The expression of selected non-ribosomal peptide synthetases in *Aspergillus fumigatus* is controlled by the availability of free iron

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Received 7 April 2005; received in revised form 12 May 2005; accepted 16 May 2005

First published online 31 May 2005

Edited by G.M. Gadd

Abstract

Three non-ribosomal peptide synthetase genes, termed *sidD*, *sidC* and *sidE*, have been identified in *Aspergillus fumigatus*. Gene expression analysis by RT-PCR confirms that expression of both *sidD* and *C* was reduced by up to 90% under iron-replete conditions indicative of a likely role in siderophore biosynthesis. *SidE* expression was less sensitive to iron levels. In addition, two proteins purified from mycelia grown under iron-limiting conditions corresponded to SidD (240 kDa) and SidC (496 kDa) as determined by MALDI ToF peptide mass fingerprinting and MALDI LIFT-ToF/ToF. Siderophore synthetases are unique in bacteria and fungi and represent an attractive target for antimicrobial chemotherapy.

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Keywords: NRPS; Iron; Siderophile; MALDI ToF; Secondary metabolites; Proteomics; 4′-PPTase

1. Introduction

The fungus *Aspergillus fumigatus* is receiving increasing attention as a significant cause of mortality in immunocompromised individuals such as bone marrow and stem cell transplant patients [1]. The near-completion of the *A. fumigatus* genome sequencing effort and resultant data [2] has greatly facilitated the search for genes which may be involved in mediating organismal pathogenicity and a number of reports have shown that either specific gene deletion or silencing may downregulate the virulence of *A. fumigatus* [3–5]. Hissen et al. [6] have convincingly argued that siderophores produced by the fungus are involved in iron acquisition from transferrin and that, at least in vitro, they may be responsible for organism growth and survival in human serum.

Most members of the family Ascomycota (e.g., *A. fumigatus*) produce hydroxamate-type siderophores. Hydroxamates can be sub-divided into four groups: rhodotorulic acid, fusarines, coprogens and ferrichromes. *A. fumigatus* produces ferrichrome class siderophores (e.g., ferricrocin), which are cyclic peptides [7]. In addition, a number of siderophores (primarily triacetylfusarinine C and ferricrocin) were purified from *A. fumigatus* culture medium after 8 h of growth in medium containing human serum [6].

In *Aspergillus nidulans*, siderophore biosynthesis commences with the N³-hydroxylation of ornithine, followed by transacylation and subsequent covalent assembly of the modified ornithine residues with or without further amino acids (e.g., serine, alanine and glycine),...
catalyzed by non-ribosomal peptide synthetases (NRPS) [8,9]. NRPS are multifunctional enzymes, which operate via a thiotemplate mechanism [10] and consist of semi-autonomous units or modules (Fig. 1), co-linearly arranged to the structure of the peptide product. A typical module minimally consists of an adenylation domain (A-domain), responsible for the activation of its cognate substrate amino acid as an amino acyl adenylate, a downstream located peptidyl carrier protein (PCP or P) or thiolation domain and an upstream positioned condensation domain (C-domain) [11]. Siderophores are predominantly released by cyclization of the peptide product, often catalyzed by cyclotrimerizing TE-domains (e.g., enterobactin, yersiniabactin) or variants of the condensation domains, termed as cyclization domains [12,13]. Furthermore, the iterative (repetitive) use of the enzyme template, as well as a non-linear structure (e.g., –P–C–P–C–), seems to be characteristic of siderophore synthetases. SidC, an NRP synthetase involved in ferricrocin biosynthesis in A. nidulans, encodes a 525.5 kDa protein comprising three adenylation, five condensation and five thiolation domains [9,14]. In spite of only three complete modules, the entire hexapeptides of the cyclic hydroxamate-type siderophores, such as triacetylfularinine C and ferricrocin are formed by repeated use of modules. Furthermore, sidA encodes an L-ornithine-N5-monoxygenase which catalyzes the first step in the siderophore biosynthesis pathway in A. nidulans [9]. Deletion of the sidA gene completely inhibited siderophore biosynthesis in A. nidulans and resulted in severely diminished fungal viability. The sidA ortholog in A. fumigatus has been recently reported and deletion mutants show a distinct reduction in virulence [3]. Ustilago maydis, an infectious agent of maize, scavenges iron using extracellular hydroxamate siderophores, where siderophore production is initiated by the sidl gene. However, analysis of siderophore mutants in U. maydis suggests that the siderophore biosynthetic pathway is not involved in the infection of maize [15]. More recently, Yuan et al. [16] have identified an NRP synthetase gene (sid2) involved in ferrichrome biosynthesis in U. maydis. Expression of sid2 was upregulated (×2.5) in low-iron compared to high-iron media.

We have detected a number of putative NRPS open reading frames in the genome of A. fumigatus and recently demonstrated in vitro NRPS (termed Pes1/SidB) activation by a functional 4'-phosphopantetheinyl transferase [17]. Evidence is now presented that at least three distinct NRPS genes are susceptible to regulation of expression by the level of free iron present in the culture medium.

2. Materials and methods

2.1. Microorganisms and culture media

Aspergillus fumigatus ATCC 26933 (obtained from the American Type Culture Collection, Manasas, VA, USA) was used in this study and maintained as spore suspensions containing 50% (v/v) glycerol at −80 °C. The fungus was grown in a mineral salt medium (pH 6.8) composed of 25 g/l glucose, 3.5 g/l (NH₄)₂SO₄, 2.0 g/l KH₂PO₄, 0.5 g/l MgSO₄ and 8 mg/l ZnSO₄ and supplemented with different concentrations of Fe(III)Cl₃, as required. A. fumigatus cultures were set up in 500 ml shaking flasks at 230 rpm, previously treated to ensure that all traces of iron were removed from the glassware [6]. Medium (250 ml) was inoculated with A. fumigatus conidia at a final concentration of 10⁷ per ml and flasks were incubated at 37 °C and 230 rpm.

2.2. Bioinformatic analyzes

Preliminary A. fumigatus sequence data were obtained from The Institute for Genomic Research website at http://www.tigr.org. The unannotated A. fumigatus genome was interrogated, via a BLAST program, for non-ribosomal peptide synthetase encoding open reading frames (ORFs) using the Acremonium chrysogenum
alpha-aminoadipyl-cysteinyl-d-valine-synthetase (ACVS) encoding gene (GenBank Accession No. E05192) and Aspergillus nidulans peptide synthetase (sidC) gene (GenBank Accession No. AY223812). Putative NRPS-encoding ORFs, from the A. fumigatus genome, were aligned to other peptide synthetases using Clustal W (http://www.ebi.ac.uk/clustalw/). Bootstrap neighbor joining trees, using the resulting alignment, were drawn using the tree finder program (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

2.3. Detection and quantification of siderophores

Siderophore production in A. fumigatus was investigated using the Chrome Azurol S (CAS) assay [18,19].

2.4. DNA/RNA isolation and RT-PCR

Filtered and washed A. fumigatus hyphae, collected at appropriate culture time-points, were crushed using a mortar and pestle under liquid nitrogen. All DNA and RNA manipulations were carried out as recently described [20]. To facilitate equal amounts of RNA for cDNA synthesis and RT-PCR, densiometric measurements of rRNA on RNA agarose gels were performed. PCR of genomic or cDNA was performed using AccuTaq polymerase (Sigma–Aldrich), 1–10 ng genomic DNA and 0.5 μM each of forward and reverse primer in a total volume of 20 μl. PCR conditions were as follows: 96 °C denaturation for 3 min; (93 °C denaturation for 30 s, 65 °C annealing for 90 s, 68 °C extension for 90 s) x 45 cycles; 68 °C extension for 7 min. PCR was performed using primers sidD-forward (5'-ACGCAACCGACTGGGTGT-3'), sidD-reverse (5'-ATTCCGTGCAGGACTCGGAT-3'); sidC-forward (5'-CCGATATTGACACATCTCTCTCC-3'), sidC-reverse (5'-CCGAATCTCTCCGGTCTC-3'), sidE-forward (5'-GAGCCAGCTGACGAGATGAT-3'), sidE-reverse (5'-GGAGCGCCTTGTATACACAC-CT-3'). The gene encoding calmodulin, which is constitutively expressed in A. fumigatus, served as a control in RT-PCR experiments and was amplified across an intron–exon boundary [21]. Optimal cDNA amplification was found to require 45 cycles of PCR. PCR-amplified DNA was electrophoresed, visualized and subjected to semi-quantitative expression analysis as recently described [20].

2.5. Protein purification

All protein purification steps were performed at 4 °C. Mycelia were harvested by filtration and washed twice in phosphate-buffered saline (PBS, pH 7.4). Washed mycelia were resuspended in Buffer A (100 mM Tris, 50 mM NaCl, 20 mM EDTA, 30 mM DTT, 44.71 mg/l PMSF, 2 mg/l DNaseI, 300 mg/l lysozyme, 21 mg/l leupeptin, 10 mg/l TLCK, 10 mg/l pepstatin A and 10% (v/v) glycerol, pH 7.3 [22]) and passed through a French press (700–1000 bar). Cell debris was removed by centrifugation at 40,000 g for 30 min. Supernatant was concentrated by Q-Sepharose™ anion-exchange chromatography (load/wash: Buffer A without NaCl; elution: Buffer A + 1 M NaCl; bed volume: 20 ml; protein load: 80 mg). Selected fractions, identified by SDS–PAGE (data not shown), containing high molecular mass proteins were further purified by gel permeation chromatography over a Superose 6 resin (10 x 300 mm; flow rate 1.0 ml/min; 100 mM Tris, 50 mM NaCl, 20 mM EDTA, 30 mM DTT and 10% (v/v) glycerol, pH 7.3.) using an AKTA purifier 100 system (Amersham Bioscience, Sweden). Protein concentrations were determined using the Bradford assay [23] with BSA as standard. SDS–PAGE was performed according to the method of Laemmli [24] using high molecular mass protein calibration (Sigma–Aldrich).

2.6. Two-dimensional electrophoresis

After gel permeation chromatography, selected protein-containing fractions were also subject to separation by 2D-PAGE. Protein (200 g) was precipitated with 5 volumes of ice-cold 100% acetone and air-dried. Pellets were resuspended in 250 μl IEF-buffer (10 mM Tris, 8 M urea, 2 M thiourea, 4% (w/v) CHAPS, 1% (v/v) Triton X-100, 65 mM DTT and 0.8% (w/v) amphotelye), loaded onto linear immobilized pH gradient (IPG) strips (13 cm, pH range: 3–10) and isoelectric focussing (IEF) was performed at 16 °C using an Ettan™IPGphorII™ instrument (Amersham Bioscience). After a conditioning step at 50 V for 10 h, 250 V was held over 15 min, followed by voltage ramping to 8000 V in 5 h, which was held for further 8 h. IPG strips were then incubated twice in 15 ml equilibration buffer (50 mM Tris, 30% (v/v) glycerol, 2% (w/v) SDS and 6 M urea, pH 6.8), supplemented with 2% (w/v) DTT and subsequently with 2.5% (w/v) iodoacetamide for 20 min. The strips were then placed on a 7.5–10% (w/w) SDS–PAGE gel and electrophoresed overnight at 80 V at 4 °C.

2.7. Mass spectrometry

Protein samples for peptide mass determination were separated by SDS–PAGE or 2D-PAGE, excised from the gels and digested with trypsin (Promega sequencing grade; 5–20 μg, overnight). The resultant peptide mixtures were extracted from gel pieces, mixed with saturated α-cyano-4-hydroxycinnamic acid as previously described [17] and mixtures (0.5 μl) applied to MTP 384 ground steel mass spectrometry targets (Bruker) and allowed to dry. Matrix Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry (MALDI ToF MS) was performed using a Bruker ultraflex
LIFT-ToF/ToF (UK). Determination of tryptic fingerprints was carried out on different spots of one sample, and device parameters, especially laser power, adjusted until an optimal signal/noise ratio was found. Mass spectra with high signal intensity were subjected to LIFT-ToF/ToF analysis [25]. Spectra were processed using FlexAnalysis software (Bruker), using tryptic fragments of trypsin for internal calibration of spectra. Database searches and sequence comparisons were carried out via Mascot in-house server (MatrixScience) and Biotools (Bruker), respectively.

3. Results and discussion

3.1. Molecular characterization and phylogenetic analysis of three NRPS-encoding genes sidD, C and E

After interrogation of the A. fumigatus genome by BLAST analysis, using the ACVS- and A. nidulans peptide synthetase (sidC) encoding genes, several ORFs, encoding NRPSs were identified. AfusidD and C (GenBank Accession Nos. DQ013888 and DQ011870) clustered with known and proposed siderophore synthetases upon phylogenetic analysis (data not shown). According to the modular organization (Fig. 1) and strong resemblance, sidD (GenBank Accession No. DQ011871) is likely to be an ortholog of sid2, a putative siderophore synthetase encoding gene of Aspergillus oryzae (GenBank Accession No. AB087617). Both sequences showed 75% identity (85% similarity). Like sid2, sidD encodes a bi-modular NRPS consisting of two adenylation, two thiolation and one condensation modules (Table 1) in AniSidC and AfuSidC. Thus, similarity: 71%). The 13,551 bp ORF of AfusidC predicted a protein of 4517 amino acids (molecular mass: 496 kDa). AfuSidC clusters together with both AniSidC and Sid2, a ferrichrome siderophore peptide synthetase gene by U. maydis (GenBank Accession No. U62738), indicating a functional relationship. Similar amino acid codes were found for the corresponding adenylation modules (Table 1) in AniSidC and AfuSidC. Thus, almost the same amino acids were predicted to be

Comparison with putative ortholog sequences Sid2 (A. oryzae) and SidC (A. nidulans) according to the NRPS prediction blast server ([28]; http://raynam.chm.jhu.edu/~nrps/instructions.html).
recognized and activated by the adenylation domains of module one (alanine) and three (glycine or serine) of both genes according to NRPS adenylation domain prediction [28]. Alanine, glycine and serine might be charged by the domains of AfuSidC, which are thereby constituents of the hydroxamate siderophore ferricrocin, produced by \textit{A. fumigatus} [32].

\textit{SidE} comprises a 6330 bp ORF and encoded a theoretical bi-molecular NRPS-protein of 2109 amino acids. Like AfuSidD, this hypothetical protein (SidE) appeared to contain two adenylation and thiolation domains and one condensation domain (Fig. 1). SidE shared 24\% amino acid identity (42\% similarity) with AfuSidC and both proteins may be derived from a common ancestor. In addition, SidE clustered with \textit{A. nidulans} SidC and Sid2 of \textit{U. maydis} following phylogenetic analysis whereby 25\% identity/42\% similarity and 23\% identity/40\% similarity, respectively, is evident. While no prediction was possible for the substrate amino acid encoded by module 1 of SidE, module 2 contains the eight-amino acid non-linear motif, DVYFTGGV, and predicted incorporation of valine (Table 1) [28].

### 3.2. Siderophore production

Fig. 2 shows that optimal siderophore production occurred when \textit{A. fumigatus} was grown in the absence of iron. Furthermore, significantly higher siderophore amounts were apparent at the 72 h time-point following culture under iron-free conditions compared to earlier time-points ($p < 0.05$). Significant diminution of siderophore levels was apparent ($p < 0.05$) under 20 $\mu$M iron conditions (up to 2-fold), although total levels present at 72 h time-point were approximately 50\% of those evident under iron-free conditions. Low siderophore production was evident, and minimal differences in siderophore levels were detectable, at any time-point, under high iron (300 $\mu$M) culture conditions. Moreover, maximum siderophore productions under high iron conditions were between 2- and 15-fold less than those observed under iron free or low iron culture conditions.

It has recently been demonstrated that \textit{sidA} expression in \textit{A. fumigatus} is upregulated under conditions of iron starvation (12–24 h culture time) [3]. Moreover, dramatic increases in the levels of extracellular TAFC and intracellular desferriferricrocin were concomitantly observed [3]. Hissen et al. [6] have demonstrated that siderophore production by \textit{A. fumigatus} is significantly greater in the presence of holo-transferrin, as opposed to serum, in minimal essential medium (MEM) over a 19-h incubation time and that Fe(III) (20 $\mu$M) already causes iron limitation which results in induction of enhanced siderophore formation and excretion. In the present study, where minimal media containing glucose and several salts were supplemented with different concentrations of Fe(III), this finding was confirmed. These data clearly demonstrate that siderophore production is enhanced in the absence of free iron.

### 3.3. Expression analysis of \textit{sidD}, \textit{C} and \textit{E}

Expression of \textit{sidD}, \textit{C} and \textit{E} was assessed by semi-quantitative RT-PCR analysis. rRNA loading was used as a control to ensure equal amounts of RNA used for cDNA synthesis (Fig. 3A). The presence of genomic DNA was excluded by both DNAse treatment of isolated RNA prior to RT-PCR and analysis of the size difference between the genomic and cDNA amplicon of \textit{calm} from which introns have been excised (Fig. 3B). It can be seen that \textit{sidD} and \textit{C} expression was evident at all time points during \textit{A. fumigatus} growth from $T = 24–72$ h in the absence of free iron and that diminution of expression occurs both under low (20 $\mu$M) and high (300 $\mu$M) iron conditions (Fig. 3C and D). Expression of \textit{sidD} diminished by between 25\% and 60\% under low iron conditions and by up to 90\% under high iron conditions (24 and 48 h), upon comparison to \textit{sidD} expression in the absence of free iron. Although \textit{sidD} exhibits 75\% sequence identity with \textit{sid2} of \textit{A. oryzae}, no information is available on the expression pattern of \textit{sid2} and so a comparative analysis was not possible.
Expression of $sidC$ appeared to be maintained under low iron conditions compared to that of $sidD$, but was reduced by approximately 65–80% under high iron conditions (24 and 48 h) relative to expression in iron-free culture conditions. Yuan et al. [16] have observed a similar phenomenon when $U. maydis$ was cultured in low iron medium whereby a 2.5-fold increase in $sid2$ expression was determined by Northern hybridization. Interestingly, expression of both $sidD$ and $C$ appeared to be upregulated under high iron conditions at the 72 h time-point, possibly due to depletion of iron in the medium as fungal growth proceeds. Constitutive and constant expression of $sidE$ was observed under all experimental conditions, except during the early growth phase under high iron conditions (24 h) where a 65% decrease in expression is evident relative to culture in the absence of iron at 24 h. Although previous work has also shown that the expression of $A. nidulans sidC$ is negatively regulated by the presence of iron (10 $\mu$M) [33], the results presented here are the first demonstration that the expression of up to three NRPS-encoding genes in $A. fumigatus$ is differentially regulated by iron availability.
3.4. Protein expression analysis

Protein extracts of *A. fumigatus* cultures grown with and without free iron over a time course of 3 days were prepared and subjected to SDS–PAGE analysis. Two proteins, one of approximate molecular mass 200 kDa, termed high molecular mass protein (HMMP)1, and another of 300–400 kDa, termed HMMP2, were predominantly expressed at 48 h, in the absence of iron (Fig. 4A). Expression of HMMP1 was still evident at 72 h, moreover, this protein also seems to appear at a later time-point (72 h) in the high-iron containing medium. The protein expression profile of HMMP1 indicated resemblance to the transcript course of *sidD*, which encodes a 210 kDa NRP synthetase (Fig. 3). HMMP1 (approx. 200 kDa) was partially purified by means of
Table 2

<table>
<thead>
<tr>
<th>Parent mass/SidD-MH + (monoisotopic)</th>
<th>Actual sequence from LIFT spectrum</th>
<th>Theoretical SidD amino acid sequence (domain of origin)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1186.608/1187.621</td>
<td>LQNXQXXVXXK</td>
<td>LQMAQQGVAK (A1)</td>
</tr>
<tr>
<td>1522.773/1523.654</td>
<td>YXIXEADSAXRK</td>
<td>YAIVEDSGDKR (C1)</td>
</tr>
<tr>
<td>1424.750/1424.758</td>
<td>AQMRDFERXXR</td>
<td>AQMRFVFRVR (C1)</td>
</tr>
<tr>
<td>2344.243/2344.136</td>
<td>TXAAXIXXXXPLXXXHXXPPPXX</td>
<td>TAAAIFEDPSWLVAGHEGYPGR (A1)</td>
</tr>
</tbody>
</table>

Highlighted amino acids are identical in both actual and predicted SidD-derived peptides. Peptides originate from either adenylation (A) or condensation (C) domain 1 of SidD.

...of biotin or fluorescently labeled coenzyme A analogs, in association with functional 4'-phosphopantetheinyl transferase activity, to label native NRPS and thereby facilitate purification [34]. Such an approach may prove useful for the purification of additional, low abundance, peptide synthetases (e.g., SidE) in *A. fumigatus*.

In summary, the iron-mediated, differential expression of two NRPS has been demonstrated in *A. fumigatus*. Furthermore, two proteins, namely SidD and C, involved in siderophore biosynthesis have been purified, analyzed by SDS-PAGE/2D-PAGE and identified by MALDI-ToF/MALDI LIFT-ToF/ToF mass spectrometry. This work furthers our understanding of siderophore biosynthesis in this organism and offers new insights in iron regulation of gene expression in *Aspergillus* spp.

**Acknowledgments**

This work was financially supported by the Higher Education Authority of Ireland under the Programme for Research in Third Level Institutions (HEA-PRTL1). Claire Neville was a recipient of a Daniel O’Connell Fellowship from NUI Maynooth. Preliminary sequence data were obtained from The Institute for Genomic Research website at http://www.tigr.org. Sequencing of *Aspergillus fumigatus* was funded by the National Institute of Allergy and Infectious Disease U01 AI 48830 to David Denning and William Nierman, the Wellcome Trust, and Fondo de Investigaciones Sanitarias.

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