Nonribosomal peptide synthesis in *Aspergillus fumigatus* and other fungi

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In fungi, nonribosomal peptide synthetases (NRP synthetases) are large multi-functional enzymes containing adenylation, thiolation (or peptidyl carrier protein, PCP) and condensation domains. These enzymes are often encoded within gene clusters. Multiple NRP synthetase ORFs have also been identified in fungi (14 in *Aspergillus fumigatus*). LeaA, a methyltransferase, is involved in secondary metabolite gene cluster regulation in *Aspergillus* spp. The NRP synthetases GliP and FtmA respectively direct the biosynthesis of the toxic metabolites gliotoxin and brevianamide F, a precursor of bioactive prenylated alkaloids. The NRP synthetase Pes1 has been shown to mediate resistance to oxidative stress, and in plant-pathogenic ascomycetes (e.g. *Cochliobolus heterostrophus*) an NRP synthetase, encoded by the *NPS6* gene, significantly contributes to virulence and resistance to oxidative stress. Adenylation (A) domains within NRP synthetases govern the specificity of amino acid incorporation into nonribosomally synthesized peptides. To date there have only been limited demonstrations of A domain specificity (e.g. *A. fumigatus* GliP and in *Beauveria bassiana*) in fungi. Indeed, only in silico prediction data are available on A domain specificity of NRP synthetases from most fungi. NRP synthetases are activated by 4'-phosphopantetheinylation of serine residues within PCP domains by 4'-phosphopantetheinyl transferases (4'-PPTases). Coenzyme A acts as the 4'-phosphopantetheine donor, and labelled coenzyme A can be used to affinity-label apo-NRP synthetases. Emerging fungal gene disruption and gene cluster expression strategies, allied to proteomic strategies, are poised to facilitate a greater understanding of the coding potential of NRP synthetases in fungi.

Introduction

Nonribosomal peptide synthesis – mechanistic aspects

Nonribosomal peptide synthesis (NRPS) is a key mechanism responsible for the biosynthesis of bioactive metabolites in bacteria and fungi (Mootz et al., 2002; Reiber et al., 2005) (Fig. 1). Nonribosomal peptide synthetase genes, generally present as part of multi-gene clusters, encode NRP synthetases (up to 2.3 MDa molecular mass) which, in turn, biosynthesize peptide products (Wiest et al., 2002; Keller et al., 2005) (Fig. 1). NRP synthetases are composed of discrete domains [adenylation (A), thiolation (T) or peptidyl carrier protein (PCP) and condensation (C) domains] which when grouped together are referred to as a single module as shown for the putative *Aspergillus fumigatus* NRP synthetase encoded by pesM (Fig. 2, Table 1). Each module is responsible for the recognition (via the A domain) and incorporation of a single amino acid into the growing peptide product. Thus, an NRP synthetase is generally composed of one or more modules and can terminate in a thioesterase domain (TE; Fig. 2) that releases the newly synthesized peptide from the enzyme (Grunewald & Marahiel, 2006; Mootz et al., 2002). Occasionally, epimerase (E) domains (responsible for L- to D- amino acid conversion) are present within the NRP synthetase. A nonlinear 8–13 aa ‘signature motif’ governs the selection of substrate amino acids by the appropriate A domain (Grunewald & Marahiel, 2006; Schwecke et al., 2006). In addition, all bacterial and fungal NRP synthetases described to date require post-translational 4’-phosphopantetheinylation to facilitate metabolite production. Here, 4’-phosphopantetheinyl transferases (4’-PPTases) catalyse the transfer of 4’-phosphopantetheine from coenzyme A (CoA) to a conserved serine residue within the thiolation domains of cognate apo-NRP synthetases to yield activated holo-NRP synthetases. These 4’-phosphopantetheinyl prosthetic groups anchor activated intermediates during NRPS, via thioester formation, and facilitate their movement between active sites within the NRP synthetase (Grunewald & Marahiel, 2006).

Most bioactive metabolites exhibit a peptidyl and/or polyketide composition along with elaborate architecture including cyclic or branched-cyclic structures and modified proteogenic or non-proteogenic amino acids. As mentioned above, NRP synthetases responsible for the biosynthesis of bioactive peptides possess a collinear modular structure, with each module responsible for the activation,
thiolation and condensation of one specific substrate amino acid (Mootz et al., 2002). In linear NRP synthetases, the three core domains are organized in the order adenylation, thiolation and condensation (A-T-C)n to form an elongation module that adds one amino acid to the growing chain. Variations on this structure include the iterative NRP synthetases characteristic of siderophore synthetases (Mootz et al., 2002; Reiber et al., 2005) or nonlinear NRP synthetases which deviate in their domain organization from the standard (A-T-C)n architecture (Fig. 2). The emergence of fungal genome sequences has led to the identification of multiple ORFs predicted to encode NRP synthetase genes (e.g. in Cochliobolus heterostrophus, Claviceps purpurea and Fusarium graminearum: Lee et al., 2005; Haarmann et al., 2005; Varga et al., 2005; Tobiasen et al., 2007).

Aspergillus fumigatus and NRPS

A. fumigatus is responsible for approximately 4% of all hospital-based deaths in Europe (Brookman & Denning, 2000). The organism is an opportunist fungal pathogen of immunocompromised patients and is the commonest aetiological agent of invasive aspergillosis (IA) (Brakhage & Langfelder, 2002). Although occurring at a lower frequency than the commonest fungal infection, candidiasis, IA results in greater mortality (as high as 60–90%) and is therefore the leading worldwide cause of death due to fungal infection (Tekaia & Latgé, 2005). IA is a major cause of illness and death among bone marrow and solid organ transplant and leukaemia patients and those with pre-existing pulmonary malfunction. In fact, aspergillosis accounts for at least 3500 deaths per annum in the USA (Kontoyiannis & Bodey, 2002). The significance of A. fumigatus as a human pathogen is reflected in the sequencing of the entire A. fumigatus genome, and the in silico annotated genome of A. fumigatus is now available at ‘CADRE’ (http://www.cadre-genomes.org.uk) (Mabey et al., 2004; Nierman et al., 2005). The genomes of both Aspergillus nidulans and Aspergillus oryzae have also been sequenced (Galagan et al., 2005; Machida et al., 2005). Although extensive in silico annotation of the A. fumigatus genome is in place, biochemical support for most gene identification is limited and a significant number of genes (approx. 5% of total) are only identified as encoding ‘hypothetical proteins’. Moreover, although many genes have been identified, the precise enzymic function of the cognate protein remains frustratingly obscure. Nowhere is this issue more problematic than in the information deficit with respect to the relationship between NRP synthetase genes/proteins and the nature of the bioactive peptidyl products biosynthesized by these multi-functional, and mechanistically elusive, enzymes.

Fig. 1. Schematic representation of the enzyme functions involved in NRPS. NRP synthetases are generally found within transcriptionally co-regulated gene clusters whereby all genes required for NRP peptide product synthesis are co-expressed. Apo-NRP synthetases are converted to the active holo-form by 4′-phosphopantetheinylation of thiolation (T or PCP) domains within the NRP synthetase. NRP peptide synthesis is further facilitated by the action of additional enzyme activities – either components of, (e.g. amino acid epimerization) or distinct from (e.g. oxidation), the NRP synthetase.

Fig. 2. Predicted module arrangement of the A. fumigatus NRP synthetase gene (pesM) (12 kb; Table 1). The gene is predicted to encode three adenylation (A), three thiolation (T) and three condensation (C) domains, in addition to one epimerase (E) and one thioesterase (TE) domain. Module 1 has a molecular mass of approximately 90 kDa.
The significance of *A. fumigatus* as a potential reservoir of bioactive, nonribosomally synthesized, peptides has been consolidated by the observation that at least 14 NRP synthetase genes/gene clusters are found within the *A. fumigatus* genome (http://www.cadre-genomes.org.uk/; Mabey et al., 2004; Nierman et al., 2005; Reiber et al., 2005; Neville et al., 2005; Reeves et al., 2006) (Table 1). However, to date only limited information has been forthcoming relating these specific NRP synthetase genes, indirectly or directly, to a specific peptide product (e.g. iron-chelating siderophores or mycotoxins) (Reiber et al., 2005; Cramer et al., 2006a; Maiya et al., 2006) (Table 1). Thus, much further work is needed to fully understand the process of NRPS in *A. fumigatus* and to investigate if nonribosomally synthesized peptides contribute to the success of this organism as a pathogen of immunocompromised individuals. Moreover, information gleaned from molecular studies of *Aspergillus* spp. will have strong resonance in other fungal species (e.g. *Magnaporthe grisea*, *Trichoderma harzianum*, *Cochliobolus heterostrophus*) of agricultural (i.e. plant pathogens) or commercial importance, which produce NRP products, for which genome sequence analysis has been completed or is currently under way (Dean et al., 2005; Vizcaino et al., 2005; Lee et al., 2005). These are also attractive models for NRPS investigations. Ultimately, such efforts will move us towards an era of confident prediction of the constituent amino acids of nonribosomally synthesized peptides from the NRP synthetase gene sequence and the role of NRPS in mediating fungal opportunistic pathogenesis.

**Global regulation and specific activation of NRPS**

**Regulation of secondary metabolism in filamentous fungi**

It is now clear that NRPS gene cluster expression, and NRP synthetase activation, are both subject to global regulation and local activation, respectively, prior to initiation of NRPS in fungi.

A novel nuclear-located protein (LeaA; 375 aa) which acts as a global regulator of secondary metabolism in the genus *Aspergillus* has been identified (Bok & Keller, 2004). It was demonstrated, using deletion (ΔleaA)/complementation studies, that the protein was required for the production of sterigmatocystin and penicillin in *A. nidulans* and gliotoxin in *A. fumigatus*. The authors also noted that LeaA was conserved in all filamentous fungi for which genome sequence data were available, and identified the presence of a conserved S-adenosylmethionine (SAM) binding site in LeaA – a feature of known nuclear protein methyltransferases. More recently, site-specific mutation analysis of the

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**Table 1. NRP synthetase genes of *A. fumigatus*, with hypothetical NRP synthetase domain architecture based on in silico analysis**

<table>
<thead>
<tr>
<th>NRPS gene</th>
<th>Domain architecture</th>
<th>CADRE ID</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pesI (pesB)</td>
<td>ATECACA</td>
<td>Afu1g10380</td>
<td>Neville et al. (2005); Nierman et al. (2005); Reeves et al. (2006)</td>
</tr>
<tr>
<td>sidC</td>
<td>ATCATCTCT</td>
<td>Afu1g17200</td>
<td>Reiber et al. (2005); Nierman et al. (2005)</td>
</tr>
<tr>
<td>sidE</td>
<td>ATCATC</td>
<td>Afu3g03350</td>
<td>Reiber et al. (2005); Nierman et al. (2005)</td>
</tr>
<tr>
<td>sidD</td>
<td>ATCATC</td>
<td>Afu3g03420</td>
<td>Reiber et al. (2005); Nierman et al. (2005)</td>
</tr>
<tr>
<td>pesF</td>
<td>ATCATC</td>
<td>Afu3g12920</td>
<td>Nierman et al. (2005)</td>
</tr>
<tr>
<td>pesG</td>
<td>ATC</td>
<td>Afu3g13730</td>
<td>Nierman et al. (2005)</td>
</tr>
<tr>
<td>pesH</td>
<td>ACATC</td>
<td>Afu3g15270</td>
<td>Nierman et al. (2005)</td>
</tr>
<tr>
<td>pesI</td>
<td>ATCATC</td>
<td>Afu5g12730</td>
<td>Nierman et al. (2005); Cramer et al. (2006b)</td>
</tr>
<tr>
<td>pesJ</td>
<td>CATC</td>
<td>Afu6g09610</td>
<td>Nierman et al. (2005)</td>
</tr>
<tr>
<td>pesK (gliP)</td>
<td>ATCATC</td>
<td>Afu6g09660</td>
<td>Nierman et al. (2005); Cramer et al. (2006a); Kupfahl et al. (2006)</td>
</tr>
<tr>
<td>pesL</td>
<td>ATC</td>
<td>Afu6g12050</td>
<td>Nierman et al. (2005)</td>
</tr>
<tr>
<td>pesM</td>
<td>ATCATC</td>
<td>Afu6g12080</td>
<td>Nierman et al. (2005)</td>
</tr>
<tr>
<td>pesN (fimA)</td>
<td>ACATC</td>
<td>Afu8g00170</td>
<td>Nierman et al. (2005); Maiya et al. (2006); Sheppard et al. (2005)</td>
</tr>
<tr>
<td>pesO</td>
<td>PKSNNCAT(DH)</td>
<td>Afu8g00540</td>
<td>Nierman et al. (2005); Sheppard et al. (2005)</td>
</tr>
</tbody>
</table>

http://mic.sgmjournals.org 1299
SAM binding site (-LDLCCGTG-) in LeaA, whereby alanines replaced the underlined G residues, resulted in an identical phenotype to ΔleaA (Bok et al., 2006a). These findings provide strong evidence to support the hypothesis (Bok & Keller, 2004, 2006a) that LeaA is a methyltransferase – possibly involved in secondary metabolite gene cluster regulation due to alterations in chromatin structure following the differential methylation of histone proteins. Clearly, the functional expression of LeaA and direct determination of methyltransferase activity would support this hypothesis.

The confirmation of LeaA as a global regulator of secondary metabolic gene cluster expression has also facilitated microarray transcription profiling of A. nidulans gene expression to explore the effects of leaA deletion and over-expression (Bok et al., 2006b; Schardl, 2006). Here, using expression analysis of wild-type (WT) and ΔleaA mutant strains, ‘secondary metabolite cluster signatures’ (corresponding to diminution of gene expression in entire secondary metabolite gene clusters compared to adjacent gene expression) have been identified (Bok et al., 2006b).

The authors then selected a single LeaA-transcriptionally regulated cluster, which comprised five ORFs and was predicted to encode an indole alkaloid-type metabolite. Mutational (ΔidiB) and comparative metabolite analysis confirmed that the cluster encoded the biosynthesis of terrequinone A, a fungal bisindolylquinone – not previously identified as an A. nidulans secondary metabolite (Bok et al., 2006b). The potential of this ‘secondary metabolite cluster signature’ strategy for secondary metabolite gene cluster annotation and genome mining is enormous in all LeaA-expressing filamentous fungi. However as noted by Schardl (2006), in Gibberella zeae PH-1 there are 37 ORFs with significant identity to LeaA it is unlikely that all function as global regulators of secondary metabolism.

**4′-PPTases and NRPS**

Post-translational phosphopantetheinylation of 4′-phosphopantetheine-dependent carrier proteins is essential for metabolite production in NRPS, polyketide biosynthesis, fatty acid biosynthesis and lysine biosynthesis. 4′-PPTase is responsible for the conversion of the peptidyl carrier protein (PCP in NRPS synthetases), acyl carrier protein (ACP in polyketide and fatty acid biosynthesis) and α-aminoadipate semialdehyde reductase (lysine biosynthesis) from an inactive apo-form to an active holo-configuration, in an Mg\(^{2+}\)-dependent reaction (Walsh et al., 1997). This priming reaction involves transferring the 4′-phosphopantetheine residue from coenzyme A to the side-chain β-hydroxyl group of the conserved serine present on the carrier protein, thereby providing the 4′-phosphopantetheine cofactor required for activation of the enzyme complex (Lambalot et al., 1996). In the case of fungal lysine biosynthesis, the 4′-PPTase catalyses the activation of the δ-aminoadipate semialdehyde reductase which, when activated, converts α-aminoacidic acid to α-aminoacidic-δ-semialdehyde in an ATP/NADPH-dependent manner (Guo et al., 2001).

4′-PPTases are classified according to their structure and carrier protein specificity and form three main groups: AcpS type, Sfp type and those present in fatty acid synthase II (Sanchez et al., 2001). AcpS type are those 4′-PPTases commonly involved in fatty acid synthesis, while Sfp type are commonly involved in secondary metabolism in both NRPS and polyketide biosynthesis (Mootz et al., 2002). 4′-PPTase sequences exhibit limited regional homology to each other, and one such homolog motif is WxxKEAxxK (Lambalot et al., 1996). Many 4′-PPTases have been identified in bacteria; however, until recently few have been found in filamentous fungi. In the case of lysine biosynthesis, 4′-PPTase-encoding genes have been identified in Saccharomyces cerevisiae and Candida albicans (Guo et al., 2001; Mootz et al., 2002). The recombinant 4′-PPTase (Lys5p) of C. albicans was shown also to activate the α-aminoadipate reaction in S. cerevisiae and Schizosaccharomyces pombe (Guo & Bhattacharjee, 2003). A 4′-PPTase was also identified in Streptomyces verticillus associated with the synthesis of the hybrid peptide-polyketide drug bleomycin (Sanchez et al., 2001).

The only characterized 4′-PPTase in A. nidulans (npgA) has been proven to be essential for penicillin biosynthesis by Kesztenman-Pereyra et al. (2003). This group identified the A. nidulans 4′-PPTase by using the Sfp 4′-PPTase sequence of Bacillus subtilis to search the A. fumigatus genome database. In that study the A. fumigatus genome was interrogated by homology searching, resulting in the identification of a putative 4′-PPTase. This putative 4′-PPTase sequence was then used, in turn, to detect the 4′-PPTase sequence in A. nidulans. More recently, Han et al. (2005) characterized a temperature-sensitive suppressor (snpA), which encodes a eukaryotic translation release factor (eRF1) that overcomes a npgA mutation in A. nidulans by facilitating nonsense mutation read-through.

As noted above, 4′-PPTases catalyse the transfer of 4′-phosphopantetheine moieties from CoA to thiolation/peptidyl carrier protein domains of NRP synthetases and polyketide synthetases (PKS); so-called apo-acyl carrier proteins (ACPs or PCPs) (Neville et al., 2005; Mootz et al., 2002). However, determination of 4′-PPTase activity is not trivial. In Saccharopolyspora erythraea, Weissman et al. (2004) confirmed the in vivo activity of a putative 4′-PPTase (SePptII) by LC-MS analysis of a recombinant ACP-TE didomain, co-expressed in Escherichia coli with SePptII. We have expressed a functional, promiscuous, Sfp-type 4′-PPTase using the baculovirus expression system, which was used to post-translationally activate, in vitro, a module of recombinant Pes1 (Pes1\(\text{TEA}\)), derived from an A. fumigatus NRP synthetase (Neville et al., 2005). Apo-NRP synthetase activation was detected by MALDI-TOF analysis whereby a 4′-phosphopantetheinylated (Ppant) peptide (Ppant-SFSAMK) from Pes1\(\text{TEA}\) was present after co-incubation of the 4′-PPTase with the recombinant NRP
synthetase fragment and CoA. In addition to confirming that the 4′-PPTase was competent to facilitate apo-NRP synthetase activation in *A. fumigatus*, evidence of promiscuity was also forthcoming via 4′-phosphopantetheinylation of recombinant α-aminoacidate reductase (Lys2p) from *Candida albicans*, a key enzyme involved in lysine biosynthesis (Guo & Bhattacharjee, 2003; Neville et al., 2005). The difficulties in assessing 4′-PPTase activity were overcome to a significant extent by La Clair et al. (2004), who described an elegant method of covalently labelling T/PCP domains of NRP synthetases (and PKS), using biotinylated or fluorescently labelled coenzyme A, in 4′-PPTase-mediated reactions.

**NRP synthetases in *A. fumigatus* and other fungi**

**NRP products as mediators of opportunistic parasitism**

Circumvention of the host immune response facilitates in vivo fungal dissemination, and recent work has demonstrated that an NRP product, the modified diketopiperazine gliotoxin, secreted by *A. fumigatus*, is capable of specifically blocking the respiratory burst in humans by inhibiting assembly of the NADPH oxidase in isolated polymorphonuclear leukocytes (Tsunawaki et al., 2004). In addition, the release of hydroxamate-type siderophores, to facilitate iron acquisition by the organism, is also essential for fungal virulence (Schrettl et al., 2004); however, precise information on the identity of siderophore biosynthetic genes in *A. fumigatus* has not been forthcoming. In fact, two independent groups reported that deletion of *A. fumigatus* *sidA*, encoding an L-ornithine N²-monooxygenase which catalyses the first committed step of hydroxamate-type siderophore biosynthesis, resulted in the complete inability to synthesize both triacetylfulusaricule C and ferricrocin (Schrettl et al., 2004; Hissen et al., 2005). Moreover, both *sidA* mutant strains were completely avirulent in murine models of IA. LeaA also plays a role in mediating the opportunistic pathogenicity of *A. fumigatus*, and Bok et al. (2005) have shown that a *leaA* mutant is essentially avirulent in an immunocompromised mouse model system.

Thus, although classically referred to as secondary metabolites, gliotoxin and siderophores, in addition to a diverse range of other bioactive components, may actually play a front-line role in organismal growth and opportunistic parasitism or pathogenicity.

Additional bioactivities, many of which are predicted to be synthesized nonribosomally, include ergot alkaloids, the tremorgenic mycotoxin verruculogen, and aspergillazines A–E, all of which have been shown to be produced by either *A. fumigatus* or *Aspergillus unilateralis* (aspergillazines A–E) (Panaccione & Coyle, 2005; Coyle & Panaccione, 2005; Kosacle et al., 2005; Capon et al., 2005). Khoufache et al. (2007) have demonstrated that verruculogen specifically modifies the electrophysiological properties of human nasal epithelial cells and speculate that it may play a role in the colonization or invasion of the airway epithelium – although direct proof remains outstanding.

**Brevianamide F, gliotoxin (precursor) and other peptide synthetases in *A. fumigatus***

To date, only two *A. fumigatus* NRP synthetases have been definitively shown to produce specific NRP peptide products. Firstly, a bimodular NRP synthetase (ftmA; CADRE ID: Afu8g00170; Table 1) from *A. fumigatus* has been shown to encode the dipeptide brevianamide F (cyclo-L-Trp-L-Pro), which is a precursor of bioactive prenylated alkaloids (e.g. fumitremorgins A, B and C and tryprostatin B) (Maiya et al., 2006). Secondly, Gardiner & Howlett (2005) identified a cluster of 12 genes in *A. fumigatus* by a comparative genomics approach, and demonstrated that the genes were co-regulated and that the timing of expression correlated with gliotoxin production. It has since been conclusively demonstrated that the gene cluster directs gliotoxin biosynthesis since disruption of the transcriptional regulator (gliZ) eliminates expression of the entire cluster and prevents gliotoxin production (Bok et al., 2006). This unambiguously confirms the role of the hypothetical gliotoxin gene cluster in gliotoxin formation. The *gliP* gene (CADRE ID: Afu6g09660; Table 1), encoding the bimodular NRP synthetase responsible for the biosynthesis of the dipeptide L-Phe-L-Ser, a precursor of gliotoxin, has been identified by gene disruption whereby a *gliP* mutant failed to produce gliotoxin (Cramer et al., 2006a; Kupfahl et al., 2006). Unexpectedly, this mutant was of equivalent pathogenicity to a WT strain of *A. fumigatus*, suggesting that gliotoxin production may not be required for virulence in immunocompromised animal models of IA (Cramer et al., 2006a; Kupfahl et al., 2006). However, an immunocompromised murine model system may not be appropriate for assessment of the role of gliotoxin in mediating *A. fumigatus* pathogenicity, and the use of non-immunocompromised model systems has been proposed (Kupfahl et al., 2006). Although gliotoxin does not appear to affect initial conidia–macrophage interactions (Cramer et al., 2006a), a reduced cytotoxic effect on macrophage-like cells and T cells, when exposed to ΔgliP as opposed to WT culture filtrates, has been observed (Kupfahl et al., 2006). Furthermore, gliotoxin may be responsible for inhibiting mast cell degranulation, since ΔgliP culture filtrate did not prevent degranulation as effectively as WT culture filtrates (Cramer et al., 2006a).

Adenylation domain specificity analysis by Balibar & Walsh (2006) has now confirmed that the first A domain of GliP is responsible for activation of L-Phe, while the second A domain activates L-Ser. Moreover, these authors have also shown that the cyclized diketopiperazine (DKP), composed of Phe-Ser, is released slowly from GliP, after biosynthesis, and postulate that further modification of the DKP may occur when bound to GliP. However, there is an information deficit on the subsequent biosynthetic mechanism used to convert this dipeptide precursor into a functional toxin, especially with respect to thiol incorporation into the gliotoxin precursor. Investigation of this unusual process is merited as it should contribute to the elucidation of the epipolythiodioxopiperazine (ETP)
biosynthetic mechanism, in *A. fumigatus* and other fungi (e.g. *Leptosphaeria maculans: Gardiner et al., 2004*), for related toxins such as sirodesmin. Although Balbar & Walsh (2006) have clearly defined the adenylation domain specificity of GlIP, the precise assignment of adenylation domain specificity of FtmA (Maiya et al., 2006) remains outstanding.

Ergot alkaloid biosynthesis, involving a dimethylallyltryptophan synthetase (DMAT synthetase; encoded on *A. fumigatus* chromosome II) has also been reported (Coyle & Panaccione, 2005); however, this pathway does not appear to involve an NRP synthetase. Another putative DMAT synthetase is present on chromosome III of *A. fumigatus*, adjacent to an NRP synthetase gene (pesI; Table 1), raising the possibility of an alternative ergot alkaloid biosynthetic pathway in the fungus. However, it should be noted that the presence of a DMAT synthetase adjacent to an NRP synthetase does not necessarily mean that these genes are part of an alternative ergot gene cluster, as DMAT synthetases can prenylate many classes of secondary metabolites. At present it is unclear if these genes are susceptible to Lea regulation and so further investigation is required (Bok et al., 2006b). Finally, the expression of pesI, ftmA and pesO (Table 1) has been demonstrated to be under the control of StuA, which is responsible for transcriptional activation during acquisition of developmental competence (Sheppard et al., 2005), suggesting NRPS functionality beyond secondary metabolism. Cramer et al. (2006b) have noted that NRPS8 (identified as pesI in Table 1) is primarily expressed in ungerminated conidia and speculated that the pesl NRP peptide product may be involved in the early stages of fungal development.

Using a bioinformatic approach we have also identified, and *in silico* analysed the domain architecture of, a number of novel NRP synthetase genes (sequenced by Nierman et al., 2005), one of which we have termed pesM (size: 12 kb) (Table 1, Fig. 2). Moreover, our bioinformatic analyses predict the domain architecture of pesM described in Fig. 2 which indicates that three adenylation domains encode the incorporation of at least three amino acids into the PesM peptide product. Thus, *A. fumigatus* NRP synthetases exhibit both similar and dissimilar domain organization to previously and recently identified NRP synthetases. Pes1 of *A. fumigatus* (Neville et al., 2005; Reeves et al., 2006; Table 1) was shown to comprise four thiolation, four adenylation and five condensation domains, with unusual core domain arrangement. Significantly, a pes1 deletion mutant in *A. fumigatus* (Δpes1) exhibits altered conidial morphology and increased sensitivity to oxidative stress due to H$_2$O$_2$ presence (Reeves et al., 2006; Table 1). Work is currently under way to identify the nature of the Pes1-encoded peptide product.

**Siderophore synthetases in *A. fumigatus* and other fungi?**

NRPS represents an important mechanism for the biosynthesis of extra- and intracellular iron-chelating siderophores which aid fungal growth (Eisendle et al., 2003) (Fig. 3). In *A. fumigatus*, studies have identified the expression of three NRP synthetase genes (sidC, D and E) (Table 1) which are, to varying extents, susceptible to regulation by the level of free iron present in the culture medium. It has been demonstrated that sidD expression is significantly upregulated under iron-limiting culture conditions (iron-free or 20 μM free iron), concomitant with siderophore production (Fig. 3), and that the cognate SidD protein is present – as determined using a combined 2D-PAGE/MALDI-TOF and TOF/TOF mass spectrometry approach (Reiber et al., 2005). Previous microarray expression studies in *S. cerevisiae* have shown that disabled ORFs can be expressed at the transcript level (Harrison et al., 2002). Thus, the functional identification of NRP synthetases, at the protein level, is important since pseudogenes may undergo transcription due to the presence of functional promoters (Vizzaino et al., 2005; Lee et al., 2005). In addition, detection of NRP synthetase gene expression can be problematic (Cramer et al., 2006b).

Relevantly, a gene (NPS6) in the plant-pathogenic fungus *Cochliobolus heterostrophus* which contributes to both fungal virulence in maize and resistance to oxidative stress has been identified (Lee et al., 2005). Deletion of NPS6 in the plant-pathogenic ascomycetes *Alternaria brassicicola*, *Cochliobolus miyabeianus*, *C. heterostrophus*, and *E. gramininarum* resulted in reduced virulence and an increased sensitivity to H$_2$O$_2$ (Oide et al., 2006). The authors further demonstrated that the application of exogenous Fe$_3$$^{3+}$ restored NPS6 mutant virulence in exposed plants and that the gene encodes extracellular hydroxamate-type siderophores. NPS6 appears to be an orthologue of sidD, which indicates that SidD (or the SidD peptide product), in part, may mediate resistance to oxidative stress in *A.

![Fig. 3. Siderophores in action. *A. fumigatus* growing on the lower half of the culture plate, under iron-free conditions, releases siderophores which scavenge Fe$_3$$^{3+}$ from the Chrome Azurol S reagent (Reiber et al., 2005), in the upper section of the plate, resulting in the red colour.](image-url)
nonribosomally synthesized, peptides also represents an emerging strategy for the reliable diagnosis of fungal infection, which, to date, has proved to be extremely difficult (Mennink-Kersten et al., 2004). We have previously demonstrated the specific detection of gliotoxin by ELISA (Fox et al., 2004), and gliotoxin can be detected by LC-MS in sera and lungs from mice suffering from experimentally induced IA (Lewis et al., 2005). Gliotoxin can also be detected in cancer patients with either proven or probable IA (Lewis et al., 2005).

Cyclic NRP products have also been proposed to be specific markers of fungal infection whereby analysis of biological fluids by mass spectrometry may yield information on specific fungal strains (Jegorov et al., 2006). One potential limitation to the diagnostic potential of NRP peptides is that very few NRP synthetases may be unique to specific Aspergillus species (Cramer et al., 2006b). Therefore, since several different Aspergillus species can cause invasive disease, the utility of using a NRP product as a diagnostic marker specifically for A. fumigatus remains unproven. Nevertheless, detection of NRP products represents a novel strategy for the diagnosis of Aspergillus infection and merits future investigation.

**Fungal NRPS specificity code and future directions**

NRP synthetase adenylation domains and substrate amino acid specificity code

NRP synthetase adenylation domains in fungi, which range in size between 468 and 549 aa, contain an 8–13 aa non-linear signature motif that governs substrate amino acid selection from the cellular pool (Mootz et al., 2002; Schwecke et al., 2006). *In silico* prediction algorithms for the substrate amino acid specificity code of NRP synthetase adenylation domains have been the subject of at least four major studies (Schwecke et al., 2006; Stachelhaus et al., 1999; Challis et al., 2000; Rausch et al., 2005), in addition to comparative analysis of adenylation domain presence in NRP synthetases and other enzymes (e.g. *α*-aminoadipate semialdehyde dehydrogenase: Di Vincenzo et al., 2005), the initial studies (Stachelhaus et al., 1999; Challis et al., 2000), although extremely comprehensive, focussed on elucidation of bacterial adenylation domain specificity, which does not appear to be predictive of fungal adenylation domain signature motifs. However, direct biochemical evidence of adenylation domain specificity in fungi is emerging. It has been conclusively demonstrated that the adenylation domain within the NRP synthetase module of a PKS-NRP hybrid synthetase in *Fusarium moniliforme* encodes homoserine, as part of the final product, fusarin C (Rees et al., 2007). Moreover, the PKS-NRP synthetase hybrid product, 2-pyridone tenellin, in *Beauveria bassiana* contains tyrosine encoded by the single adenylation domain in the NRP synthetase (Eley et al., 2007), and the adenylation domain within the *A. nidulans* NRP synthetase, TdiA, may encode acryl acid, although in this case direct proof remains to be provided (Bok et al., 2006b).

A number of groups (Reiber et al., 2005; Reeves et al., 2006; Lee et al., 2005; Schwecke et al., 2006; Cramer et al., 2006b) have attempted to assign specificity by phylogenetic analysis of adenylation domain architecture. A molecular modelling approach was also adopted by Schwecke et al. (2006). While some predictive agreement is evident between these studies, it is clear that much work is needed to establish the specificity of (non-siderophore) NRP synthetase adenylation domains. Moreover, it has been suggested that the transductive
support vector machine (TSVM) approach (Rausch et al., 2005) may not be suitable for the prediction of fungal adenylation domain signature motifs (Schwecke et al., 2006). Recent phylogenomic analysis of NRP synthetases from Aspergillus species has both confirmed many previous observations on adenylation domain relationships (Lee et al., 2005; Schwecke et al., 2006) and illustrated new ones such as the clustering of adenylation domains encoding proven and potential ETP-type toxins (Cramer et al., 2006b). Nonetheless, as a general rule, all bioinformatic predictions should be confirmed by in vitro studies (Dorrestein et al., 2006). Thus, the unambiguous assessment of fungal adenylation domain specificity requires direct biochemical analysis whereby the substrate amino acid binding specificity of individual recombinant adenylation domains is assessed by amino-acid-dependent ATP/PPi exchange reaction (Couch et al., 2004; May et al., 2005). In addition, expression of functional apo-adenylation-thiolation domains, in vitro 4′-phosphopantetheinylation to the holo-form followed by incubation with relevant amino acid substrates should facilitate the identification of the bound amino acid – by either whole protein or peptide mass spectrometry (Dorrestein et al., 2006).

Future directions and concluding remarks

Fungal, and in particular A. fumigatus, NRPs still presents many challenges in terms of understanding, exploitation and even nomenclature (see legend of Table 1). Firstly, although at least 14 NRP synthetase genes are present in A. fumigatus, the function of most of this coding potential (i.e. the biochemical nature of resultant NRP peptide products) remains unknown. In addition, the function of most additional ORFs, associated with NRP synthetase genes in clusters, remains to be fully elucidated. Finally, the amino acid signature motifs of nearly all adenylation domains within A. fumigatus and other fungal NRP synthetases must be defined. The convergence of established technologies such as genome sequencing, in silico annotation and metabolite profiling, in addition to ‘secondary metabolite cluster signatures’ (Bok et al., 2006b) combined with the application of emerging and novel genomic (e.g. fungal gene deletion and silencing: Mouyna et al., 2004; Krappmann, 2006) and proteomic strategies (i.e. recombinant adenylation-thiolation domain activation and activity analysis: Kelleher & Hicks, 2005; Neville et al., 2005; Hicks et al., 2006) sets the ideal scene for rapid and unambiguous advances in dissecting the global importance of NRPS in filamentous fungi of biomedical and biotechnological relevance.

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References


