was confirmed by Southern-blot analysis. The *A. nidulans mirA*-deletion strain was termed MIK03. To generate an *argB* auxotroph strain (WGTRAN) derived from WG355, WG355 was transformed with plasmid pTRAN3-1A. pTRAN3-1A carries a defective *argB* copy, which allowed targeted integration of a single vector copy at the *argB* locus by complementing the *argB2* mutation [43].

Construction of *S. cerevisiae* expression vectors for *mirA*, *mirB* and *mirC*

The open reading frames of *mirA*, *mirB*, and *mirC* were amplified by PCR from *A. nidulans* cDNA using the following primers: 5′-ATCTA**GGATCC**ACA<u>ATG</u>GCTCTCGACGATAT-3′ and 5′-TCAAT**AAGCTT**CGT<u>TCA</u>TGACCTCGCCTCC-3′ for *mirA*, 5′-ATCTA**GGATCC**AAC<u>ATG</u>ACGATTGGCTCCAA-3′ and 5′-TCAAT**AAGCTT**GTGT<u>CTA</u>GAACACCATTCCC-3′ for *mirB*, and 5′-ATCTA**TCTAGA**GCG<u>ATG</u>CCCCTGCTTG-AAC-3′ and 5′-TCAAT**GCATGC**GGC<u>CTA</u>TGTTCGTTTTG-

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CTC-3′ for *mirC* (restriction enzyme cleavage sites for *Bam*HI, *Hin*dIII, *Xba*I or *Sph*I, respectively, are in bold; the translation start and stop codons are underlined). After cleavage with *Bam*HI/*Hin*dIII (for *mirA* and *mirB*) or *Xba*I/*Sph*I (for *mirC*), the 1.8 kb amplification products were inserted into the identically cut yeast transformation vector YCp22Gal [23]. In the resulting vectors YCp22Gal/mirA, YCp22Gal/mirB, and YCp22Gal/mirC, the respective putative *A. nidulans* siderophore transporters are placed under control of the galactokinase (*GAL1*) promoter. All constructs were verified by DNA sequencing.

Iron-uptake assays

Siderophores were isolated from microbial cultures, as described previously, and the purity of siderophores was checked by HPLC [44]. FCH was obtained from Sigma. Siderophores were labelled with ^{55}Fe as described by Wiebe and Winkelmann [45]. Uptake studies of siderophore-bound iron by *A. nidulans* were performed as described previously [34]. For *S. cerevisiae* iron-transport assays, cells were grown overnight in minimal medium with raffinose as the carbon source (YNB-raffinose/CSM-Trp). Subsequently cells were resuspended in minimal medium with galactose as the carbon source (YNB-galactose/CSM-Trp) and grown for 5 h at 30 °C. Cells were harvested and resuspended at a final A_{600} of 0.6 in the same fresh medium containing 100 μM bathophenanthroline disulphonate (BPS). After addition of ^{55}Fe-siderophores to a final concentration of 2 μM, cells were incubated at 30 °C. Samples were withdrawn as a function of time and cells were washed on the filter of a cell harvester (Brandel, Evry, France) before scintillation counting.

Northern-blot analysis

Generally, 15 μg of total RNA was electrophoresed on 2.2 M formaldehyde gels (1.2 % agarose) and blotted on to Hybond N membranes (Amersham, Braunschweig, Germany). Hybridization probes were generated by PCR using oligonucleotides: 5′-AGCCCGGTGTGAAAAGAG-3′ and 5′-AACAGGAGGAGGATTGCGCC-3′ for *mirA* [34], 5′-GTATATCTTCGCGCAGGG-3′ and 5′-AACCCATCAACACCCGAG-3′ for *mirB*, 5′-CTGTACTTCTTTGGAGGG-3′ and 5′-AGCAAAGGTCTGAGTGACAC-3′ for *mirC*, and 5′-CGGTGATGAGGCACAGT-3′ and 5′-CGGACGTCGACATCACA-3′ for γ-actin-encoding *acnA* [46].

A. nidulans EST sequences and genomic sequences of *A. fumigatus*, *Neurospora crassa* and *C. albicans*

A. nidulans EST sequences were retrieved from http://www.genome.ou.edu/asper_blast.html. Genomic sequences of *A. fumigatus*, *N. crassa*, and *C. albicans* were retrieved from http://www.sanger.ac.uk/Projects/A_fumigatus/, http://www.genome.wi.mit.edu/annotation/fungi/neurospora/, and http://www-sequence.stanford.edu/group/candida/, respectively.

Phylogenetic-tree analysis

For phylogenetic analysis, protein sequences were aligned using Clustal W [47] and trees were calculated using PAUP* version 4.0b10 (Swofford/2002; Sinauer Associates, Inc., Sunderland, U.K.).

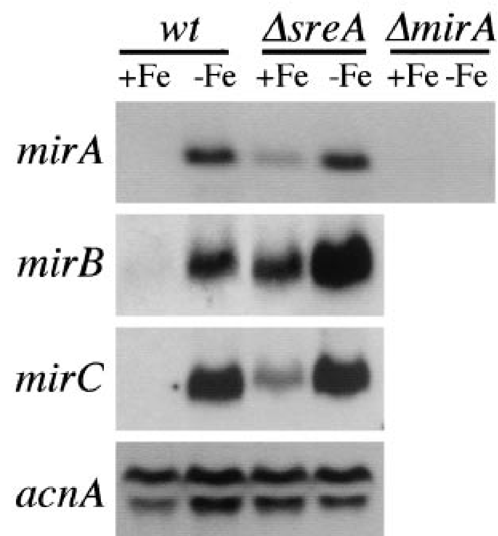

Figure 1 Expression analysis of *mirA*, *mirB* and *mirC* in *A. nidulans wt*, Δ*sreA*, and Δ*mirA* strains

Northern analysis was performed with 15 μg of total RNA isolated from strains grown for 24 h in minimal medium supplemented with 10 μM $FeSO_4$ (+Fe) or without iron addition (−Fe). As a control for loading and RNA quality, blots were hybridized with the *A. nidulans* γ-actin-encoding gene (*acnA*) [46].

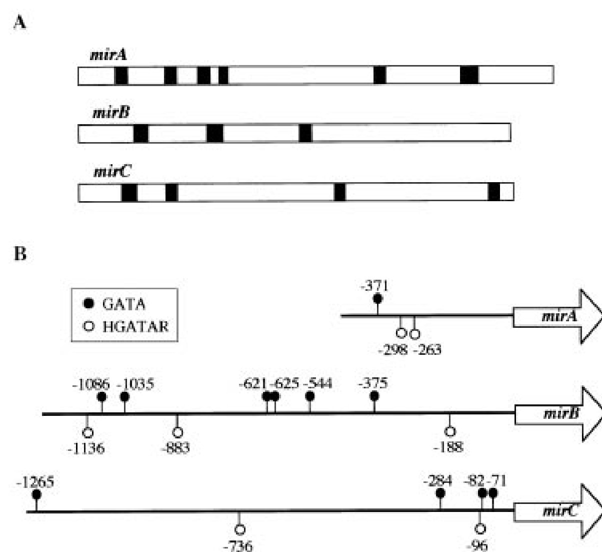

Figure 2 Comparison of exon/intron organization (A) and 5′-upstream regions (B) of *mirA*, *mirB* and *mirC*

(**A**) Exons are marked by open boxes; introns are in black. (**B**) Presence of putative binding sites for GATA transcription factors (H = A, C or G; R = A or G).

RESULTS

Isolation and characterization of *A. nidulans mirB* and *mirC*

The filamentous fungus *A. nidulans* excretes fusigen and TAF, and uses the intracellular siderophore FC as an iron-storage compound [34,35]. Biosynthesis of these siderophores as well as uptake of iron from siderophores has been shown to be repressed by iron, and derepressed under iron-replete conditions, in strains

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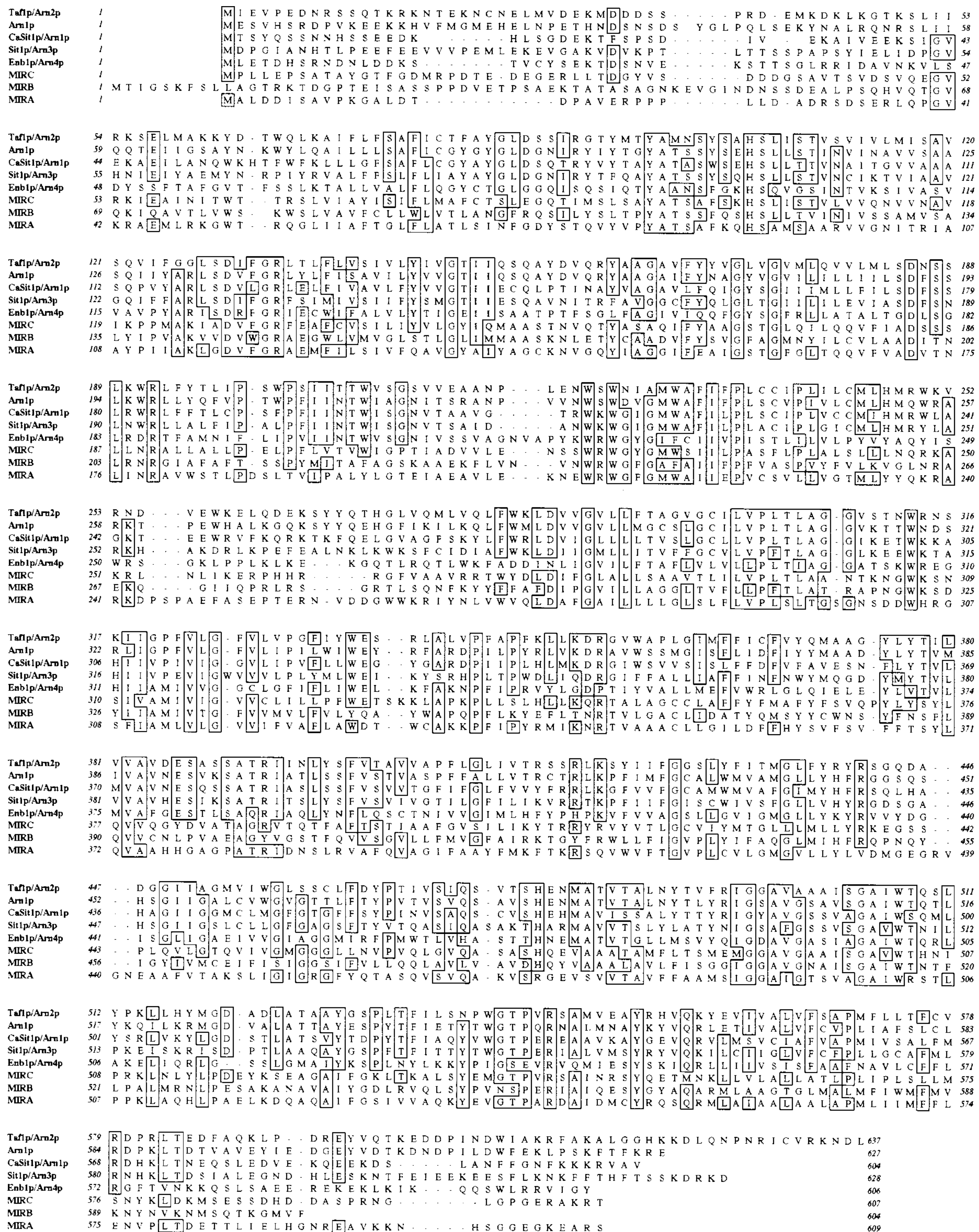

Figure 3 Sequence alignment of *A. nidulans*, *S. cerevisiae* and *C. albicans* siderophore transporters

Amino acid residues identical in at least five of the compared proteins are boxed. CaSit1p/Arn1p is found in *C. albicans*, MIRA, MIRB and MIRC in *A. nidulans*, and the remaining transporters in *S. cerevisiae*.

lacking the GATA-factor SREA [34,35]. Differential mRNA display comparing a *wt* and the Δ*sreA* strain grown under iron-replete conditions identified *mirA*, which encodes an orthologue of the *S. cerevisiae* and *C. albicans* siderophore permeases [34].

Inspection of the *A. nidulans* EST database revealed the presence of two further paralogues; the deduced amino acid sequences of EST clones y4c11a1 and r5g04a1 displayed significant but differing similarity to that of MIRA. Northern-blot

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Table 2 Amino acid identity (%) between the UMF family members of *A. nidulans*, *S. cerevisiae* and *C. albicans*

	Identity (%)						
	MIRB	MIRC	Enb1p/Arn4p	Sit1p/Arn3p	Arn1p	Taf1p/Arn3p	CaSit1p/CaArn1p
MIRA	30	34	26	28	30	28	28
MIRB		33	26	26	27	25	25
MIRC			26	31	31	30	29
Enb1p/Arn4p				32	32	32	34
Sit1p/Arn3p					32	44	49
Arn1p						57	51
Taf1p/Arn3p							45

analysis (Figure 1), using the inserts of this partial cDNA sequences as probes, proved that under iron-replete conditions expression of both corresponding genes is repressed in the *wt* and about 30 % derepressed in the Δ*sreA* strain. The entire genes, termed *mirB* and *mirC*, were isolated from a cosmid library provided by the Fungal Genetic Stock Center [48] and subsequently characterized. According to the mapping information of the hybridizing cosmid clones, *mirB* (cosmid clones W29C01, W13G10) and *mirC* (cosmid clones W26A03, W28D04, W04C10, W13G03, W15E05, W15G06) are localized on chromosome V and IV, respectively. The genomic sequences of *mirB* (GenBank accession number AY131330) and *mirC* (GenBank accession number AY135152) were determined directly from cosmid W29C01 and cosmid W26A03, respectively. The entire cDNA sequences were analysed by reverse transcription PCR using the rapid amplification of cDNA ends protocol according to Frohman et al. [49]. Comparison of cDNA and genomic sequences revealed the presence of three introns in *mirB* and four introns in *mirC*. The length, the 5′- and 3′-boundaries of the introns as well as the putative lariat formation sites perfectly match the features of fungal introns [50]. The determined length of the 5′-untranslated regions of the transcripts of *mirB* and *mirC* are 42 and 276 nt, respectively. The 3′-untranslated regions of *mirB* and *mirC* have been found to be 152 and 407 nt, respectively. A comparison of the exon/intron organization of *mirA*, *mirB* and *mirC* is shown in Figure 2. The deduced coding regions of 604 (66.2 kDa) and 607 (66.3 kDa) amino acids, respectively, display significant similarity to MIRA and the siderophore transporters from *S. cerevisiae* and *C. albicans* (Figure 3). The individual amino acid identities between the various UMF transporters are given in Table 2. Despite the limited amino acid identity of MIRA, MIRB and MIRC with the other UMF members of 26–34 %, all these proteins show similar hydropathy profiles, suggesting a conserved topology (results not shown). Topology analysis (http://bioweb.pasteur.fr/seqanal/interfaces/toppred.html) predicts 13–15 membrane-spanning domains in the different UMF members but remains highly speculative (results not shown).

Siderophore uptake by a Δ*mirA* *A. nidulans* strain

Exemplary for this transporter family, *mirA* was disrupted by replacement with the *argB* gene in an *argB*⁻-*A. nidulans* strain as described in the Experimental section. Correct disruption was verified by PCR and Southern-blot analysis (results not shown). Furthermore, Northern-blot analysis confirmed lack of *mirA* transcription in the deletion strain Δ*mirA* (Figure 1).

Compared with the *wt* and *argB*⁻ strains, Δ*mirA* showed no growth defects under iron-replete or iron-depleted conditions, on solid or in liquid media. Similarly, the regulation of intracellular and extracellular siderophore synthesis, growth on several ferri-siderophores as sole iron source (TAF, FC, FCH, and EB), and the resistance to various iron chelator [BPS, EDTA, ethylenediamine-*N*,*N*′-bis-(*o*-hydroxyphenyl)acetic acid (EDDHA), and 2,2′-dipyridyl] were not affected in Δ*mirA* (results not shown). Moreover, sensitivity tests to the antibiotic phleomycin, which indicates the intracellular iron availability [35], did not show any differences between Δ*mirA*, *wt*, and *argB*⁻ strains. Taken together, these data indicated that *mirA* either does not encode an essential transporter for siderophores, and/or that *A. nidulans* possesses an alternative, siderophore-independent, iron uptake system.

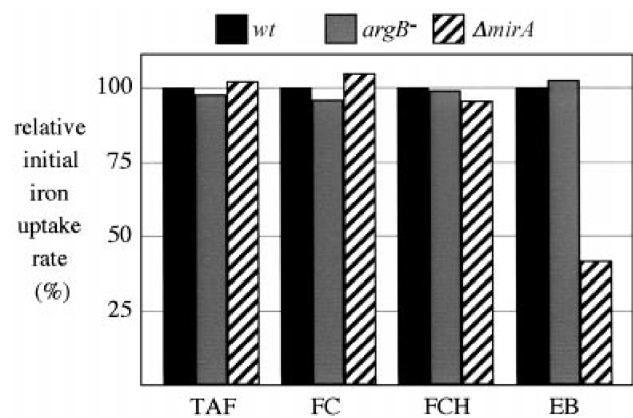

Figure 4 Initial uptake of iron from FCH, FC, TAF and EB by *A. nidulans* *wt*, *argB*⁻ and Δ*mirA* strains grown under iron-depleted conditions

Assays were performed as described in the Experimental section. Samples were prepared in triplicate, and the standard deviation did not exceed 15%.

Using short-term ^{55}Fe(III)-uptake assays, we have shown previously that *A. nidulans* is able to take up iron from the native siderophores TAF and FC as well as from various heterologous siderophores, e.g. EB, which is produced by several genera of *Enterobacteriaceae* [34]. Application of this assay showed that the initial iron uptake from EB was reduced to 40 % in the Δ*mirA* strain, compared with *wt* and *argB*⁻, whereas the iron uptake from TAF, FCH and FC was not affected (Figure 4). This suggested that MIRA transports EB. The residual uptake of EB by Δ*mirA* may be due in part to: (i) cross chelation by the homologous siderophore TAF, (ii) an alternative iron-uptake system, and/or (iii) uptake of EB by additional siderophore transporters with the same or overlapping specificity.

Expression of *mirA*, *mirB* and *mirC* in *S. cerevisiae*

Recently, two independent reports proved that it is possible to analyse the function of the *C. albicans* siderophore transporter CaSit1p/CaArn1p in a *S. cerevisiae* strain lacking all four known siderophore transporters (Arn1p, Arn2p/Taf1p, Sit1p, and Enb1p) as well as Fet3p, which is an essential component of the reductive iron uptake [23,29]. Importantly, comparison of the specificities examined in the heterologous and native systems did not show any differences [23]. Expression of the cDNA sequences of *mirA*, *mirB* and *mirC* under control of the *GAL1* promoter in such a strain (PHY14) showed that MIRA transports EB exclusively and that MIRB transports TAF

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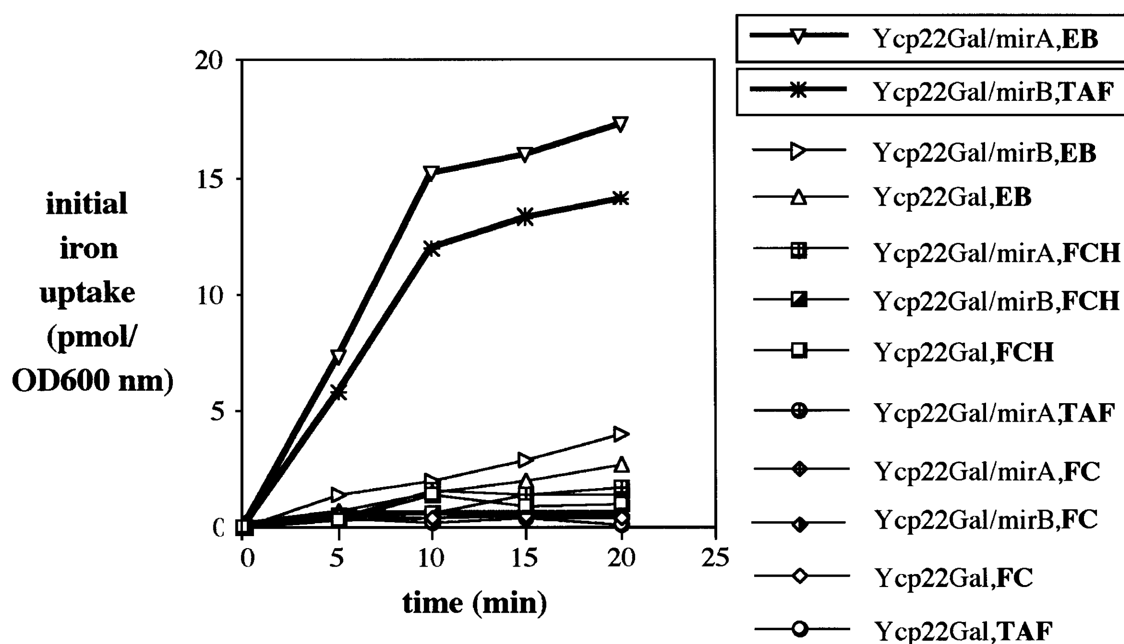

Figure 5 ***A. nidulans* siderophore-transporter-mediated iron uptake in *S. cerevisiae***

S. cerevisiae Phy14 was transformed with Ycp22Gal, Ycp22Gal/mirA or Ycp22Gal/mirB. Iron-uptake assays from the siderophores FCH, FC, EB and TAF were performed in triplicate as described in the Experimental section. The transformant type and siderophore (bold) combinations are indicated in the key on the right-hand side of the Figure. The standard deviation did not exceed 10%. OD600 nm = A_{600}.

specifically (Figure 5). In contrast, the presence of the control vector in the yeast strain or expression of *mirC* (results not shown) did not lead to significant uptake of iron from the tested siderophores.

DISCUSSION

Under iron starvation, *A. nidulans* excretes two siderophores, fusigen and TAF, and utilizes these native, as well as various heterologous, siderophores [34,35]. Lack of the GATA transcription factor SREA leads to derepression of siderophore biosynthesis and uptake of ferri-siderophores under iron-replete conditions. Differential mRNA display identified the first non-yeast member of the family 16 (UMF) of the major facilitator superfamily [51], an orthologue of the *S. cerevisiae* and *C. albicans* siderophore permeases [34]. In the present study we demonstrated that *A. nidulans* possesses, in addition to *mirA*, at least two further iron- and SREA-regulated paralogues, *mirB* and *mirC*. *mirA* and *mirC* are localized on chromosome IV, whereas *mirB* is localized on linkage group V. With respect to the number and position of introns, *mirA*, *mirB*, and *mirC* display completely different exon/intron organizations (Figure 2A). The 5′-upstream regions of all three genes contain several HGATAR motifs (Figure 2B), which represent putative binding sites for GATA factors like SREA [52]. Also of note is the fact that simple GATA sequences, not following the HGATAR consensus motif, have been found to serve as binding sites for GATA factors *in vivo*, e.g. binding sites in the *sid1* promoter for the *Ustilago maydis* SREA homologue URBS1 [53]. Northern-blot analysis proved that expression of *mirA*, *mirB* and *mirC* is repressed by iron in the *wt* strain, and approximately 30 % derepressed in the Δ*sreA* strain, under iron-replete conditions (Figure 1). These data are consistent with partial derepression of siderophore uptake under iron-replete conditions observed in short-term uptake assays in a Δ*sreA* strain [34]. Furthermore, the partial derepression indicates that additional, SREA-independent iron-regulatory mechanisms exist in *A. nidulans*. In this respect it is interesting to note that an *FRE*-orthologous putative metalloreductase-encoding gene of *A. nidulans* has recently been found to be SREA-independently iron-regulated [54]. A conceivable further iron-regulatory mechanism could involve an orthologue to *S. cerevisiae* AFT1p, but similar sequences seem to be missing in the genomes of *A. nidulans*, *A. fumigatus* and *N. crassa*.

Deletion of *mirA* in *A. nidulans* did not result in any obvious growth phenotype; the tested features included growth on solid and in liquid media under iron-replete as well as iron-depleted conditions, regulation and amount of siderophore production, sensitivity to various chelators for ferric and ferrous iron (BPS, EDTA, EDDHA, and 2,2′-dipyridyl), growth on ferrisiderophores (TAF, FC, FCH and EB) as sole iron source, and resistance to the antibiotic phleomycin, which indicates intracellular iron availability due to activation by iron [35]. These results indicate that *mirA* does not encode an essential component of the iron assimilatory system under the conditions used.

Short-term ^{55}Fe(III)-uptake assays demonstrated that MIRA deficiency results in a decrease in EB uptake by 60 %, while utilization of TAF, FC, and FCH was not affected. We have shown previously that *A. nidulans* can utilize the catecholate type siderophore EB, which is produced by several *Enterobacteriaceae*, and the presented data suggest that MIRA is the responsible transporter. Both unaffected growth on ferric EB as sole iron source and 40 % residual EB uptake in the MIRA-deficient strain indicated that *A. nidulans* possesses an MIRA-independent mechanism to utilize EB-chelated iron. These results disclose the limitations of investigation of siderophore transport in *A. nidulans*, and other filamentous fungi, at the moment, and the exact reason for the residual uptake of iron from EB in the

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Δ*mirA* strain is unknown. Moreover, it could not be excluded that MIRA transports additional siderophores, which might be masked by the existence of further transporters with redundant substrate specificity.

The functional analysis of siderophore-transporter-encoding genes in *A. nidulans* is hampered for the following reasons. (i) So far a siderophore-biosynthesis-negative strain is not available, which would prevent cross-chelation of tested siderophores with the native siderophores TAF and fusigen. (ii) The exact number of putative siderophore transporters is not known, and the lack of a complete genomic sequence, and the presence of introns in most genes, makes the exact prediction of genes difficult. Consequently, the analysis of the specificity of transporters is possibly handicapped by the existence of transporters with the same or overlapping specificity siderophores. (iii) *A. nidulans* might possess alternative high-affinity iron-uptake systems. Notably, we have recently identified an iron-regulated *FRE* orthologue in *A. nidulans* [54]. So far it is not known if the complete reductive iron-uptake system exists in this fungus but it is striking that database searches of the genomes of the closly related fungi *A. fumigatus* and *N. crassa* revealed putative *FET3* and *FTR1* orthologues (H. Haas, unpublished work).

Due to these limitations, the putative *Aspergillus* transporters were further analysed heterologously in *S. cerevisae*. This approach has been successfully applied for the functional analysis of *C. albicans* CaArn1p/CaSit1p, whereby the substrate specificities obtained in the heterologous system reflected the native substrate profile [23,29]. *Saccharomyces* does not produce any siderophores, but has multiple well-characterized transport systems that can utilize siderophores secreted by other organisms; additionally, strains lacking the responsible high-affinity iron-uptake systems are available [12–17]. Functional analysis of the *Aspergillus* transporters in yeast confirmed that MIRA transports exclusively EB and showed that MIRB is specific for TAF. Remarkably, the major *S. cerevisiae* transporters for EB (Enb1p/Arn4p) and TAF (Taf1p/Arn2p) also display the strictest substrate specificity, wheras Arn1p and Sit1p/Arn3p are less specific. Arn1p and Sit1p/Arn3p of *S. cerevisiae* and *C. albicans* CaArn1p/CaSit1p transport TAF, albeit at a very reduced rate that is limiting for growth [17,23]. With respect to sequence similarity and regulation of expression, *mirC* displays typical features of a further *A. nidulans* siderophore transporter. The failure to detect any transport activity for MIRC might have three reasons: (i) MIRC transports a heterologous siderophore, which was not tested, (ii) the uptake mechanism mediated by *Aspergillus* MIRC is incompatible with the heterologous yeast system (it is important to note that in addition to successful analysis of the *C. albicans* siderophore transporter, Arn1p/Sit1p in yeast, there is at least one other example of lack of functional expression due to unknown reasons [30]) and, (iii) MIRC does not transport siderophores. Notably *S. cerevisiae* possesses, in addition to the siderophore transporters Enb1p/Arn4p, Taf1p/Arn2p, Sit1p/Arn3p and Arn1p, two further UMF proteins, YKR106w and Ycl073cp, which do not seem to be involved in siderophore transport [58]. Unfortunately all attempts to generate a *mirC*-deficient *A. nidulans* strain have failed so far, leaving the function of MIRC unclear.

Phylogenetic analysis (Figure 6) of the available sequences of *S. cerevisiae*, *C. albicans* and *A. nidulans* did not show clustering of functional similar siderophore transporters (e.g. MIRA and Enb1p/Arn4p, or MIRB and Taf1p/Arn2p) but revealed a higher level of similarity between the *Aspergillus* transporters MIRA, MIRB, and MIRC. These data suggest that the paralogous siderophore transporters of *A. nidulans* and *S. cerevisiae* are 'in-paralogues' [55] which arose after the split of these two species. Consequently, the substrate specificity of *Aspergillus* transporters cannot simply be deduced by *in silico* comparison with the respective *S. cerevisiae* homologues, but requires experimental determination. Furthermore, comparison of the amino acid sequences of the known siderophore transporters did not reveal obvious candidates for amino acid residues or protein domains involved in the discrimination of substrates.

S. cerevisiae possesses four siderophore transporters, while in *C. albicans* only a single one has been detected. It has been suggested that a lack of siderophore biosynthesis is the driving force behind the diversity in siderophore transporters within a species [29]. We have shown that the siderophore producer *A. nidulans* has the ability to utilize the native siderophores TAF and FC as well as various heterologous siderophores, e.g. EB, ferrirubin, and FCH. Like the uptake of TAF and EB, utilization of iron bound to FCH, FC and ferrirubin by *A. nidulans* seems to be non-reductive because it is not inhibited by BPS (results not shown). This indicates the presence of at least one further siderophore transporter besides MIRA and MIRB. The inspection of the genomes of the *N. crassa* and *A. fumigatus*, close relatives of *A. nidulans*, suggests the presence of two and seven putative orthologous siderophore-transporter-encoding genes, respectively. Taken together, these data show that producers of siderophores can also contain multiple siderophore transporters. Competition for heterologous siderophores was suggested to play an important role in microbial systems. In this respect, it is remarkable that *A. nidulans*, despite inherent siderophore production, is able to specifically utilize the catecholate-type siderophore EB, which is produced exclusively by various *Enterobacteriaceae*.

As already emphasized by Ardon et al. [29], the ability to study siderophore transport in such a well-defined system as *S. cerevisiae* facilitates the analysis of the recognition properties of siderophore transporters and might also be helpful in the development of siderophore analogues with antimicrobial

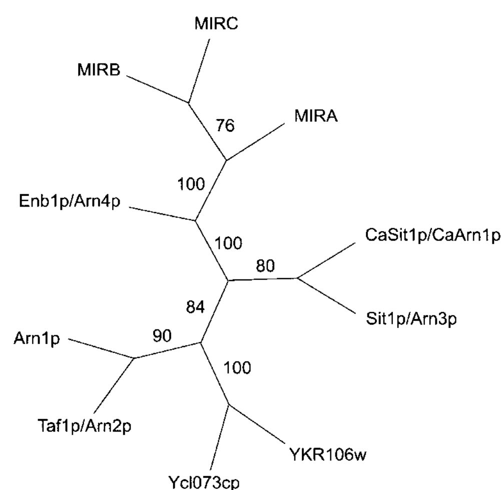

Figure 6 Phylogenetic tree of the siderophore transporters from *A. nidulans*, *S. cerevisiae* and *C. albicans*

Sequences were aligned using Clustal W [47] and trees were calculated using PAUP*4.0b10. Numbers indicate the bootstrap probability values of observing the branch topology shown (1000 resamplings; a value of 100 indicates the maximum of probability that the split is certain). The given tree was calculated with the 'neighbour joining' algorithm; the 'maximum parsimony' analysis resulted in exactly the same topology. YKR106w and Ycl073cp are *S. cerevisiae* paralogues with unknown function.

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properties [5]. The determination of the substrate specificities of an increasing number of transporters should, moreover, facilitate the identification of amino acid residues that are important for substrate recognition.

We thank Dr P. Punt for providing vector pTRAN3-1A. We are grateful to Dr B. A. Roe, Dr D. Kupfer, Dr H. Zhu, Dr J. Gray, Dr S. Clifton, Dr R. Prade, Dr J. Loros and Dr J. Dunlap for the information supplied by the *A. nidulans* cDNA sequencing project, the Whitehead Institute/MIT Center for Genome Research (Cambridge, MA, U.S.A.) for access to the *N. crassa* genome sequence, the Stanford Genome Technology Center (Palo Alto, CA, U.S.A.) for access to the *C. albicans* genome sequence, and the Sanger Institute (Cambridge, U.K.) and its collaborators, Dr David Denning and Dr Andrew Brass at the University of Manchester (Manchester, U.K.), for access to the *A. fumigatus* genome sequence. We acknowledge the excellent technical assistance of Ms M. Gerads (Düsseldorf, Germany). This work was supported by Austrian Science Foundation grant FWF-P13202-MOB (to H.H.) and Austrian National Bank (OENB) grant 8750 (to H.H.).

REFERENCES

1 Bagg, A. and Neilands, J. B. (1987) Molecular mechanism of regulation of siderophore-mediated iron assimilation. Microbiol. Rev. **51**, 509–518
2 Leong, S. A. and Winkelmann, G. (1998) Molecular biology of iron transport in fungi. Met. Ions Biol. Syst. **35**, 147–186
3 De Luca, N. G. and Wood, P. M. (2000) Iron uptake by fungi: contrasted mechanisms with internal or external reduction. Adv. Microb. Physiol. **43**, 39–74
4 Weinberg, E. D. (1999) The role of iron in protozoan and fungal infectious diseases. J. Eukaryotic Microbiol. **46**, 231–238
5 Roosenberg, J. M., Lin, Y. M., Lu, Y. and Miller, M. J. (2000) Studies and syntheses of siderophores, microbial iron chelators, and analogs as potential drug delivery agents. Curr. Med. Chem. **7**, 159–197
6 Lesuisse, E. and Labbe, P. (1989) Reductive and non-reductive mechanisms of iron assimilation by the yeast *Saccharomyces cerevisiae*. J. Gen. Microbiol. **135**, 257–263
7 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. U.S.A. **89**, 3869–3873
8 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
9 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
10 Askwith, C., Eide, D., Van Ho, A., Bernard, P. S., Li, L., Davis-Kaplan, S., Sipe, D. M. and Kaplan, J. (1994) The *FET3* gene of *S. cerevisiae* encodes a multicopper oxidase required for ferrous iron uptake. Cell (Cambridge, Mass.) **76**, 403–410
11 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 (Washington, D.C.) **271**, 1552–1557
12 Yun, C. W., Ferea, T., Rashford, J., Ardon, O., Brown, P. O., Botstein, D., Kaplan, J. and Philpott, C. C. (2000) Desferrioxamine-mediated iron uptake in *Saccharomyces cerevisiae*. Evidence for two pathways of iron uptake. J. Biol. Chem. **275**, 10709–10715
13 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. Microbiology **144**, 3455–3462
14 Heymann, P., Ernst, J. F. and 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**, 301–306
15 Heymann, P., Ernst, J. F. and Winkelmann, G. (2000) A gene of the major facilitator superfamily encodes a transporter for enterobactin (Enb1p) in *Saccharomyces cerevisiae*. Biometals **13**, 65–72
16 Heymann, P., Ernst, J. F. and Winkelmann, G. (2000) Identification and substrate specificity of a ferrichrome-type siderophore transporter (Arn1p) in *Saccharomyces cerevisiae*. FEMS Microbiol. Lett. **186**, 221–227
17 Yun, C. W., Tiedeman, J. S., Moore, R. E. and Philpott, C. C. (2000) Siderophore-iron uptake in *Saccharomyces cerevisiae*. Identification of ferrichrome and fusarinine transporters. J. Biol. Chem. **275**, 16354–16359
18 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
19 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
20 Robertson, L. S., Causton, H. C., Young, R. A. and Fink, G. R. (2000) The yeast A kinases differentially regulate iron uptake and respiratory function. Proc. Natl. Acad. Sci. U.S.A. **97**, 5984–5988
21 Lesuisse, E., Blaiseau, P. L., Dancis, A. and Camadro, J. M. (2001) Siderophore uptake and use by the yeast *Saccharomyces cerevisiae*. Microbiology **147**, 289–298
22 Weissman, Z., Shemer, R. and Kornitzer, D. (2002) Deletion of the copper transporter CaCCC2 reveals two distinct pathways for iron acquisition in *Candida albicans*. Mol. Microbiol. **44**, 1551–1560
23 Heymann, P., Gerads, M., Schaller, M., Dromer, F., Winkelmann, G. and Ernst, J. F. (2002) The siderophore iron transporter of *Candida albicans* (Sit1p/Arn1p) mediates uptake of ferrichrome-type siderophores and is required for epithelial invasion. Infect. Immun. **70**, 5246–5255
24 Eck, R., Hundt, S., Hartl, A., Roemer, E. and Kunkel, W. (1999) A multicopper oxidase gene from *Candida albicans*: cloning, characterization and disruption. Microbiology **145**, 2415–2422
25 Ramanan, N. and Wang, Y. (2000) A high-affinity iron permease essential for *Candida albicans* virulence. Science (Washington, D.C.) **288**, 1062–1064
26 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 *TUP1* regulator. Microbiology **148**, 29–40
27 Ismail, A., Bedell, G. W. and Lupan, D. M. (1985) Siderophore production by the pathogenic yeast, *Candida albicans*. Biochem. Biophys. Res. Commun. **130**, 885–891
28 Sweet, S. P. and Douglas, L. J. (1991) Effect of iron concentration on siderophore synthesis and pigment production by *Candida albicans*. FEMS Microbiol. Lett. **64**, 87–91
29 Ardon, O., Bussey, H., Philpott, C., Ward, D. M., Davis-Kaplan, S., Verroneau, S., Jiang, B. and Kaplan, J. (2001) Identification of a *Candida albicans* ferrichrome transporter and its characterization by expression in *Saccharomyces cerevisiae*. J. Biol. Chem. **276**, 43049–43055
30 Lesuisse, E., Knight, S. A., Camadro, J. M. and Dancis, A. (2002) Siderophore uptake by *Candida albicans*: effect of serum treatment and comparison with *Saccharomyces cerevisiae*. Yeast **19**, 329–340
31 Hu, C. J., Bai, C., Zheng, X. D., Wang, Y. M. and Wang, Y. (2002) Characterization and functional analysis of the siderophore-Fe transporter CaArn1p in *Candida albicans*. J. Biol. Chem. **277**, 30598–30605
32 Latge, J. P. (1999) *Aspergillus fumigatus* and aspergillosis. Clin. Microbiol. Rev. **12**, 310–350
33 Brookman, J. L. and Denning, D. W. (2000) Molecular genetics in *Aspergillus fumigatus*. Curr. Opin. Microbiol. **3**, 468–474
34 Oberegger, H., Schoeser, M., Zadra, I., Abt, B. and Haas, H. (2001) SREA is involved in regulation of siderophore biosynthesis, utilization and uptake in *Aspergillus nidulans*. Mol. Microbiol. **41**, 1077–1089
35 Haas, H., Zadra, I., Stoffler, 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
36 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
37 Pontecorvo, G., Roper, J. A., Hemmons, L. M., MacDonald, K. D. and Bufton, A. W. J. (1953) The genetics of *Aspergillus nidulans*. Adv. Genet. **5**, 141–238
38 Payne, S. M. (1994) Detection, isolation, and characterization of siderophores. Methods Enzymol. **235**, 329–344
39 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
40 Zadra, I., Abt, B., Parson, W. and Haas, H. (2000) *xylP* promoter-based expression system and its use for antisense downregulation of the *Penicillium chrysogenum* nitrogen regulator NRE. Appl. Environ. Microbiol. **66**, 4810–4816
41 Upshall, A., Gilbert, T., Saari, G., O'Hara, P. J., Weglenski, P., Berse, B., Miller, K. and Timberlake, W. E. (1986) Molecular analysis of the *argB* gene of *Aspergillus nidulans*. Mol. Gen. Genet. **204**, 349–354
42 Tilburn, J., Sarkar, S., Widdick, D. A., Espeso, E. A., Orejas, M., Mungroo, J., Penalva, M. A. and Arst, Jr, H. N. (1995) The *Aspergillus* PacC zinc finger transcription factor mediates regulation of both acid- and alkaline-expressed genes by ambient pH. EMBO J. **14**, 779–790
43 Punt, P. J., Greaves, P. A., Kuyvenhoven, A., van Deutekom, J. C., Kinghorn, J. R., Pouwels, P. H. and van den Hondel, C. A. (1991) A twin-reporter vector for simultaneous analysis of expression signals of divergently transcribed, contiguous genes in filamentous fungi. Gene **104**, 119–122
44 Konetschny-Rapp, S., Huschka, H. G., Winkelmann, G. and Jung, G. (1988) High-performance liquid chromatography of siderophores from fungi. Biol. Met. **1**, 9–17

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45 Wiebe, C. and Winkelmann, G. (1975) Kinetic studies on the specificity of chelate-iron uptake in *Aspergillus*. J. Bacteriol. **123**, 837–842
46 Fidel, S., Doonan, J. H. and Morris, N. R. (1988) *Aspergillus nidulans* contains a single actin gene which has unique intron locations and encodes a γ-actin. Gene **70**, 283–293
47 Higgins, D. G., Thompson, J. D. and Gibson, T. J. (1996) Using CLUSTAL for multiple sequence alignments. Methods Enzymol. **266**, 383–402
48 Brody, H., Griffith, J., Cuticchia, A. J., Arnold, J. and Timberlake, W. E. (1991) Chromosome-specific recombinant DNA libraries from the fungus *Aspergillus nidulans*. Nucleic Acids Res. **19**, 3105–3109
49 Frohman, M. A., Dush, M. K. and Martin, G. R. (1988) Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. Proc. Natl. Acad. Sci. U.S.A. **85**, 8998–9002
50 Ballance, D. J. (1986) Sequences important for gene expression in filamentous fungi. Yeast **2**, 229–236
51 Pao, S. S., Paulsen, I. T. and Saier, Jr, M. H. (1998) Major facilitator superfamily. Microbiol. Mol. Biol. Rev. **62**, 1–34
52 Scazzocchio, C. (2000) The fungal GATA factors. Curr. Opin. Microbiol. **3**, 126–131
53 An, Z., Mei, B., Yuan, W. M. and Leong, S. A. (1997) The distal GATA sequences of the *sid1* promoter of *Ustilago maydis* mediate iron repression of siderophore production and interact directly with Urbs1, a GATA family transcription factor. EMBO J. **16**, 1742–1750
54 Oberegger, H., Schoeser, M., Zadra, I., Schrettl, M., Parson, W. and Haas, H. (2002) Regulation of *freA, acoA, lysF* and *cycA* expression by iron availability in *A. nidulans*. Appl. Env. Microbiol. **68**, 5769–5772
55 Remm, M., Storm, C. E. and Sonnhammer, E. L. (2001) Automatic clustering of orthologs and in-paralogs from pairwise species comparisons. J. Mol. Biol. **314**, 1041–1052
56 Brakhage, A. A. and Turner, G. (1992) L-lysine repression of penicillin biosynthesis and the expression of penicillin biosynthesis genes *acvA* and *ipnA* in *Aspergillus nidulans*. FEMS Microbiol. Lett. **77**, 123–127
57 Gietz, R. D. and Schiestl, R. H. (1991) Applications of high efficiency lithium acetate transformation of intact yeast cells using single-stranded nucleic acids as carrier. Yeast **7**, 253–263
58 Winkelmann, G. (2001) Siderophore transport in Fungi. In Microbial Transport Systems (Winkelmann, G., ed.), pp. 463–479, Wiley-VCH, Weinheim

Received 29 October 2002/12 December 2002; accepted 17 December 2002
Published as BJ Immediate Publication 17 December 2002, DOI 10.1042/BJ20021685

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