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Ferrichrome in *Schizosaccharomyces pombe* – an iron transport and iron storage compound

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Abstract

Schizosaccharomyces pombe has been assumed not to produce siderophores. Nevertheless, the genomic sequence of this fission yeast revealed the presence of siderophore biosynthetic genes for hydroxamates. Applying a bioassay based on an *Aspergillus nidulans* strain deficient in siderophore biosynthesis, and using reversed-phase HPLC and mass spectrometry analysis, we demonstrate that *S. pombe* excretes and accumulates intracellularly the hydroxamate-type siderophore ferrichrome. Under iron-limiting conditions, the cellular ferrichrome pool was present in the desferri-form, while under iron-rich conditions, in the ferri-form. In contrast to *S. pombe*, hydroxamate-type siderophores could not be detected in two other yeast species, *Saccharomyces cerevisiae* and *Candida albicans*.

Introduction

Iron is one of the most important nutrients of almost all organisms because of its essential metabolic role as redox cofactor for numerous enzymes, e.g. in heme moieties and iron-sulfur clusters. Although iron is abundant in the environment, it is rarely present in a soluble form in an aerobic environment, because oxidation processes lead to insoluble ferric hydroxides. To satisfy the iron need, fungi have developed various high-affinity mechanisms of iron aquisition (reviewed in Van Ho *et al.* 2002; Haas 2003; Leong & Winkelmann 1998), including (i) solubilization of iron by enzymatic reduction of ferric iron and subsequent uptake of ferrous iron by high affinity transporters, (ii) uptake of heme-iron, and (iii) mobilization of iron by siderophores. Siderophores are low molecular mass, organic, ferric iron-specific chelators, which are produced and excreted in the desferri-form mainly during iron starvation. With exception of carboxylates produced by zygomycetes (e.g. rhizoferrin produced by various Mucorales), fungal siderophores belong to the hydroxamate-type (van der Helm and Winkelmann 1994). After chelation of extracellular iron, the iron of the ferri-form is recovered by cells either by a reductive system or by specific transporters able to internalize the siderophore-iron complex (reviewed in Winkelmann 2001).

In order to warrant a steady supply of iron, cells need, in addition to uptake systems, mechanisms for the storage of this metal ion, obtained under iron-sufficient growth conditions. Free accessible iron is a devastating metal because of its ability to catalyze reactive oxygen species via the Haber-Weiss/Fenton reaction, thereby damaging almost every type of molecule found in living cells including sugars, amino acids, phospholipids, DNA bases and organic acids. Thus, iron storage compounds may also be regarded as iron detoxifiers. In animals, plants and some bacteria, iron is stored as ferritin, phytoferritin or bacterioferritin, respectively. With the exception of zygomycetes, ferritin-like molecules have not been detected among fungi (reviewed in Matzanke 1994). In contrast, most ascomycetes and basidiomycetes contain hydroxamate-type siderophores as iron storage molecules.

Some fungal species possess more than one high-affinity iron uptake systems, e.g. the basidiomycete *Ustilago maydis* utilizes reductive iron assimilation, excretes the siderophores ferrichrome and ferrichrome A, and harbours ferrichrome intracellularly (Leong & Winkelmann 1998; Ardon *et al.* 1998). In contrast, the ascomycete *Aspergillus nidulans* lacks reductive iron assimilation – it excretes the siderophores fusigen and triacetylfusarinine C as well as accumulates desferricrocin as a cellular iron storage compound (Oberegger *et al.* 2001; Eisendle *et al.* 2003; Oberegger *et al.* 2003). Similarly, *Neurospora crassa* excretes coprogen and contains ferricrocin intracellularly (Matzanke *et al.* 1987; 1988). As shown in *N. crassa* and *A. nidulans*, subsequent to uptake, the iron bound to the extracellular siderophore is transferred to cellular storages of desferri-ferricrocin synthesized during iron depleted growth (Eisendle *et al.* 2003). Recently, it has been demonstrated that lack of the intracellular siderophore ferricrocin causes oxidative stress in *Aspergillus nidulans* and decreases asexual sporulation (Eisendle *et al.* 2003). The leading eukaryotic model organism, *Saccharomyces cerevisiae*, represents an exception among microorganism because it lacks the ability to synthesize siderophores (Neilands 1995), although it is able to utilize the iron bound to siderophores produced by other microorganisms by reductive iron assimilation or specific siderophore-iron transporters (Lesuisse *et al.* 1998, Heymann *et al.* 1999; 2000; Yun *et al.* 2000). Consistently, the genome of *Saccharomyces* lacks orthologs to siderophore biosynthetic genes of *U. maydis* and *A. nidulans* (Haas 2003). As in the case of *S. cerevisiae*, *Schizosaccharomyces pombe* was assumed to lack a siderophore system (Neilands *et al.* 1987). This fission yeast has been shown to utilize reductive iron assimilation and the expression of the genes involved is regulated by the GATA-transcription factor Fep1, which displays significant similarity to the regulators of siderophore biosynthesis of *U. maydis*, *A. nidulans*, *P. chrysogenum* and *N. crassa* (Askwith & Kaplan 1997; Pelletier *et al.* 2002; Voisard *et al.* 1993; Zhou *et al.* 1998; Oberegger *et al,* 2002; Haas *et al.* 1997; 1999). Furthermore, the *S. pombe* genome contains three genes displaying significant sequence similarity to siderophore transporter-encoding genes of *S. cerevisiae*, *C. albicans* and *A. nidulans* (Kosman 2003; Haas *et al.* 2003). Recently it has been shown that these three genes are Fep1-mediated iron-regulated and that two of the encoding gene products, Str1 and Str2, transport siderophores including ferrichrome (Pelletier *et al.* 2003). Despite of the inability of past attempts to detect intracellular siderophore production (Neilands *et al.* 1987), two genes were identified in the genome sequence of the fission yeast, displaying significant similarity to genes involved in biosynthesis of hydroxamate-type siderophores (Haas 2003), which appears to contradict reports of missing siderophore synthesis. In this study we demonstrate that *S. pombe* not only has siderophore biosynthetic genes, but we also demonstrate that it produces both extracellular and intracellular hydroxamate-type siderophores.

Materials and methods

Strains and growth conditions

The *S. pombe* strain used in this study was Urs Leupold 972h- (DSM 70576), the genome sequence of which has recently been analyzed (Wood *et al.* 2002). The strain was grown for 72 h at 30 °C in liquid Edinburg minimal medium (EMM) according to Moreno *et al.* (1991). The +Fe-EMM medium contained 30 μM $FeSO_4$, for preparation of the low iron medium (−Fe-EMM), iron was omitted. The *C. albicans* (CBS5982) and *S. cerevisiae* strains used were also grown in +Fe-EMM or −Fe-EMM.

For production of conidia, the Δ*sidA A. nidulans* strain SIA05 (*argB2; bgA0; biA1,* Δ*sidA::argB*), which lacks siderophore biosynthesis (Eisendle *et al.* 2003), was grown at 37 °C on *Aspergillus* minimal medium(AMM)-agar plates according to Pontecorvo *et al.* (1953) containing 1% glucose as the carbon source, 20 mM glutamine as the nitrogen source, 20 μg/l biotin, and 10 μM triacetylfusarinine C. For the Bioassay, 10^4 conidia were point inoculated on -Fe-EMM- agar plates in close vicinity to different yeast strains. Alternatively, 10^4 conidia were point inoculated on 6-well tissue culture test plates (TPP, Switzerland) containing 5 ml solid -Fe-AMM and supernatant of the respective yeast strain grown for 48 h in liquid -Fe-EMM.

Purification, identification and quantification of siderophores

Crude identification of extracellular siderophore production was performed using the chrome azurol S liquid assay (Schwyn & Neilands 1987). Characterization and quantification of extracellular and cellular siderophores was performed by reversed-phase HPLC

chromatography according to Heymann *et al.* (2000) and Oberegger *et al.* (2001). Samples were analyzed with and without the addition of $FeCl_3$ to a final concentration of 0.5 mM, allowing the discrimination between the ferri- and desferri-form of the siderophores, as photometric determination at 435 nm records only ferri-siderophores.

Mass spectrometry

Positive FAB spectra were measured in a glycerol pTSA matrix on a Finnigan TSQ70 instrument.

Results

Putative siderophore biosynthetic genes of S. pombe – SPAC23G3.02c (encoding CAB72227) and SPAC23G3.03 (encoding CAB72228)

The two *S. pombe* genes *SPAC23G3.02c* encoding protein CAB72227 and *SPAC23G3.03* encoding CAB72228 could be involved in siderophore biosynthesis (Haas 2003). CAB72228 displays significant similarity to various fungal ornithine N^5-monooxygenases, which catalyzes the first committed enzymatic step in siderophore biosynthesis. The highest degree of similarity was found to *A. nidulans* SidA with 34.4% amino acid identity, furthermore significant similarity was found to *U. maydis* Sid1 (Mei *et al.*, 1993), *Pseudomonas aeruginosa* PvdA (Visca *et al.*, 1994) and the IucD, the lysine N^6-hydroxylase of *Escherichia coli* (Herrero *et al.*, 1988). An alignment of CAB72228 and related monooxygenases has been previously published (Eisendle *et al.* 2003). CAB72228 contains all three signatures typical for ω-amino acid hydroxylases (Stehr *et al.*, 1998): (i) a putative flavine adenine dinucleotide (FAD)-binding domain (motive GXGXXG); remarkably, this signature shows a typical replacement of the last glycine to proline – an unique feature of hydroxylases involved in siderophore biosynthesis; (ii) a putative nicotinamide adenine dinucleotide phosphate (NADP)-binding domain (motive GXGXX(G/A)); and (iii) a domain proposed to be involved in substrate binding (motive $D(X)_3(L/F)ATGY(X)_4(H/P)$).

CAB72227 shows features that have normally been found in nonribosomal peptide synthetases. Such enzymes are exceptionally large multifunctional proteins with a modular construction able to assemble compounds from a remarkable range of proteinogenic and nonproteinogenic precursors (Kleinkauf and Von Dohren, 1996; Weber and Marahiel, 2001). Each module contains an adenylation domain, a condensation domain and a peptidyl carrier domain which contains phosphopantetheine as a covalently linked cofactor. NCBI BLAST searches (http://www.ncbi.nlm.nih.gov/blast/) identified three complete modules plus three additional peptidyl carrier domain/condensation domain-units in CAB72227 (Figure 1). With respect to amino acid similarity and modular organization, CAB72227 displays the highest similarity to *Um*-Sid2, the ferrichrome peptide synthetase of *U. maydis*, and *An*-SidC, the ferricrocin peptide synthetase of *A. nidulans*. *An*-SidC contains three complete modules plus two additional peptidyl carrier domain/condensation domain-units, and *Um*-Sid2 is predicted to comprise three complete modules plus one additional peptidyl carrier domain and one partial condensation domain. Interestingly, the order of the domains in module 2 and 3 of CAB72227 is changed compared to *Um*-Sid2 and *An*-SidC. A comparison of the modular structure of CAB72227, *Um*-Sid2 and *An*-SidC is shown in Figure 1.

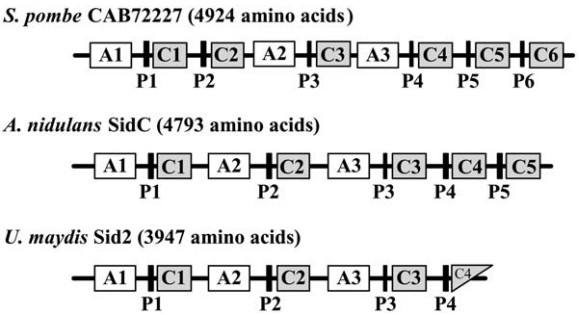

Figure 1. Modular organization of *S. pombe* CAB72227 *A. nidulans* SidC and *U. maydis* Sid2. A, C and P refer to the adenylation, condensation and peptidyl carrier domain, respectively.

The genes encoding CAB72227 and CAB72228 are located on chromosome I and are transcribed divergently from a common intergenic region in the length of 1497 base pairs (GenBank accession number AL138854). Organization of genes in clusters is in most cases indicative of an involvement of the gene products in a common pathway. Noteworthy, the *U. maydis* orthologs of the genes encoding CAB72227 and CAB72228, *Um-sid1 and Um-sid2*, show the same genetic structure (Yuan *et al.* 2001).

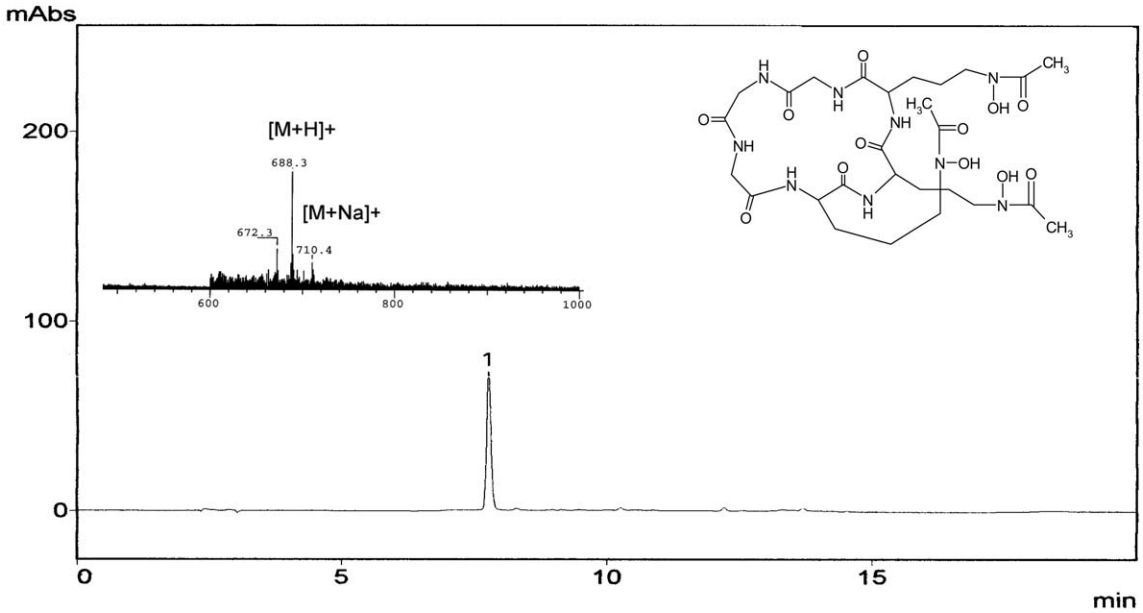

Figure 2. HPLC chromatogram of ferrichrome (ferric form) isolated from *S. pombe* cells using a detector wavelength of 435 nm. The corresponding FAB mass spectrum of the intracellular desferrichrome and the structural formula are schown as inserts. The molecular ion m/z = 688.3 was assigned to $[M+H]^+$ of the iron-free ferrichrome and m/z = 710 was assigned to $[M+Na]^+$.

Table 1. Signature sequences of putative adenylation domains of *S. pombe* CAB72227, *A. nidulans* SidC and *U. maydis* Sid2 (Amino acid residues identical in at least three signature sequences are shadded).

NRPS	Module	Signature sequence position								
		235	236	239	278	299	301	322	330	331
*Sp*CAAB72227	A1	D	V	F	T	I	I	A	I	H
	A2*									
	A3	D	V	L	D	I	G	F	I	G
*An*SidC	A1	D	P	M	M	W	M	A	I	N
	A2	D	V	Q	H	T	I	T	V	V
	A3	D	P	L	S	T	G	A	I	G
*Um*Sid2	A1	D	L	M	L	I	G	L	L	F
	A2	D	V	L	S	I	G	A	I	G
	A3	D	V	I	D	M	G	A	I	G

*A2 of CAB72227 shows poor alignment with the respective domain of *B. brevis* GrsA, which makes determination of the signature sequence impossible. According to the information given in GenBank (AL138854) the nucleotide sequence in the corresponding region might contain mistakes which presents a possible explanation for the low similarity.

Analysis of siderophores

In order to analyze whether *S. pombe* produces siderophores, this yeast was grown for 72 h at 30 °C in liquid EMM with (+FE-EMM) and without iron supplementation (−Fe-EMM). In order to enrich for putative siderophores, the supernatant was first centrifuged at 10 000 g and then was adsorbed to Amberlite XAD-16 resin (CWG). Subsequently the eluate was analysed by reversed-phase HPLC as describe previously (Konetschny-Rapp *et al.* 1988; Oberegger *et al.* 2001). This analysis (Fig. 2) indicated that *S. pombe* contains intracellular desferrichrome, determined as ferrichrome after addition of iron (Rt = 7.385 min). Mass spectrometry confirmed the identity: of purified desferrichrome by assigning the positive ions, $[M+H]^+ = 688.3$ Da and $[M+Na]^+ = 710$ m/z (Fig-

ure 2, insert). During iron-replete conditions, however, this siderophore was present primarily in the ferric form, whereby the amount of siderophores stored in the cells during iron-replete and iron depleted conditions did not vary significantly (data not shown). When larger amounts of culture supernatant were concentrated and analyzed by HPLC after 48 h of incubation, only small amounts of desferrichrome could be detected extracellulary which corresponded to about 1% of the total intracelluar content.

For the analysis of extracellular siderophores, a bioassay based on the *A. nidulans* Δ*sidA* strain was initially performed. This strain, which lacks the L-ornithine N^5-monooxygenase-encoding gene *sidA*, is not able to grow due to the absence of an alternative high-affinity iron uptake system unless it is externally supplemented with siderophores, e.g. ferricrocin, triacetylfusarinine or fusarines (Eisendle *et al.* 2003). When Δ*sidA* conidia were point-inoculated on −FE-EMM agar plates in close vicinty of *S. pombe* cells, Δ*sidA*-hyphe were found to grow towards the *S. pombe* colony, which indicates cross-feeding with siderophores (Figure 3A). In contrast, no cross-feeding of Δ*sidA* was found by *C. albicans* or *S. cerevisiae* (data not shown). A variation of this assay – point inoculation of 10^4 Δ*sidA* conidia on −Fe-AMM agar plates supplemented with the supernatant of −Fe-EMM-48h-cultures of *S. pombe*, *C. albicans* or *S. cerevisiae*, respectively, yielded the same result: growth stimulation of Δ*sidA* occurred only by *S. pombe* compounds that were excreted into the medium (Figure 3B). HPLC-analysis of the *S. pombe* supernatant indicated low amounts of ferrichrome, which was confirmed by mass spectrometry. Remarkably, the amount of extracellular siderophores excreted did not vary significantly between iron-replete and iron depleted growth as demonstrated by the *Aspergillus* bioassay (data not shown).

Discussion

This study clearly demonstrates that *S. pombe* not only accumulates intracellularly the hydroxamate-type siderophore ferrichrome but also excretes this siderophore, although to a small extent. Similar to *N. crassa* and *A. nidulans* (Matzanke *et al.* 1988; Oberegger *et al.* 2001), *S. pombe* was found to accumulate the intracellular siderophore in the desferri-form during iron-limiting conditions, whereas during iron-rich conditions, only the ferri-form was found within the cells. These data suggest that *S. pombe* utilizes ferrichrome as an iron storage molecule. As suggested for *A. nidulans*, the accumulation of the desferri-ferrichrome during iron starvation could represent a protective mechanism to avoid the iron toxic effects. Interestingly, in *S. pombe* the production of extracellular and intracellular ferrichrome was not found to be upregulated during iron starvation. This stands in contrast to most other fungi (Haas *et al.* 2003). Importantly, the bioassay performed as shown in Figure 3A strongly indicates that the extracellular ferrichrome is excreted rather than released by lysis of cells.

S. pombe seems to possess only one nonribosomal peptide synthetase, CAB72227, which is likely to be involved in formation of the intracellular siderophore ferrichrome. CAB72227 displays significant similarity to ferrichrome synthetase *Um*-Sid2 of *U. maydis* and the ferricrocin synthetase *An*-SidC of *A. nidulans* (Figure 1). At first sight, the modular structure of CAB72227 suggests that it synthesizes a tripeptide. Alternatively it might be speculated that it is responsible for the formation of a larger peptide *via* repeated use of the complete modules and further involvement of the incomplete modules. A similar mechanism was proposed for the synthesis of ferrichrome by the *Um*-Sid2 and ferricrocin by *An*-SidC (Yuan *et al.* 2001; Eisendle *et al.* 2003). Based on structural data of the phenylalanine adenylation domain of gramicidine synthetase I of *Bacillus brevis* (GrsA), nine residues, termed the 'signature sequence', have been proposed to play a major role in defining substrate specificity for incorporation of amino acids (Stachelhaus *et al.*, 1999; Challis *et al.*, 2000). Using a predictive program (http://raynam.chm.jhu.edu/∼nrps/), the amino acid specificity of the three modules of CAB72227 could not be definitely assessed. Nevertheless, the signature sequences of the adenylation domains of CAB72227 display remarkable similarity to that of *An*-SidC and *Um*-Sid2, as shown in Table 2. The structure of the ferrichrome, cyclic Gly_3-(N^5-acetyl-N^5-hydroxyornithine)$_3$, is identical to that of ferricrocin with the exception of the replacement of one glycine by a serine residue. Thus, the similar signature sequences in CAB72227, *An*-SidC and *Um*-Sid2 might indicate an identical specificity for common precursors, i.e. glycine or N^5-acetyl-N^5-hydroxyornithine. The amino acid sequence of *S. pombe* CAB72227 suggests that it might be the nonribosomal peptide synthetase involved in ferrichrome synthesis. CAB72228 is very likely to be the ornithine N^5-monooxygenase, which is essential for biosynthesis of all hydroxamate-

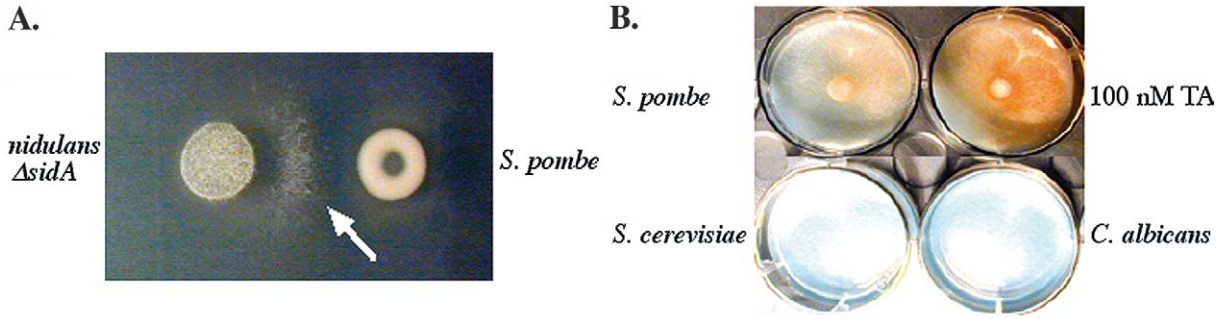

Figure 3. *A. nidulans* Δ*sidA*-bioassay for detection of excreted siderophores. **A.** *S. pombe* was grown for 24 h at 30 °C in liquid −Fe-EMM and 5 μl of the respective cell suspension were spotted in close vicinity to 10^4 point-inoculated conidia of *A. nidulans* Δ*sidA* on a −Fe-EMM agar plate. The arrow marks the region of *A. nidulans* Δ*sidA* mycelia formed due to siderophore cross-feeding by *S. pombe*. **B.** *S. pombe*, *C. albicans* and *S. cerevisiae* were grown for 48 h at 30 °C in liquid −Fe-EMM, 200 ml of the culture supernatant was freeze-dryed and resuspended in 4 ml of water. 100 μl were applied to a bioassay using *A. nidulans* Δ*sidA* as indicator for production of hydroxamate-type siderophores in 6-well tissue culture test plates containing 5 ml of solid −Fe-AMM agar per well. As a control, growth of *A. nidulans* Δ*sidA* was stimulated with 100 nM triacetylfusarinine C (TAFC).

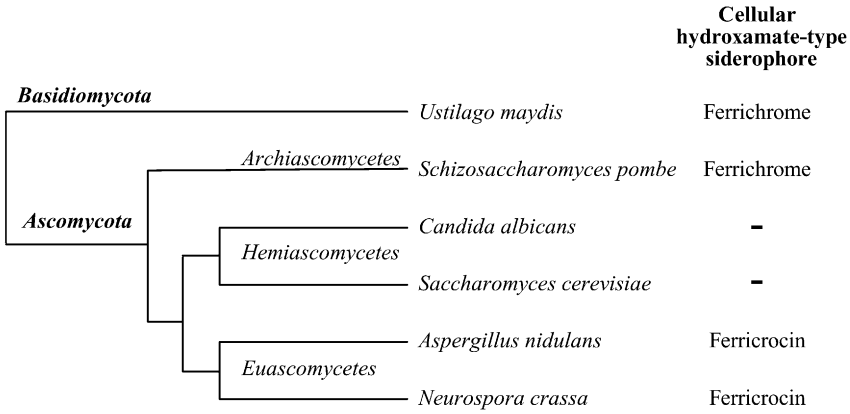

Figure 4. Phylogenetic tree of selected fungal species according to Berbee *et al.* (2000) and Heckman *et al.* (2001).

type siderophores. Whether this is the case, remains to be proved by further genetic and biochemical analysis.

It was noteworthy that the *A. nidulans* Δ*sidA*-bioassay, which demonstrated the excretion of siderophores by *S. pombe*, did not show siderophore excretion for *S. cerevisiae* and *C. albicans*. The genome sequences of these two yeast species lack orthologs to genes encoding ornithine N^5-monooxygenase and nonribosomal peptide synthetase (Haas 2003). Therefore, it seems very likely that these two yeast species indeed cannot synthesize hydroxamate-type siderophores. This is generally accepted for *S. cerevisiae* (Neilands 1995). In contrast, for *C. albicans* the synthesis of siderophores has been reported but the compound has not been chemically characterized (Ismail *et al.* 1985). Remarkably, the siderophore transporter *Ca*Arn1p/*Ca*Sit1p has recently been shown to be required for epithelial invasion and penetration by *C. albicans*, but not for systemic infection (Heymann *et al.* 2002). Possibly, the siderophore produced by *C. albicans* belongs to a chemically different class – in this case it cannot be utilized by *A. nidulans*, as shown by the bioassay.

S. pombe is one of the model fungi for the molecular analysis of iron homeostasis in fungi. Many publications stated that the fission yeast, like the budding yeast, is not able to synthesize siderophores. Our work in this manuscript demonstrates that *S. pombe* is with respect to iron homeostasis more similar to most other fungi than to *S. cerevisiae*. From a phylogenetic point of view (Fig. 4), it is interesting that the archiascomycote *S. pombe* utilizes the same cellular siderophore as the basidiomycete *U. maydis*. In contrast, most euascomycetes, e.g *A. nidulans and N. crassa*, use ferricrocin, while the hemiascomycetes *C.*

albicans and *S. cerevisiae* seem to have lost the ability to synthesize hydroxamate type siderophores.

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