Chapter 6

Derepression of siderophore production increases expression of genes in heme biosynthesis but does not increase intracellular heme levels in Aspergillus niger

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Abstract

To improve heterologous peroxidase production elevated levels of intracellular heme are desired. However, the biosynthesis pathway is highly regulated to ensure sufficient heme levels, but to avoid toxic levels of heme and/or its highly reactive intermediates. To generate elevated heme levels in *Aspergillus niger* we have studied the role of the iron-specific transcription factor SreA in heme biosynthesis and iron uptake. Deletion of *sreA* in *A. niger* resulted in increased sensitivity towards phleomycin and high iron concentrations, colonies appear orange/red in colour under these high iron conditions. Accumulation of heme pathway intermediates uroporphyrinogen III, coproporphyrinogen III and protoporphyrin IX was observed by HPLC analysis and Northern analysis demonstrated upregulation of several heme biosynthesis genes in Δ*sreA*. However, unlike the *Aspergillus fumigatus* counterpart no obvious increase in heme content was observed despite increased siderophore synthesis in *A. niger*.

Genome mining revealed the presence of several genes relevant for synthesis and utilization of siderophores, but those related to the widespread extracellular siderophore triacetylfusarinine C is absent in the *A. niger* genome. Reverse phase HPLC confirmed the absence of triacetylfusarinine C, and demonstrated that *A. niger* produces one dominant intracellular siderophore, ferrichrome and three extracellular siderophores of which the major siderophore is coprogen B. The upregulation of the heme biosynthesis pathway genes, accumulation of heme intermediates, and increased intracellular siderophore levels indicate that iron uptake is indeed enhanced in the *A. niger sreA* mutant. The lack of elevated heme levels therefore suggests limitations or control mechanisms at protein level of coproporphyrinogen III oxidase and/or ferrochelatase, as was also observed in *A. niger* strains overexpressing heme biosynthesis genes.

Introduction

For the heterologous production of heme-containing peroxidases in *Aspergilli*, the co-factor heme is suggested to be a limiting factor. Supplementation of a heme source was found to improve their production (Andersen et al. 1992; Conesa et al. 2000; Elrod et al. 1997), but details on the exact mechanism on how this improvement is achieved are unclear. Moreover, supplementation of a heme source is not preferred for the production of heme-containing peroxidases on an industrial scale.

The ideal approach to yield more available heme for peroxidase production would be to increase intracellular heme availability. Elrod et al. (1997) showed that improvement of

Coprinus cinereus peroxidase production was achieved by overexpression of heme biosynthesis genes in *Aspergillus oryzae* (Elrod, et al., 1997). A four-fold overexpression of both *hemA* and *hemB* resulted in an accumulation of uroporphyrinogen III, although peroxidase levels were still relatively low (Elrod, et al., 1997). Our recent results with strains overexpressing individual heme biosynthetic pathway genes further demonstrate the difficulty of increasing intracellular heme levels (Franken et al. 2013).

An alternative and possibly more efficient approach to achieve elevated levels of intracellular heme could be found by influencing iron uptake. Iron is an essential molecule to almost every form of life, being involved in a multitude of processes like biosynthesis of DNA, electron transport, synthesis of amino acids, sterols and lipids (Haas 2012; Philpott 2006). In many of these processes the iron is incorporated in a heme molecule. As such, iron could be major player in the regulation of heme biosynthesis and its role in fungi has been extensively reviewed earlier (Haas et al. 2008; Philpott 2006).

Although iron is one of the most abundant metals on earth, it is mostly present in its oxidized ferric state (Fe^{3+}) (Philpott 2006), forming particles of ferric hydroxides that have a very low solubility (Haas 2012; Philpott 2006). As a result, the bioavailability of iron is low. Therefore organisms have developed various mechanisms to sequester available iron from its environment but at the same time prevent iron toxicity. Under conditions of iron limitation, fungi can use high-affinity iron uptake systems. These systems consist of a reductive iron assimilation that is initiated by reduction of ferric iron by metalloreductases present at the plasma membrane, and/or non-reductive iron assimilation that occurs through the action of siderophores (iron-chelators). When iron is readily available, the high-affinity uptake systems are not expressed and iron is sequestered through low affinity transporters with low specificity (Haas 2012; Philpott 2006).

Iron homeostasis in both *Aspergillus fumigatus* and *Aspergillus nidulans* was found to be predominantly under control of the transcription factors SreA and HapX. 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, the *hapX* is de-repressed and subsequently represses *sreA* expression thereby inhibiting iron dependent pathways, including the heme biosynthesis pathway. SreA repression derepresses siderophore synthesis and thus facilitates increased iron uptake. Besides transcriptionally controlled, both SreA and HapX are also posttranslationally regulated by iron, to ensure a rapid adaptation to changing iron concentrations so that iron homeostasis can be maintained (Haas 2012; Hortschansky et al. 2007; Schrettl et al. 2008). Deletion of *sreA* in *A. fumigatus* results 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). As a result, the Δ*sreA*-strain contained increased heme content and accumulated significant amounts of heme intermediates (Schrettl et al. 2008). Based on these results, a Δ*sreA* strain would therefore be an attractive alternative to increase heme biosynthesis in *A. niger* as well. In this study, we have constructed and analysed the *sreA* deletion strain as a potential target to increase heme biosynthesis in *A. niger*.

Materials and methods

Strains and culture conditions

Aspergillus niger N402 (*cspA1* derivative of ATCC9029 (Bos et al. 1988)) and the *pyrG*$^{-}$ derivative of this strain, AB4.1 (van Hartingsveldt et al. 1987), were used during this study. *Aspergillus* strains were grown on minimal medium (MM) (Bennet and Lasure 1991) or on complete medium (CM) consisting of minimal medium with the addition of 10 g l^{-1} yeast extract and 5 g l^{-1} casamino acids. When required, plates were supplemented with hygromycin (100 μg ml^{-1}) and caffeine (500 μg 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. Transformation of DH5α was performed according to the heat shock protocol as previously described (Inoue et al. 1990).

Potential deletion strains were pre-screened after 48 h of growth on MM containing 10 μg/ml phleomycin. After verification, the deletion strain was analysed into more detail for sensitivity towards phleomycin, hydrogen peroxide, and iron ($FeSO_4$). Voriconazole sensitivity was analysed by E-test strips (containing a gradient in voriconazole, AB Biodisk) according to the instructions of the manufacturer and analysed after 24 h and 45 h of growth.

Molecular biological techniques

Chromosomal DNA of *A. niger* was isolated as described by Kolar *et al.* (1988). Southern analysis was performed according to Sambrook and Russel (2001). α-^{32}P-dCTP-labelled probes were synthesized using Rediprime II DNA Labelling System (Amersham Pharmacia Biotech) according to the instructions of the manufacturer. Restriction enzymes were obtained from Invitrogen, New England Biolabs and Fermentas and used according to instructions of the manufacturer. Ligation of DNA fragments was performed using the

Rapid DNA ligation Kit (Fermentas). When required, fragments were dephosphorylated using Shrimp Alkaline Phosphatase (Fermentas). Sequencing was performed by Service XS (Leiden, The Netherlands).

Construction of sreA deletion plasmid

For the construction of the *sreA* deletion plasmid, N402 genomic DNA was used as a template. The 5' flanking region was amplified as a 2.0 kb fragment introducing *Not*I and *Xba*I restriction sites at the 5' and 3' end respectively using the primers pSreA 1Fw (5'-ATAAGAATGCGGCCGCGCTTCCACAACATCCCAAATAG-3') and pSreA 2rev (5'-tgctctagaCCAGTGACAAAGTCCCCAGAT-3'). The 3'flanking region was amplified as a 1.1 kb fragment introducing *Xho*I and *Kpn*I restriction sites at the 5' and 3' end respectively using the primers pSreA 3Fw (5'-ccgctcgagGTCCTAGAACTGTTTATGCTGC-3') and pSreA 4rev (5'-ggggtaccTTAACCGACGCAATGCATAGCT-3'). Fragments were cloned into pBluescript SKII (Stratagene) using the introduced restriction sites. Clones were verified by restriction analysis followed by sequencing of correct clones. Cloning of the deletion construct was continued by the insertion of the Hygromycin B selection marker from pAN7.1 (Punt et al. 1987) into the pBluescript SKII-3' flanking region as *Xba*I–*Xho*I, followed by the insertion of the 5' flanking region as *Not*I-*Xba*I to yield the plasmid p∆*sreA*. The plasmid was linearized prior to transformation using *Eco*RV.

Transformation of A. niger

Transformations were performed according to Meyer *et al* (2010) with the following modifications. 100 ml cultures inoculated with $1 \cdot 10^8$ conidia were grown at 30 °C at 250 rpm for 12-16 h. 200 mg lysing enzymes (Sigma-Aldrich) were dissolved in 10 ml SMC and set to pH 5.6. Protoplast formation was performed at 37 °C and 120 rpm and was verified every 30 min by microscopy. 100 µl of protoplasts were mixed with 10 µl of DNA solution (5 to 10 µg), and 25 µl of freshly made PEG buffer. Top-agarose was added to a volume of 40 ml, mixed and poured onto two 15 cm selective transformation plates.

Northern analyses

Northern analysis was performed on the same samples as described for the heme and porphyrin quantification (see below). Equal amounts of RNA were loaded and expression levels of *hemA, hemB, hemF, hemH, met1 (first gene in siroheme synthesis)* were examined and actin was used to correct for loading differences.

Siderophore and heme analysis

N402 and Δ*sreA* strains were grown overnight at 200 rpm at 30 °C in 200 ml MM according to Pontecorvo et al. (1953), containing 20 mM glutamine as nitrogen source, 0 μM, 1 μM, 10 μM or 100 μM $FeSO_4$. Intracellular and extracellular siderophores were extracted as described previously (Oberegger, 2001). Extracellular siderophores were iron-saturated by incubating 1 ml culture supernatant with 100 μl of 72 mM $FeSO_4$. The mixture was subsequently mixed with 200 μl Phenol in a new tube, followed by vortexing and centrifuging at max speed. Siderophores were subsequently extracted from the water phase using 300 μl diethylether (Fluka).

Intracellular siderophores were extracted by grinding 50 mg freeze dried mycelium. The mycelium was suspended in 1.8 ml sodium phosphate buffer (pH 7.5) followed by 30 min incubation on ice. After centrifugation at max speed, 800 μl extract was saturated with 100 μl of 72 mM $FeSO_4$ followed by the extraction procedure described above for the extracellular siderophores. Extracts were analysed by reverse-phase HPLC (Schrettl et al. 2007) and mass spectrometric analysis (Gsaller et al. 2013) as previously described.

Heme concentrations and potential accumulation of porphyrins were determined by HPLC with UV and fluorescence detection and normalized to the sample protein content according to the method of Bonkovsky *et al* (1986). PPIX (Frontier scientific) and porphyrin acid chromatographic marker kit (Frontier scientific) were used as standards.

Results and discussion

Iron has an important role in heme biosynthesis. Increased heme levels were obtained by interfering with control of iron uptake by deleting the transcriptional repressor SreA in *A. fumigatus* and *A. nidulans* (Oberegger et al. 2001; Schrettl et al. 2008). Based on the results obtained in these studies, we constructed a Δ*sreA* (An01g02370) strain in *A. niger* N402. Transformants were pre-screened for increased sensitivity towards phleomycin as was described for the *A. fumigatus* Δ*sreA* strain, using MM and MM containing 10 μg ml^{-1} Phleomycin. $Phleo^S$ transformants were analyzed by Southern analysis and correct transformants were obtained (data not shown). One of the correct deletion strains, BR27#29, was selected for further studies and designated Δ*sreA*.

Like its *A. fumigatus* counterpart, the *A. niger* Δ*sreA* strain was found to be more sensitive towards phleomycin and higher concentrations of iron (Figure 6.1). Under high iron conditions, a reduced growth on solid media was observed for Δ*sreA* and the colonies

display an orange/reddish colouring likely caused by the accumulation of iron, heme and intracellular siderophores (Haas 2012; Schrettl et al. 2010; Schrettl et al. 2008). No significant change in sensitivity was observed towards hydrogen peroxide (Figure 6.1) or voriconazole (data not shown), which would otherwise have been indicative for increased levels of heme dependent peroxidase and cytochrome P450 activity respectively.

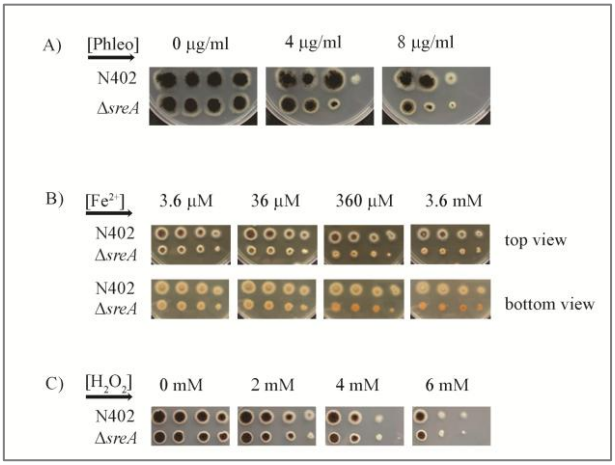

Figure 6.1: phenotypic analysis of Δ*sreA*. A) Deletion of *sreA* in *A. niger* results in increased sensitivity towards phleomycin. B) High iron conditions result in decreased growth of Δ*sreA* and development of orange/reddish colour, clearly observed from a bottom view (second row) C) No altered sensitivity was observed for Δ*sreA towards* H_2O_2.

The Δ*sreA* strain was subsequently analyzed for expression levels of several heme biosynthesis genes and for heme- and porphyrin-content. Similar as the *A. fumigatus* Δ*sreA*, 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) is elevated (Figure 6.2). Following Northern analysis, heme and porphyrin content were determined for the *A. niger* Δ*sreA*. In line with the observed upregulation of *hemA* and *hemF,* accumulation of ΣC8-C4 and PPIX was observed. However, no significantly increased heme content was observed under the conditions tested (Figure 6.3) in the *A. niger* Δ*sreA*. The observed upregulation on *hemF* and *hemH* expression does not appear to be sufficient to increase heme levels, indicating (additional) limitations at protein level as was observed earlier (Franken et al. 2013). Also potential regulation on aminolevulinic acid synthase (*hemA*) via putative heme regulatory motifs (Elrod et al. 2000; Franken et al. 2011) could contribute to the absence of increased heme levels.

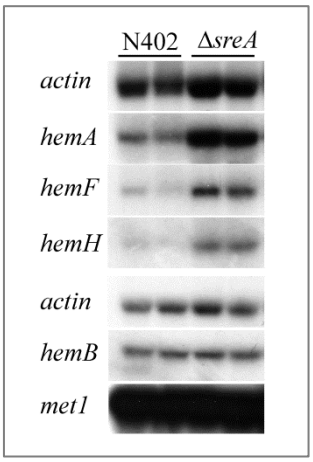

Figure 6.2: Northern analysis of N402 and Δ*sreA* results in upregulation of predominantly *hemF* and *hemH.* A minor increase is also observed for *hemA.* Strains were analysed after 24h of growth on MM containing 0.01% casamino acids at 30 °C. Equal amounts of RNA (10 µg) were loaded and the expression levels of *hemA, hemB, hemF, hemH* and *met1* were analysed. Expression levels of *actin* were used to correct for loading differences.

As the observed results appear to deviate from the results of the *A. fumigatus* and *A. nidulans sreA* deletion strains, the *A. niger* genome was analysed in more detail for genes involved in iron uptake and their regulation as reviewed recently for *A. fumigatus* (Haas 2012). BLASTP analysis (Table 6.1) demonstrated that homologues are present for all the genes described by Haas (2012) except for *sidG* encoding fusarinine C–acetyl coenzyme A–N2-transacetylase (Schrettl et al. 2007) and *estB* (encoding triacetylfusarinine C esterase (Kragl et al. 2007)). The genome mining shows that, as was described for *A. fumigatus* and *A. nidulans* (Haas 2012; Schrettl et al. 2010; Schrettl et al. 2008), also in *A. niger* several iron uptake and regulation genes are clustered, although this clustering is somewhat different between the various *Aspergillus* species (Table 6.1; Figure 6.4). The majority of the genes involved in siderophore biosynthesis are divided in three clusters in *A. fumigatus* and *A. nidulans* (Haas 2012; Schrettl et al. 2010; Schrettl et al. 2008). In *A. niger,* however, only two main clusters are identified. The cluster containing *sidI* and *sidC* appears to be conserved, although in *A. nidulans* this cluster also contains a *sidF* like gene (AN0608). The second cluster in *A. niger* forms a 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.*

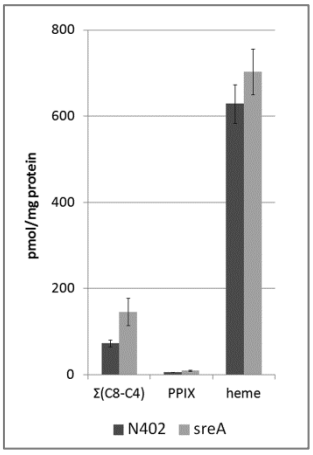

Figure 6.3: Quantification of heme and porphyrin content in N402 and Δ*sreA* demonstrates the Δ*sreA* strain does not accumulate heme, but rather contains elevated levels of porphyrins. Strains were analysed after 24h of growth on MM containing 0.01% casamino acids at 30 °C.

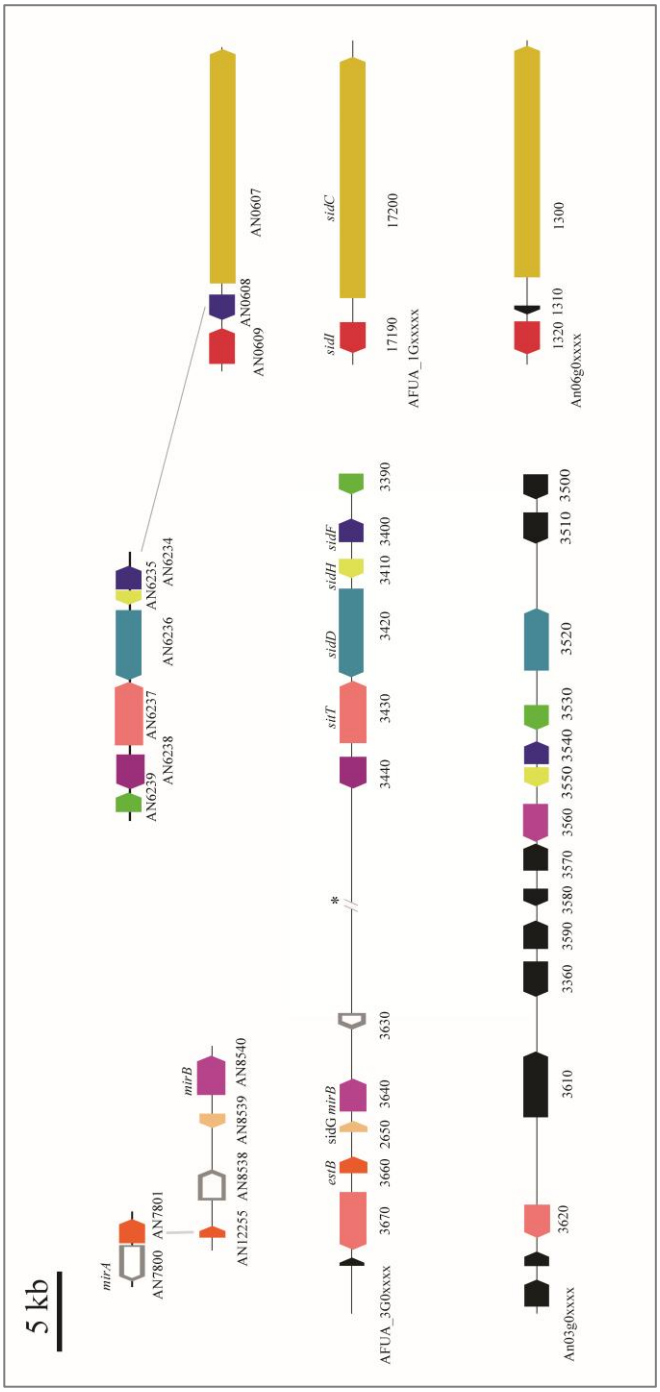

Figure 6.4: localization of siderophore synthesis and transporter genes of *A. niger CBS 513. 88, A. nidulans FGSC A4* and *A. fumigatus Af293*. Although several genes are localized in clusters in all three organisms, the clustering is not preserved. Homologous genes are indicated in identical colour. Black arrows: genes present in the other genomes but not clustered. Grey open arrows: gene only present within this organism. *, region between AFUA_3G3440 and AFUA_3G3630 spans 56 kb.

Table 6.1: 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)*. N.P. = not present, * corresponding protein used for BlastP. Homologous *A. fumigatus* proteins have been analysed as well and results indicate identity towards 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 grey. BlastP of *A. nidulans mirA* results yields *A. fumigatus mirC* as most similar protein.

gene name	*A. fumigatus (E-value/max identity)*	*A. nidulans (E-value/max identity, compared to protein xx)*	*A. niger (E-value/max identity, compared to protein xx)*	*Accession version*
		Siderophore biosynthesis		
SidA (Schrettl et al. 2004)	*AFUA_2G07680**	*AN5823.2 (0.0/78%)*	*An05g00220 (0.0/81%)*	*XP_001390596.2*
SidC (Reiber et al. 2005)	*AFUA_1G17200**	*AN0607.2 (0.0/55%)*	*An06g01300 (0.0/61%)*	*XP_001390952.1*
SidD (Reiber et al. 2005)	*AFUA_3G03420**	*AN6236.2 (0.0/64%)*	*An03g03520 (0.0/66%)*	*XP_001390234.2*
SidF (Schrettl et al. 2008)	*AFUA_3G03400**	*AN6234.2 (0.0/80%)*	*An03g03540 (0.0/63%)*	*XP_001390236.1*
		AN0608.2 (1.00E-146/49%)		
SidG (Schrettl et al. 2008)	*AFUA_3G03650**	*AN8539.2 (3E-76/58%)*	*N.P.*	
SidH (Yasmin et al. 2012)	*AFUA_3G03410**	*AN6235.2 (2.00E-141/85%)*	*An03g03550 (8.00E-103/56%)*	*XP_001390237.1*
SidI (Schrettl et al. 2010)	*AFUA_1G17190**	*AN0609.2 (0.0/71%)*	*An06g01320 (0.0/86%)*	*XM_001390917.1*
SidL (Blatzer et al. 2011b)	*AFUA_1G04450**	*AN0455.2 (0.0/63%)*	*An01g03300 (0.0/67%)*	*XP_001388762.2*
PptA (Allen et al. 2011; Jorgensen et al. 2011)	*AFUA_2G08590**	*AN6140.2 (2.00E-128/57%)*	*An12g03950* [1] *(4.00E-107/51%)*	*XP_001395469.1*
EstB (Kragl et al. 2007)	*AFUA_3G03660 **	*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*

gene name	A. fumigatus (E-value/max identity)	A. nidulans (E-value/max identity, compared to protein xx)	A. niger (E-value/max identity, compared to protein xx)	Accession version
Siderophore Iron Transporter (SIT)				
MirA (Oberegger et al. 2001)		AN7800		
MirC (Power et al. 2006)	AFUA_2G05730 * (1.00E-93/34%, AN7800)	AN7485.2 (0.0/78%, MirC)	An02g14190 (0.0/81%, MirC) (2.00E-92/34%, AN7800)	XP_001400550.1
MirB (Liu et al. 2010)	AFUA_3G03640 *	AN8540 (0.0/55%, MirB) (1E-177/46%, mirD)	An03g03560 (0.0/52%, MirB) (0.0/54%, MirD)	XP_001390238.1
MirD (Schrettl et al. 2010)	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
		AN8365 (0.0/50%, MirB) (0.0/52%, MirD) AN3160 (0.0/47%, MirB) (0.0/49%, MirD)		
SitT (Schrettl et al. 2008)	AFUA_3G03430*	AN6237.2(0.0/82%, SitT) (0.0/55%	An03g03620(0.0/78%, SitT) (0.0/54%, AFUA_3G03670)	XM_001390207.1
	AFUA_3G03670 (0.0/55%, SitT)	AN404.2 (0.0/55%) (0.0/66%, AFUA_3G03670)		

gene name	*A. fumigatus (E-value/max identity)*	*A. nidulans (E-value/max identity, compared to protein xx)*	*A. niger (E-value/max identity, compared to protein xx)*	*Accession version*
		Reductive iron assimilation		
FetC (Schrettl et al. 2008)	*AFUA_5G03790**	*N.P.*	*An01g08960 (0.0/54%)*	*XP_001389317.1*
FreB (Blatzer et al. 2011a; Liu et al. 2010)	*AFUA_1G17270**	*AN6400.2 (0.0/50%)*	*An11g00220 (0.0/58%)*	*XP_001394026.2*
FtrA (Schrettl et al. 2008)	*AFUA_5G03800**	*N.P.*	*An16g01130 (2.00E-164/64%)*	*XP_001397426.2*
		Regulatory proteins		
AcuM (Liu et al. 2010)	*AFUA_2G12330**	*AN6293 (0.0/68%)*	*An02g04370 (0.0/72%)*	*XP_001399574.2*
MpkA (Valiante et al. 2008)	*AFUA_4G13720**	*AN5666 (2.00E-148/85%)*	*An01g09520 (0.0/95%)*	*XP_001389372.1*
PacC (Amich et al. 2010)	*AFUA_3G11970**	*AN2855 (0.0/68%)*	*An02g07890 (0.0/75%)*	*XP_001399922.1*
SrbA (Willger et al. 2008)	*AFUA_2G01260**	*AN7661.2 (0.0/69%)*	*An03g05170 (0.0/73%)*	*XP_001390398.2*
SreA (Schrettl et al. 2008)	*AFUA_5G11260**	*AN8667 (0.0/64%)*	*An01g02370 (0.0/69%)*	*XP_001388675.1*
HapX (Tanaka et al. 2002)	*AFUA_5G03920**	*AN8251 (0.0/71%)*	*An09g06280 (0.0/76%)*	*XP_001393942.1*

The Δ*sreA* strain was also examined for the actual production of siderophores by reversed phase HPLC under iron sufficient and iron limited conditions (Figure 6.5). Under iron sufficient conditions ($[Fe^{2+}] \geq 10\mu M$), *A. niger* N402 produces basically no detectable intracellular siderophores and only small amounts of extracellular siderophores. When iron however is scarce ($[Fe^{2+}] \leq 1\mu M$), biosynthesis of intra- and extracellular siderophores is increased. The results confirmed the absence of the siderophore triacetylfusarinine C (TAFC) in *A. niger*, which is the siderophore present in *A. fumigatus* and *A. nidulans*. *A. niger* was found to produce mainly three extracellular siderophores (Figure 6.5.) High-resolution mass spectrometric analysis of the major iron-saturated siderophore gave a molecular masses of m/z $(M-2H+Fe)^+$ = 780.3022 perfectly matching coprogen B ($C_{33}H_{52}N_6O_{12}Fe$, calculated molecular mass 780.2987). Levels of extracellular siderophores decrease with increasing iron concentrations in both N402 and Δ*sreA*, but levels are always higher in Δ*sreA.*

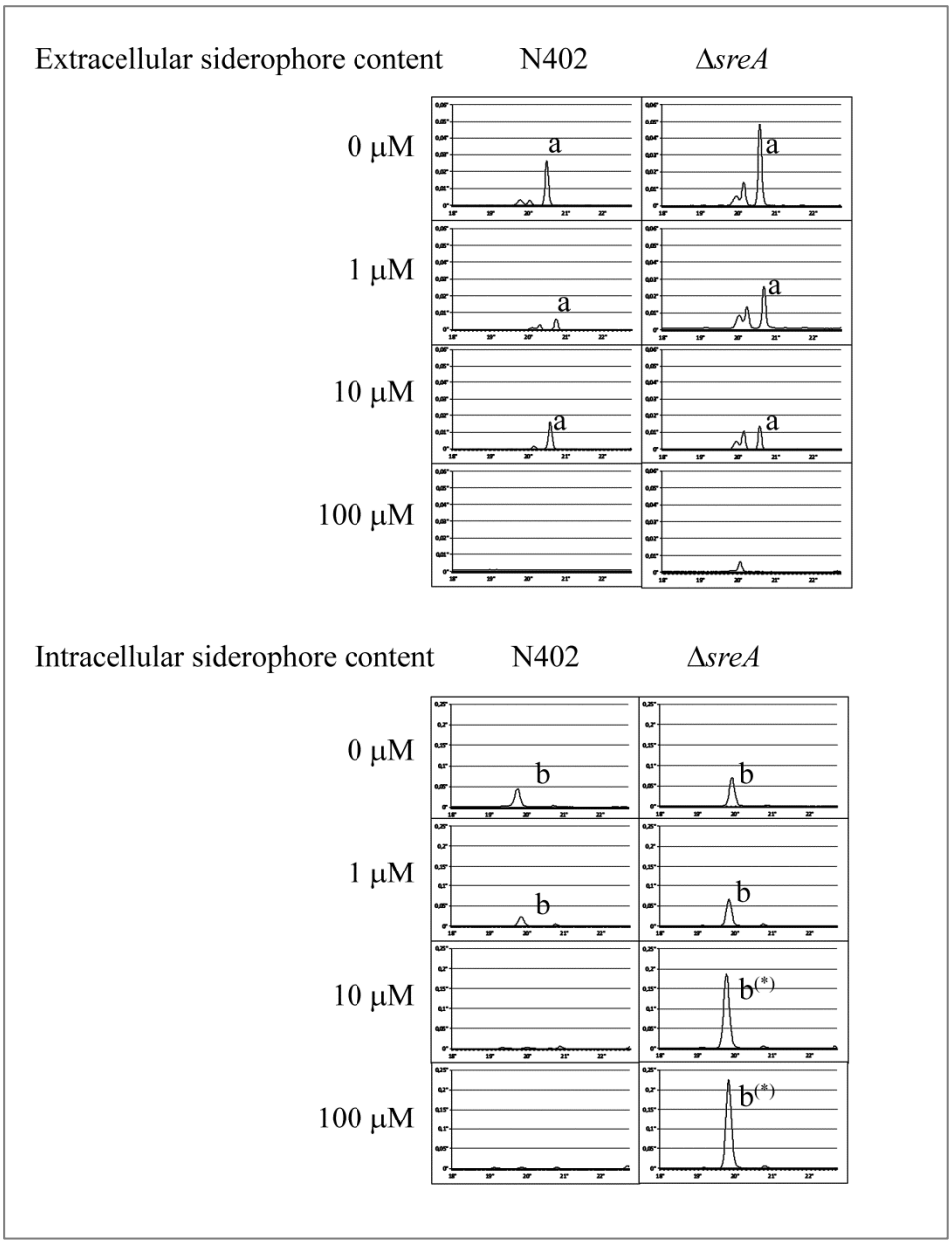

Figure 6.5: Siderophore production in *A. niger* N402 and Δ*sreA*. Under iron deprived conditions, *A. niger* produces three types of siderophores and the levels are increased in Δ*sreA* of which the major siderophore was identified as coprogen B (a). Upon increased iron concentrations, the concentration of extracellular siderophore decreases in both N402 and Δ*sreA.* The intracellular siderophore, identifies as ferrichrome (b), is also elevated in Δ*sreA* at low iron concentrations, but increases with higher iron concentrations. (*) ferrichrome is present in ferri-form instead of the otherwise desferri-form.

Besides extracellular siderophores, *A. niger* produces basically one intracellular siderophore, (Figure 6.5). High-resolution mass spectrometry of the iron-saturated intracellular siderophore gave a molecular masses of m/z $(M\text{-}2H+Fe)^+$ = 741.2426 matching ferrichrome ($C_{27}H_{43}N_9O_{12}Fe$, calculated molecular mass 741.2375). Under iron limiting conditions, the level of intracellular siderophores is moderately increased compared to N402 and present in the desferri-form (iron-free siderophore). With increasing iron concentrations its concentration decreases in N402, but increases in Δ*sreA* where it then is present in the ferri-form (siderophore containing iron).

The upregulation of heme biosynthesis genes in combination with elevated levels of ferrichrome in ferri-form indicates that also in *A. niger,* intracellular iron levels are in fact increased. SreA deletion not only derepresses siderophore synthesis, but also siderophore uptake. Due to this potentially increased siderophore uptake and increase in ferrichrome, increased extracellular siderophore synthesis might actually be masked. Like in *A. fumigatus,* the genes required for the high affinity reductive iron assimilation (RIA) are conserved in *A. niger,* although absent in *A. nidulans* (Haas 2003; Philpott 2006). As SreA also controls iron uptake through RIA (Haas 2003), this route may also be induced to some degree in the *A. niger* Δ*sreA*. Intracellular siderophores could serve as Fe^{3+} storage molecules to reduce the potential toxic effects of accumulating iron.

Taken together, deletion of *sreA* alone is not sufficient to achieve elevated heme levels in a wild-type *A. niger* strain. Despite increased iron uptake, upregulation of the heme biosynthetic pathway and accumulation of intermediates, regulation of the pathway prohibits increased heme concentrations probably by acting on the proteins ferrochelatase and/or coproporphyrinogen III oxidase. Further research on regulation and activity of these proteins may contribute to our understanding of heme biosynthesis and subsequent approaches for improving intracellular heme levels.

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