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Genome mining and functional genomics for siderophore production in *Aspergillus niger*

Angelique C. W. Franken, Beatrix E. Lechner, Ernst R. Werner, Hubertus Haas, B. Christien Lokman, Arthur F. J. Ram, Cees A. M. J. J. van den Hondel, Sandra de Weert and Peter J. Punt

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Abstract

Iron is an essential metal for many organisms, but the biologically relevant form of iron is scarce because of rapid oxidation resulting in low solubility. Simultaneously, excessive accumulation of iron is toxic. Consequently, iron uptake is a highly controlled process. In most fungal species, siderophores play a central role in iron handling. Siderophores are small iron-specific chelators that can be secreted to scavenge environmental iron or bind intracellular iron with high affinity. A second high-affinity iron uptake mechanism is reductive iron assimilation (RIA). As shown in *Aspergillus fumigatus* and *Aspergillus nidulans*, synthesis of siderophores in *Aspergilli* is predominantly under control of the transcription factors SreA and HapX, which are connected by a negative transcriptional feedback loop. Abolishing this fine-tuned regulation corroborates iron homeostasis, including heme biosynthesis, which could be biotechnologically of interest, e.g. the heterologous production of heme-dependent peroxidases. *Aspergillus niger* genome inspection identified orthologues of several genes relevant for RIA and siderophore metabolism, as well as *sreA* and *hapX*. Interestingly, genes related to synthesis of the common fungal extracellular siderophore triacetylfusarinine C were absent. Reverse-phase high-performance liquid chromatography (HPLC) confirmed the absence of triacetylfusarinine C, and demonstrated that the major secreted siderophores of *A. niger* are coprogen B and ferrichrome, which is also the dominant intracellular siderophore. In *A. niger* wild type grown under iron-replete conditions, the expression of genes involved in coprogen biosynthesis and RIA was low in the exponential growth phase but significantly induced during ascospore germination. Deletion of *sreA* in *A. niger* resulted

Corresponding author. P. J. Punt, Institute of Biology Leiden, Leiden University, Molecular Microbiology and Biotechnology, Sylviusweg 72, 2333 BE Leiden, the Netherlands and TNO Microbiology & Systems Biology, Utrechtseweg 48, 3704 HE, Zeist, the Netherlands. Tel.: +31 715 274 745; Fax: +31 715 274 900; E-mail: peter.punt@tno.nl or p.j.punt@biology.leidenuniv.nl

Angelique C. W. Franken, PhD has recently obtained her PhD degree at Leiden University where she studied heme biosynthesis in *Aspergillus niger* for the purpose of improved heterologous peroxidase production.

Beatrix E. Lechner holds a Master degree in Microbiology (Leopold–Franzens-University Innsbruck) and is completing her PhD degree at the Innsbruck Medical University on fungal adaptation to iron starvation.

Ernst R. Werner, DSc is a professor at Innsbruck Medical University. His research interest is the impact of the immune system on metabolism, with an analytical biochemistry background and a focus on tetrahydrobiopterin and ether lipids.

Hubertus Haas, PhD is a professor at the Innsbruck Medical University. His research interest is fungal metabolism at the molecular level with respect to biotechnology and virulence with a focus on iron and secondary metabolism.

B. Christien Lokman, PhD is leading lector in the technology and life sciences area at the HAN, is responsible for contract research at the HAN BioCentre and for the Masters course in Molecular Life Sciences.

Arthur F. J. Ram, PhD is head of the department of Molecular Microbiology and Biotechnology at Leiden University. The research is related to deciphering molecular mechanisms in filamentous fungi related to changing environmental or industrial conditions.

Cees A. M. J. J. van den Hondel, PhD is Emeritus professor Molecular Genetics and Microbiology at the University of Leiden. Since 1981 till present he is performing Molecular-Genetical, Cell-Biological and Biotechnological research on filamentous fungi especially *Aspergillus* species.

Sandra de Weert, PhD is head of the department of Process development Microbiology at Koppert B.V. Her research is related to optimal bioreactor growth of microorganisms and metabolite production and detection for the development of products in the field of biological crop protection.

Peter J. Punt, PhD is senior scientist in Industrial Biotechnology at TNO Microbiology & Systems Biology. He is also professor at Leiden University in this research field appointed by Lorentz van Iterson Fund TNO (LIFT). His research focuses on fungal biotechnology using molecular genetics and systems biology tools.

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in elevated iron uptake and increased cellular ferrichrome accumulation. Increased sensitivity toward phleomycin and high iron concentration reflected the toxic effects of excessive iron uptake. Moreover, SreA-deficiency resulted in increased accumulation of heme intermediates, but no significant increase in heme content. Together with the upregulation of several heme biosynthesis genes, these results reveal a complex heme regulatory mechanism.

Keywords: Aspergillus; *iron; heme biosynthesis; SreA; siderophore; transciptome analysis*

INTRODUCTION

Although iron is one of the most abundant metals on earth, it is mostly present in its oxidized ferric state (Fe^{3+}) [1], forming particles of ferric hydroxides that have a very low solubility [1, 2]. As a result, the bioavailability of iron is low. Therefore, organisms have developed various mechanisms to sequester available iron from its environment but also prevent iron toxicity, which has been extensively described by Haas *et al.* [3]. In brief, the mechanisms that fungi can use consist of (i) low-affinity iron uptake through transporters that not only transport iron but also other metals like copper and zinc, (ii) heme uptake and iron utilization via heme oxygenase-like proteins, which are present in many organisms, (iii) reductive iron assimilation (RIA), which transports iron though the formation of a high-affinity transport complex (a ferrioxidase and an iron permease [1]), whereby the ferrous iron is initially oxidized to ferric (Fe^{3+}) iron, followed by transport into the cytosol [4] and finally (iv) siderophore-mediated iron uptake [3]. Siderophore-mediated iron uptake is a mechanism used by many organisms, and siderophores can be classified into three groups: hydroxamates, carboxylates and aryl caps. Fungi mainly synthesize siderophores belonging to the hydroxamate type (the exception being rhizoferrin) [3, 5] (Table 1). The hydroxamate siderophores can be further grouped into the four structural families: rhodothorulic acid, fusarinines, coprogens and ferrichromes. These hydroxamate-type siderophores are all derived from the nonproteinogenic amino acid ornithine [3, 5] (Table 1). It also becomes clear from Table 1 that different member types of siderophores can be synthesized by different *Aspergilli*.

Synthesis of siderophores is initiated with N^5-hydroxylation of ornithine by L-ornithine N^5-monooxygenase, followed by N^5-acylation of N^5-hydroxyornithine (Figure 1). At this point biosynthesis pathways divide depending on the choice of the acyl group and therefore the siderophore type to be synthesized. The third step in siderophore biosynthesis covalently links the hydroxamates, which is accomplished by nonribosomal peptide synthetases (NRPSs) [3]. Inactivation of 4'-phosphopantetheinyl transferase (*npgA/pptA*) inactivated all NRPSs and polyketide synthases in *Aspergillus nidulans*, which resulted in abolished siderophore synthesis and lysine auxotrophy [9]. Recently, a *pptA* deletion strain was also examined in *Aspergillus niger* [10]. The resulting deletion strain displayed a similar phenotype as the *A. nidulans npgA/pptA* deletion strain [9, 10], supporting its involvement in siderophore and lysine biosynthesis in *A. niger* as well.

Heme biosynthesis is one of the bottlenecks for commercial peroxidase production [11–13]. In attempts to increase heme biosynthesis by enhanced iron uptake, we identified siderophore biosynthesis as a potential target in *Aspergilli*. Hence, our interest to analyze siderophore production in *A. niger* in more detail.

Iron uptake and homeostasis in both *Aspergillus fumigatus* and *A. nidulans* is predominantly under control of the transcription factors SreA and HapX [14, 15]. When iron is available in sufficient amounts, SreA represses the high-affinity uptake systems. SreA also acts as the repressor for *hapX* under iron sufficiency. When iron is limited, *hapX* is derepressed and subsequently represses *sreA* expression and iron-dependent pathways, including the heme biosynthesis pathway. Deletion of *sreA* in *A. fumigatus* [14] resulted in an increased iron uptake under iron sufficient conditions and upregulation of the heme biosynthesis genes *hemA* (coding for 5'-aminolevulinic acid synthase) and *hemH* (coding for ferrochelatase), leading to increased porphyrin and heme content. Here we show that SreA repression derepresses siderophore synthesis, increased iron uptake and resulted in a change in cellular accumulation of heme intermediates.

MATERIALS AND METHODS

Strains and culture conditions

Aspergillus niger N402 (*cspA1* derivative of ATCC9029 [16]) and the *pyrG*$^-$ derivative of this strain, AB4.1 [17], were used during this study.

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Table I: Classification of fungal siderophores

Main group	Structural family	Siderophore	Produced by (examples)	References
Carboxylates		Rhizzoferrin	*Rhizopus* spp; *Mucor* spp; *Phycomyces* spp; *Mycotypha* spp etc.	[3, 5]
Hydroxamate	Rhodothorulic acid	Rhodothorulic acid	*Rhodothorula* spp; *Rhodosporidium* spp; *Leucosporidium* spp; *Sporidiobolus* spp; *Sporobolomyces* spp	[3, 5]
	Coprogens	Coprogen	*Penicillium* spp; *Neurospora crassa Curvularia spp*	[3, 5] [6]
		Coprogen B	*Fusarium dimerum*	
			A. niger	
		Thiornicin	*Epicoccum purpurescens*	
		Neocoprogen I	*Curvularia lunata*	
		Neocoprogen II	*Curvularia lunata*	
	Fusarinines	Fusarinine C	*Agaricus bisporus*	[3, 5]
			Fusarium spp	
			Giberella spp	
		Triacetylfusarinine C	*A. fumigatus*; *A. nidulans*	
			Penicillium spp	
	Ferrichromes	Ferrichrome	*Ustilago sphearogena*	[3, 5, 7, 8] [6]
			A. niger	
		Ferrichrome A	*Ustilago* spp	
		Ferricrocin	*A. nidulans*; *A. fumigatus*	
		Ferrichrysin	*Aspergillys oryzae*	
		Ferrirhodin	*Fusarium sacchari*	

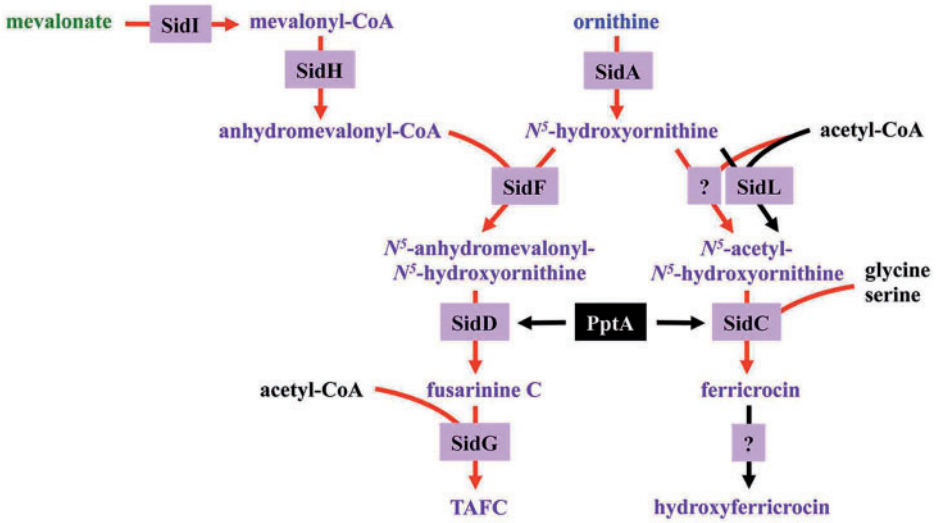

Figure 1: Siderophore biosynthesis pathway as described for *A. fumigatus* (adapted from Haas [2]). Enzymatic steps transcriptionally upregulated in *A. fumigatus* during iron starvation are marked by gray (red) arrows. The enzymes and genes are described in more detail in Table 2. (A colour version of this figure is available online at: http://bfg.oxfordjournals.org)

Aspergillus strains were grown on minimal medium (MM) [18] or on complete medium (CM) consisting of MM with the addition of $10\,g\,l^{-1}$ yeast extract and $5\,g\,l^{-1}$ casamino acids and described in detail in Franken [6]. When required, plates were supplemented with hygromycin ($100\,\mu g\,ml^{-1}$) and caffeine ($500\,\mu g\,ml^{-1}$). Conidiospores were obtained by harvesting conidia from a CM plate after 4–6 days growth at 30°C, using $0.9\,g\,l^{-1}$ (w/v) NaCl solution and stored at 4°C.

Escherichia coli DH5α was used for the amplification of recombinant DNA. The *sreA* deletion plasmid

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was constructed as described by Franken [6]. Standard molecular techniques were applied as described earlier [6]. Transformation of DH5α was performed according to the heat shock protocol as previously described [19]. *Aspergillus niger* transformations were performed according to Meyer *et al*. [20].

Genome mining and transcriptome analysis

Genome mining was performed by using Blastp analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi) of proteins involved in iron homeostasis of *A. fumigatus Af293* (taxid:330879) and *A. nidulans FGSC A4* (taxid:227321) as described earlier [2] against *A. niger CBS 513.88* (taxid:425011) and comparative and cluster analysis using Sybil [21]. Specific expression patterns of the genes identified by genome mining in *A. niger* were established by using publically available transcriptome data sets [22–26] and specified here using the conditions described by Meyer *et al.* [22]. Two kb upstream sequence of siderophore and heme synthesis and regulatory genes were analyzed for the presence of putative transcription factor binding sites for SrbA (ATCRKRYSAT [27]), SreA(ATCWGATAA[27]) and HapX (CCAAT[15]).

Heme (intermediate) and siderophore analyses

Aspergillus niger strains N402 and Δ*sreA* were cultivated as biological duplicates in standard MM at 200 rpm at 30°C for 24 h. Heme concentrations and potential accumulation of porphyrins were determined by high-performance liquid chromatography (HPLC) with UV and fluorescence detection and normalized to the sample protein content according to the method of Bonkovsky *et al.* [28]. Protoporphyrin IX (PPIX; Frontier scientific) and porphyrin acid chromatographic marker kit (Frontier scientific) were used as standards. Analytical duplicates were analyzed. Siderophore analysis was performed on N402 and Δ*sreA* strains grown overnight at 200 rpm at 30°C in 200 ml MM according to Pontecorvo [29], containing 20 mM glutamine as nitrogen source plus 0 μM, 1 μM, 10 μM or 100 μM $FeSO_4$. Intracellular and extracellular siderophores were extracted as described previously [30] and analyzed by reverse-phase HPLC [31] and mass spectrometric analysis [32].

RESULTS AND DISCUSSION

Genome mining for siderophore synthesis in *Aspergillus* species

The *A. niger* genome was analyzed in more detail for genes involved in iron uptake and its regulation as reviewed recently for *A. fumigatus* [2]. BLASTP analysis (Table 2) demonstrated that homologues are present for all the genes described by Haas [2] except for *sidG* (encoding fusarinine C–acetyl coenzyme A–N2-transacetylase [31]) and *estB* (encoding triacetylfusarinine C esterase [33]). BLASTP analysis also showed that many of the iron uptake and regulation genes were clustered in *A. niger*, as was described for *A. fumigatus* and *A. nidulans* [2, 14, 34]. This clustering, however, was clearly different between the various *Aspergillus* species (Table 2 and Figure 2). The majority of the genes involved in siderophore biosynthesis were divided in three clusters in *A. fumigatus* and *A. nidulans* [2, 14, 34]. In *A. niger* only two main clusters were identified. One cluster containing *sidI* and *sidC* appeared to be conserved, although in *A. nidulans* this cluster also contained a *sidF*-like gene (AN0608). The second cluster in *A. niger* is formed by the combination of *sidD*, *sidF* and *sidH* together with a homologue of *mirB*. In *A. fumigatus*, *mirB* is located in a different cluster together with *estB* and *sidG*, which is present about 56 kb apart from the *sidDFH* cluster on the same chromosome. Possibly part of this region between these two clusters was lost in *A. niger*, resulting in absence of, amongst others, *estB* and *sidG*.

Further analysis of the *A. niger* NRPS genes *sidC* and *sidD* present in these clusters showed that *A. niger* SidD shows the highest similarity to homologues from *Aspergillus* species (Supplementary Table S1). It displays 66% overall identity to *A. fumigatus* SidD (the closest functionally analyzed enzyme), which is involved in the biosynthesis of fusarinine C, the precursor of triacetylfusarinine C (TAFC). The *A. niger* SidD, however, is most likely responsible for production of coprogen B (see section 'Siderophore synthesis in *A. niger*' below). Notably, the next closest protein with an overall identity of 65% is *Penicillium chrysogenum* (Supplementary Table S1), which produces the extracellular siderophore coprogen [47]. A similar result was obtained for SidC. *Aspergillus nidulans* and *A. fumigatus* are known to produce ferricrocin. But, despite a high degree of homology, the *A. niger* SidC is likely involved in the synthesis of ferrichrome (see section 'Siderophore synthesis in *A. niger*'), whereas

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the closest analyzed non-*Aspergillus* SidC homologue present in *Fusarium sacchari*, which has an overall identity of 51%, is involved in the biosynthesis of the ferrichrome-type siderophore ferrirhodin [7]. These data suggest that highly similar enzymes can produce structurally different siderophores and that genome data alone are not sufficient to predict the exact siderophore type produced.

Furthermore, the BLASTP analysis indicated an expansion of homologues putatively involved in RIA in *A. niger* compared with *A. fumigatus*. RIA requires the functional ferrioxidase FetC and ferripermease FtrA [1, 3] at the plasma membrane. Three putative homologues of *ftrA* and *fetC*, respectively, are present in *A. niger*, which was earlier observed by Haas *et al*. [3]. The homologue with the highest similarity of *A. fumigatus* FetC, is clustered with one of the two highly similar homologues to *A. fumigatus ftrA*, sharing a common promoter region in both species. The four most homologous ferric reductases are not clustered with these two genes. Amongst the other homologues in *A. niger* also a second gene cluster was identified which also carries a ferric reductase (An15g05500). In *Saccharomyces cerevisiae*, the FetC/FtrA homologues Fet3p/Ftr1p mediate RIA, while their paralogues, Fet5p/Fth1p, mediate vacuolar export of stored iron [48]. Therefore, the paralogues in *A. niger* could also be involved in vacuolar handling of iron. In *A. nidulans*, these genes are completely lacking, corroborating with the lack of RIA in this species [6].

Expression analysis of siderophore-related genes in *A. niger*

To analyze whether the genes involved in siderophore biosynthesis show coregulated expression, we analyzed the expression of the genes identified by genome mining in *A. niger* using publically available transcriptome data sets [22–26]. From this analysis, it became clear that expression of most of the genes described in Table 2 was low under most cultivation conditions, including controlled batch fermentation under a variety of carbon and nitrogen sources. Several of the identified genes showed hardly any expression in any of the conditions tested.

In germinating spores, however, the genes required for the synthesis of the extracellular siderophore as well as RIA showed a significantly increased expression level (Table 2). Interestingly, only the RIA gene cluster with the highest homology to the *A. fumigatus fetC/ftrA* gene cluster was significantly induced during germination. Besides the gene-products known to be involved in synthesis of the extracellular siderophores (SidA, SidF, SidD, SidI and SidH), an esterase-like protein (An03g03530), also present in the *A. nidulans* and *A. fumigatus* clusters, was induced under these conditions (Table 2 and Figure 2), suggesting its role in extracellular siderophore biosynthesis.

The cluster genes *sitT* and *mirB*, suggested to be involved in siderophore transport in related fungal species [14], were also induced during germination. Therefore, these transporters could putatively be involved in coprogen B transport. In all cases, within 2 h after germination expression reached steady state levels. These results suggest that initial iron uptake is achieved through RIA, while simultaneously siderophores are synthesized. At later time-points, sufficient levels of siderophores may be present to maintain iron homeostasis, allowing reduced expression of the required genes. Expression of unrelated genes and genes required for intracellular siderophore synthesis and uptake is more or less constitutive (Table 2 and Figure 2). In line with these data, uptake of extracellular iron was shown to partially compensate for the lack of intracellular siderophore stores during germination in *A. fumigatus* [30]. To further explore the mechanism of coregulation of the various iron pathway genes, 2 kb upstream sequence of the various genes was analyzed for the presence of potentially relevant transcription factor binding sites (Supplementary Table S2 and Figure 2). We observed that almost all genes present in the siderophore gene clusters (and hapX itself) show putative binding sites for SreA and HapX (Supplementary Table S2 and Figure 2), while this is not the case for unclustered siderophore genes and the heme biosynthetic genes. Several of the clustered genes also show SrbA consensus binding sites. These findings further indicate the genes present in the siderophore cluster are under control of these transcription factors.

Siderophore synthesis in *A. niger*

As shown in previous studies in *A. nidulans* and *A. fumigatus*, iron blocks the production of siderophores via SreA-mediated transcriptional repression, i.e. SreA deficiency results in increased siderophore production during iron-replete conditions [14, 30]. Based on these results, a Δ*sreA* strain (An01g02370) was constructed in *A. niger* N402 [6].

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Table 2: BLASTP analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi) of proteins involved in iron homeostasis of *A. fumigatus* Af293 (taxid:330879) and *A. nidulans* FGSC A4 (taxid:227321) against *A. niger* CBS 513.88 (taxid:425011)

Gene name	Function	*A. fumigatus* (E-value/max identity)	*A. nidulans* (E-value/max identity, compared with protein xx)	*A. niger* (E-value/max identity, compared with protein xx)	*A. niger* accession version	Expression level germlings[a]	Expression level exponential phase[a]
Siderophore biosynthesis							
SidA [35]	Ornithine monooxygenase	AFUA_2G07680[b]	AN5823.2 (0.0/78%)	An05g00220 (0.0/81%)	XP_001390596.2	5.5	1
SidC [36]	Ferricrocin/ferrichrome NRPS	AFUA_1G17200[b]	AN0607.2 (0.0/55%)	An06g01300 (0.0/61%)	XP_001390952.1	1.8	1.8
SidD [36]	Fusarinine C/coprogen NRPS	AFUA_3G03420[b]	AN6236.2 (0.0/64%)	An03g03520 (0.0/66%)	XP_001390234.2	2.3	0.2
SidF [14]	Transacylase	AFUA_3G03400[b]	AN6234.2 (0.0/80%)	An03g03540 (0.0/63%)	XP_001390236.1	2.6	0.3
			AN0608.2 (1.00E–146/49%)				
SidG [14]	Transacetylase	AFUA_3G03650[b]	AN8539.2 (3E–76/58%)	N.P.			
SidH [37]	Mevalonyl-CoA hydratase	AFUA_3G03410[b]	AN6235.2 (2.00E–141/85%)	An03g03550 (8.00E–103/56%)	XP_001390237.1	2.5	0.3
SidI [34]	Mevalonyl-CoA ligase	AFUA_1G17190[b]	AN0609.2 (0.0/71%)	An06g01320 (0.0/86%)	XM_001390917.1	1	0.1
SidL [38]	Transacetylase	AFUA_1G04450[b]	AN0455.2 (0.0/63%)	An01g03300 (0.0/67%)	XP_001388762.2	0.7	0.8
NpgA/PptA [10, 39]	Phosphopantetheinyl transferase	AFUA_2G08590[b]	AN6140.2 (2.00E–128/57%)	An12g03950 (4.00E–107/51%)	XP_001395469.1	0.2	0.2
EstB [33]	Triacetylfusarinine C esterase	AFUA_3G03660[b]	AN7801.2 (6.00E–67/44%)	N.P.			
			AN12255 (1.00E–124/61%)				
Lipase/Esterase		AFUA_3G03390	AN6239.2 (1.00E–170/67%)	An03g03530 (5.00E–180/69%)	XM_001390198.2	5.4	0.5
Siderophore iron transporter							
MirA [30]	Enterobactin transporter		AN7800				
MirC [40]	Siderophore transporter	AFUA_2G05730[b] (1.00E–93/34%, AN7800)	AN7485.2 (0.0/78%, MirC)	An02g14190 (0.0/81%, MirC) (2.00E–92/34%, AN7800)	XP_001400550.1	1.4	1.8
MirB [41]	Siderophore transporter	AFUA_3G03640[b]	AN8540 (0.0/55%, MirB) (1E–177/46%, mirD)	An03g03560 (0.0/52%, MirB) (0.0/54%, MirD)	XP_001390238.1	11	4.5
MirD [34]	Siderophore transporter	AFUA_3G03440 (0.0/47%, MirB)	AN6238 (1E–172/45%, MirB) (0.0/73%, MirD)	An07g06240 (1E–165/ 44%,MirB) (0.0/55%, MirD)	XP_001400550.1	1	6.5
			AN8365 (0.0/50%, MirB) (0.0/52%, MirD)				
			AN3160 (0.0/47%, MirB) (0.0/49%, MirD)				
SitT [14]	ABC transporter	AFUA_3G03430[b]	AN6237.2(0.0/82%,SitT) (0.0/55%	An03g03620(0.0/78%, SitT) (0.0/54%, AFUA.3G03670)	XM_001390207.1	4.5	0.5
		AFUA_3G03670 (0.0/55%, SitT)	AN404.2 (0.0/55%) (0.0/66%, AFUA.3G03670)				

(continued)

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Table 2: Continued

Gene name	Function	*A. fumigatus* (E-value/max identity)	*A. nidulans* (E-value/max identity, compared with protein xx)	*A. niger* (E-value/max identity, compared with protein xx)	*A. niger* accession version	Expression level germlings[a]	Expression level exponential phase[a]
Reductive iron assimilation							
FetC [14]	Ferroxidase	AFUA_5G03790[b]	N.P.	An01g08960 (0.0/54%)	XP_001389317.1	4.8	0.3
				An15g05520 (0.0/52%)	XP_001397081.2	0.1	0.1
				An14g05370 (9e−122/43%)	XP_001401160.2	0.2	0.2
FreB [41, 42]	Ferric reductase	AFUA_1G17270[b]	AN6400.2 (0.0/50%)	An11g00220 (0.0/58%)	XP_001394026.2	1.1	0.6
				An16g01150 (7e−142/35%)	XP_001397428.1	0.2	0.2
				An13g02180 (8e−114/31%)	XP_001396356.1	0.2	0.2
				An10g00310 (9e−114/32%)	XP_001397079.2	0.2	0.3
FtrA [14]	Iron permease	AFUA_5G03800[b]	N.P.	An16g01130 (1.00E−164/64%)	XP_001397426.2	0.1	0.1
				An01g089500 (9e−156/65%)	XP_001389316.1	4	1
				An15g05510 (4e−132/58%)	XP_001397080.1	0.1	0.1
Regulatory proteins							
AcuM [41]	Zn2Cys6 TF; Repression of iron uptake including siderophore biosynthesis and RIA	AFUA_2G12330[b]	AN6293 (0.0/68%)	An02g04370 (0.0/72%)	XP_001399574.2	0.4	0.4
MpkA [43]	MAP kinase A; Repression of siderophore biosynthesis	AFUA_4G13720[b]	AN5666 (2.00E−148/85%)	An01g09520 (0.0/95%)	XP_001389372.1	1.1	2.3
PacC [44]	Cys2His2)3 TF; Activation of TAFC biosynthesis in alkaline pH	AFUA_3G11970[b]	AN2855 (0.0/68%)	An02g07890 (0.0/75%)	XP_001399922.1	0.2	0.2
SrbA [45]	bHLH-LZ TF; Activation of iron uptake including SB and RIA	AFUA_2G01260[b]	AN7661.2 (0.0/69%)	An03g05170 (0.0/73%)	XP_001390398.2	3.8	1.6
SreA [14]	GATA TF; Repression of iron uptake including siderophore biosynthesis and RIA	AFUA_5G11260[b]	AN8667 (0.0/64%)	An01g02370 (0.0/69%)	XP_001388675.1	0.3	0.5
HapX [46]	bZip-TF; Repression of iron consumption, activation of iron uptake	AFUA_5G03920[b]	AN8251 (0.0/71%)	An09g06280 (0.0/76%)	XP_001393942.1	0.8	1.5

N.P. = not present.

[a]Expression level in percent relative to actin (An15g00560) expression levels of *A. niger* N402, grown on maltose in exponential growth [23]. Dark (green) indicate > 4-fold induction, light (green) > 2-fold induction in germinating spores.

[b]Corresponding protein used for BLASTP. Homologous *A. fumigatus* proteins have been analyzed as well and results indicate identity toward the specific protein after E-value and max identity between brackets. Proteins for which the genes are clustered in the different genomes are coded in shades of gray. BLASTP of *A. nidulans mirA* results yields *A. fumigatus mirC* as most similar protein. (A colour version of this figure is available online at: http://bfg.oxfordjournals.org)

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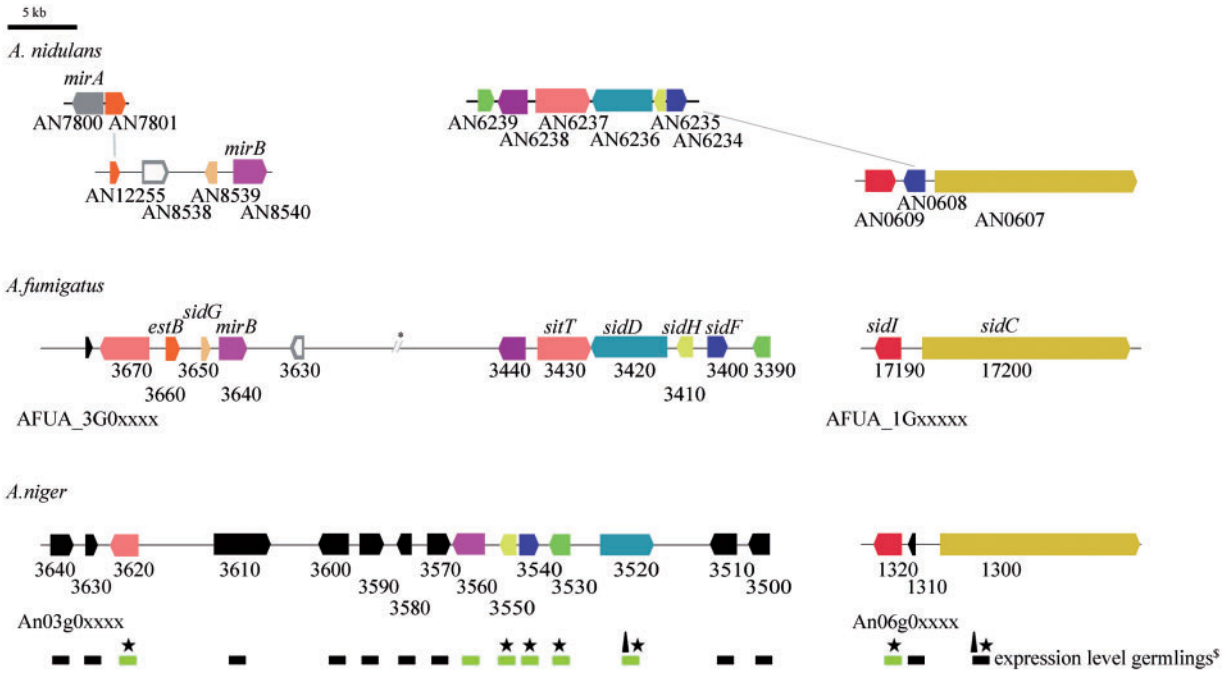

Figure 2: Genomic localization of siderophore synthesis and transporter genes of *A. nidulans FGSC A4*, *A. fumigatus Af293* and *A. niger CBS 513. 88*. Although several genes are localized in clusters in all three organisms, the clustering is not preserved. Homologous genes are indicated in identical color. Black arrows: genes present in the other genomes but not clustered. Gray open arrows: gene only present within this organism. *, region between AFUA_3G3440 and AFUA_3G3630 spans 56 kb containing only unclustered genes unrelated to siderophore biosynthesis; $, fold change expression level in germinating spores versus exponentional phase, Light gray (green) rectangle >2-fold, black rectangle: no significant change. Presence of (one or more) putative transcription factor binding sites in siderophore-related genes are indicated for SreA (★) and SrbA (▲). (A colour version of this figure is available online at: http://bfg.oxfordjournals.org)

Like its *A. fumigatus* counterpart, the *A. niger* Δ*sreA* strain was found to be more sensitive toward phleomycin and higher concentrations of iron (results not shown, [6]). Under high iron conditions, a reduced growth on solid media was observed for the Δ*sreA* strain, and the colonies displayed an orange/reddish coloring likely caused by the accumulation of iron, heme and intracellular siderophores [2, 14, 34]. No significant change in sensitivity was observed toward hydrogen peroxide or voriconazole [6], which would otherwise have been indicative for increased levels of heme-dependent peroxidase and cytochrome P450 activity, respectively.

The *A. niger* N402 and Δ*sreA* strain were examined for the actual production of siderophores by reversed phase HPLC under iron-sufficient and iron-limited conditions (Figure 3). Under iron-sufficient conditions ($[Fe^{2+}] = 30\,\mu M$), *A. niger* N402 produced basically no detectable intracellular siderophores and only small amounts of extracellular siderophores, consistent with the expression data. When iron, however, is scarce ($[Fe^{2+}] \leq 1\,\mu M$), biosynthesis of intra- and extracellular siderophores was induced. Reverse-phase HPLC demonstrated that *A. niger* synthesizes one intracellular siderophore (Figure 3), which was determined by high-resolution mass spectrometry to be ferrichrome ($C_{27}H_{43}N_9O_{12}Fe$, molecular masses of m/z $(M-2H+Fe)^+ = 741.2426$) [6], whereas ferricrocin is known to be the intracellular siderophore produced in *A. fumigatus* and *A. nidulans* [3]. Analysis of the extracellular siderophores by high-resolution mass spectrometric analysis yielded a molecular masses of m/z $(M-2H+Fe)^+ = 780.3022$ for the major iron-saturated siderophore, perfectly matching coprogen B ($C_{33}H_{52}N_6O_{12}Fe$, calculated molecular mass 780.2987) [6]. A second peak was determined to be secreted ferrichrome. At this point, no siderophore could be assigned to the third small peak observed. Deletion of *sreA* in *A. niger* resulted in moderately increased levels of extracellular ferrichrome and coprogen B and significantly increased

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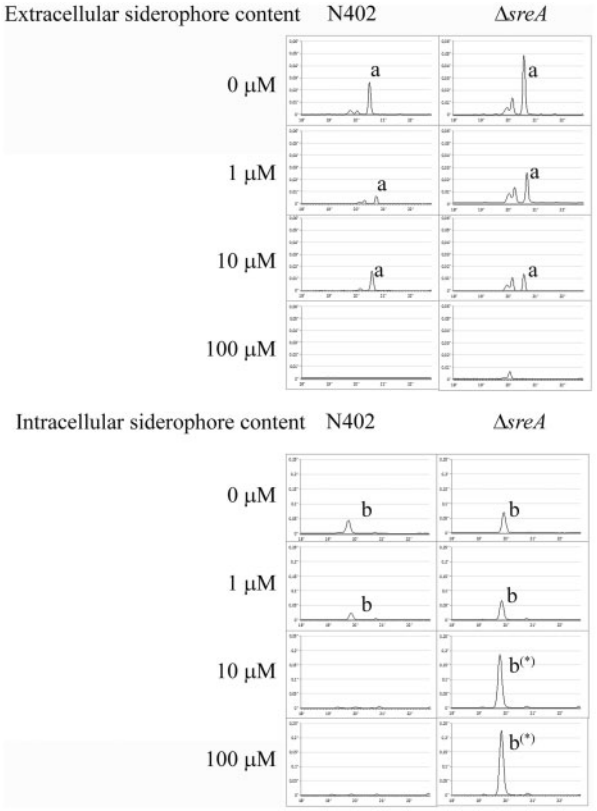

Figure 3: HPLC analysis of siderophore production in *A. niger* N402 and Δ*sreA* strains under iron-deprived and iron-sufficient conditions. Under iron deprivation, *A. niger* produced one intracellular siderophore [ferrichrome (b)] and three types of extracellular siderophores [coprogen B (a); secreted ferrichrome (b) and a third, unidentified siderophore]. Increasing iron concentrations led to decreasing levels of extracellular siderophore content in both N402 and Δ*sreA*. Increasing iron concentrations also decreased intracellular siderophore content in N402, but elevated levels of intracellular ferrichrome in ferri-form (b*) were detected in Δ*sreA* strain synthesis.

intracellular ferrichrome levels (Figure 3). The difference in the measured response of extra- and intracellular siderophore levels can be explained by the fact that during iron-replete conditions, extracellular siderophores chelate iron are then a substrate of enhanced iron uptake through enhanced RIA and siderophore transporters, such as MirB, which are also derepressed in the Δ*sreA* mutant strain. In other words, the full upregulation of extracellular siderophore production is not visible at the metabolite level because of the simultaneous elimination by increased cellular uptake. As a consequence of the increased iron uptake mediated by extracellular siderophores as well as RIA, the mutant shows a dramatic increase of cellular accumulation of iron-saturated ferrichrome.

Table 3: Quantification of heme and porphyrin content in N402

Heme intermediate	N402	sreA
Uroporhyrinogen III (C8)	0 ± 0	4 ± 0
Coproporporphyrinogen III (C4)	44 ± 5	81 ± 15
Σ(C8–C4)	73 ± 8	146 ± 32
PPIX	6 ± 0	9 ± 1
Heme	629 ± 45	703 ± 53

Note: Strains were cultivated as biological duplicates in MM for 24 h followed by protein extraction and heme (intermediate) analysis. Values are given in pmol/mg protein.

Heme biosynthesis in *sreA*

Although siderophore analysis provided an indication that intracellular iron levels were elevated, no significantly increased heme content was observed under the conditions tested in the *A. niger* Δ*sreA*. However, accumulation of the heme intermediates uroporphyrinogen III and coproporphyrinogen III including all decarboxylated intermediates (designated ΣC8–C4) was observed (Table 3). The Δ*sreA* strain was also analyzed for expression levels of several heme biosynthesis genes. Similar to *A. fumigatus* Δ*sreA*, and in line with the observed accumulation of heme intermediates, Northern analysis demonstrated upregulation of the first (*hemA*) and last step (*hemH*) of heme biosynthesis, but in *A. niger* also the expression of *hemF* (coding for coproporphyrinogen III oxidase) was elevated. In contrast, expression of *hemB* (coding for 5′-aminolevulinicacid dehydratase) was unaltered [6]. As observed earlier, these results suggest that other regulatory mechanisms than increased expression of the heme biosynthetic pathway genes regulate heme accumulation [49].

Conclusion

Despite a high degree of homology among different *Aspergilli*, the presented study reveals considerable variations regarding iron homeostasis among different *Aspergillus* species. In contrast to *A. nidulans* and *A. fumigatus* that both produce TAFC as major extracellular siderophore, *A. niger* lacks genes specifically required for TAFC synthesis and utilization and, in agreement secretes different siderophores, produces

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coprogen B and ferrichrome. Ferrichrome is also the major intracellular siderophore of *A. niger*, which again contrasts *A. nidulans* and *A. fumigatus* that both use ferricrocin for intracellular iron handling. Genome mining combined with transcriptional profiling suggested the presence of functional RIA in *A. niger*, similar to *A. fumigatus* but contrasting *A. nidulans*. Interestingly, transcriptional profiling indicated for the first time an increase in RIA- as well as siderophore-mediated iron acquisition during ascospore germination. Similar to *A. fumigatus*, SreA deficiency derepressed RIA- and siderophore-mediated iron acquisition during iron sufficiency, leading to increased cellular ferrisiderophore accumulation, increased iron toxicity and increased susceptibility to the iron-activated drug phleomycin. In contrast to *A. fumigatus*, however, SreA deficiency did not upregulate the cellular content in heme but of heme biosynthesis intermediates, indicating a complex regulatory mechanism operating. The presence of multiple FetC/FtrA paralogues may indicate reuse of vacuolar iron stores in *A. niger*. Further research will be required to complete our understanding of iron homeostasis and its links to heme metabolism in *A. niger*.

SUPPLEMENTARY DATA

Supplementary data are available online at http://bib.oxfordjournals.org/.

Key points

- In contrast to *A. nidulans* and *A. fumigatus*, *A. niger* does not produce triacetylfusarinine and ferricrocin but coprogen and ferrichrome as major siderophores.
- Extracellular siderophore biosynthesis and RIA are highly induced during ascospore germination in *A. niger*.
- Excessive iron uptake caused by SreA deficiency increases the cellular accumulation of heme intermediates but not of heme.
- Siderophore biosynthesis and other iron-related pathways are commonly regulated in *A. niger*.

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