

Review

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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,

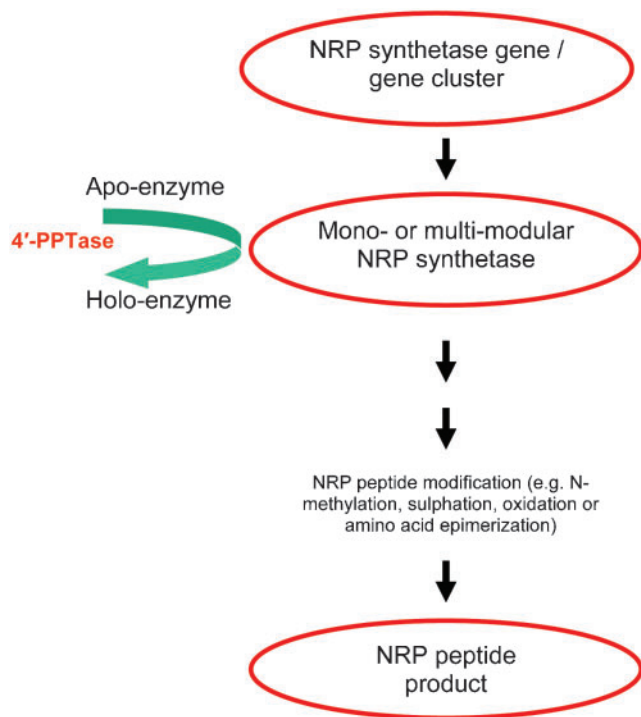


Fig. 1. Schematic representation of the enzyme functions involved in NRPS. NRP synthetases are generally found within transcriptionally co-regulated gene clusters whereby all genes required for NRP peptide product synthesis are co-expressed. Apo-NRP synthetases are converted to the active holo-form by 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.

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 opportunistic 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 (Tekaiia & 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.

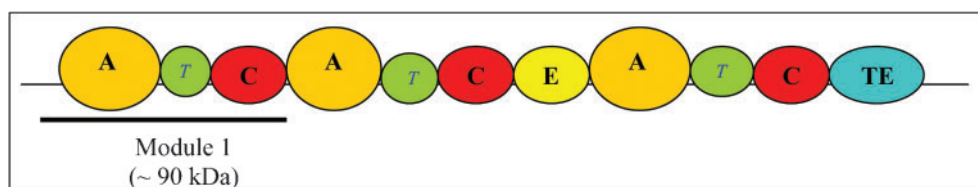


Fig. 2. Predicted module arrangement of the *A. fumigatus* NRP synthetase gene (*pesM*) (12 kb; Table 1). The gene is predicted to encode three adenylation (A), three thiolation (T) and three condensation (C) domains, in addition to one epimerase (E) and one thioesterase (TE) domain. Module 1 has a molecular 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 ($\Delta leaA$)/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

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

Key: A, adenylation domain; T, thiolation domain; C, condensation domain; E, epimerase domain; TE, thioesterase domain; DH, COG3320, putative dehydrogenase domain of multifunctional NRPS; C*, partial condensation domain; PKS, polyketide synthetase; N, incomplete domain, not identifiable. Afu6g09660, gliotoxin precursor synthetase (Cramer *et al.*, 2006a; Kupfahl *et al.*, 2006); Afu8g00170, brevianamide F synthetase (Maiya *et al.*, 2006); Afu8g00540 appears to be a hybrid PKS/NRP synthetase. A putative ORF, Afu8g01220, contains a single adenylation domain but is excluded from the table. At least four other 'NRP synthetase-like' proteins, lacking C domains, are present in the genome of *A. fumigatus* but not shown in the table: (i) *pesP*, A domain only, Afu4g14440; (ii) *pesQ*, comprising AT(DH)(DH) domains, Afu5g10120; (iii) *pesR*, comprising AT(DH)(DS) domains, Afu6g03480 (DS: cysteine desulfurase domain); and (iv) *pesS*, comprising AT(DH) domains, Afu8g01640. Note: the nomenclature of *A. fumigatus* NRP synthetases has yet to be finalized. Cramer *et al.* (2006b) refer to the above NRP synthetases as NRPS1-14. Although we initially referred to *pesB* as *pesI*, we have subsequently sought to conform to the *Aspergillus* community recommendation on gene nomenclature and avoid numerical annotation (<http://www.aspergillus.org.uk/>).

NRPS gene	Domain architecture	CADRE ID	Reference
<i>pesI</i> (<i>pesB</i>)	ATECACACATECTCT	Afu1g10380	Neville <i>et al.</i> (2005); Nierman <i>et al.</i> (2005); Reeves <i>et al.</i> (2006)
<i>sidC</i>	ATCATCATCTCTC	Afu1g17200	Reiber <i>et al.</i> (2005); Nierman <i>et al.</i> (2005)
<i>sidE</i>	ATCATC	Afu3g03350	Reiber <i>et al.</i> (2005); Nierman <i>et al.</i> (2005)
<i>sidD</i>	ATCATC	Afu3g03420	Reiber <i>et al.</i> (2005); Nierman <i>et al.</i> (2005)
<i>pesF</i>	ATCATCT	Afu3g12920	Nierman <i>et al.</i> (2005)
<i>pesG</i>	ATC	Afu3g13730	Nierman <i>et al.</i> (2005)
<i>pesH</i>	ACATC	Afu3g15270	Nierman <i>et al.</i> (2005)
<i>pesI</i>	ATCATECATCETCATCATCATEC	Afu5g12730	Nierman <i>et al.</i> (2005); Cramer <i>et al.</i> (2006b)
<i>pesJ</i>	C*ATC	Afu6g09610	Nierman <i>et al.</i> (2005)
<i>pesK</i> (<i>gliP</i>)	ATCATCT	Afu6g09660	Nierman <i>et al.</i> (2005); Cramer <i>et al.</i> (2006a); Kupfahl <i>et al.</i> (2006)
<i>pesL</i>	ATC	Afu6g12050	Nierman <i>et al.</i> (2005)
<i>pesM</i>	ATCATCEATC(TE)	Afu6g12080	Nierman <i>et al.</i> (2005)
<i>pesN</i> (<i>ftmA</i>)	ACATC	Afu8g00170	Nierman <i>et al.</i> (2005); Maiya <i>et al.</i> (2006); Sheppard <i>et al.</i> (2005)
<i>pesO</i>	PKSNNCAT(DH)	Afu8g00540	Nierman <i>et al.</i> (2005); Sheppard <i>et al.</i> (2005)

SAM binding site (-LDLGCGTG-) in *LeaA*, whereby alanines replaced the underlined G residues, resulted in an identical phenotype to $\Delta leaA$ (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 $\Delta leaA$ 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 ($\Delta tdiB$) 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 NRP 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 -aminoadipic acid to

α -aminoadipic- 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 homology 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 Keszenman-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 an *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_{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_{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 -aminoadipate 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 N^5 -monooxygenase which catalyses the first committed step of hydroxamate-type siderophore biosynthesis, resulted in the complete inability to synthesize both triacetylfulvarinine 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 bioactives, 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; Kosalec *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.*, 2006c). 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 Δ *gliP* as opposed to WT culture filtrates, has been observed (Kupfahl *et al.*, 2006). Furthermore, gliotoxin may be responsible for inhibiting mast cell degranulation, since Δ *gliP* 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 Balibar & 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 (*pesF*; 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 LeaA regulation and so further investigation is required (Bok *et al.*, 2006b). Finally, the expression of *pes1*, *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 *pesI* 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. *PesI* 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 *pesI* deletion mutant in *A. fumigatus* ($\Delta pesI$) exhibits altered conidial morphology and increased sensitivity to oxidative stress due to H₂O₂ presence (Reeves *et al.*, 2006; Table 1). Work is currently under way to identify the nature of the *PesI*-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 μ M 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 (Vizcaino *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 miyabeanus*, *C. heterostrophus*, and *F. graminearum* resulted in reduced virulence and an increased sensitivity to H₂O₂ (Oide *et al.*, 2006). The authors further demonstrated that the application of exogenous Fe³⁺ 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.*

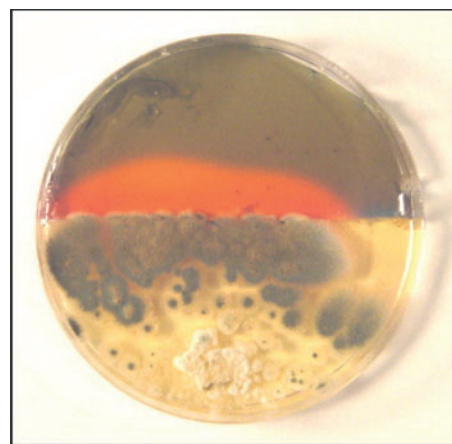


Fig. 3. Siderophores in action. *A. fumigatus* growing on the lower half of the culture plate, under iron-free conditions, releases siderophores which scavenge Fe³⁺ from the Chrome Azurol S reagent (Reiber *et al.*, 2005), in the upper section of the plate, resulting in the red colour.

fumigatus and contribute to the virulence of the fungus. Moreover, deletion of *sidC* in *A. nidulans* resulted in the inability to produce the siderophore ferricrocin and in upregulation of certain anti-oxidative enzymes (e.g. catalase and superoxide dismutase) (Eisendle *et al.*, 2003). More recently it has been shown that NPS2/SidC is responsible for the biosynthesis of ferricrocin in *F. graminearum* (Tobiasen *et al.*, 2007). Based on gene expression analysis, protein identification and siderophore detection, we have hypothesized that the NRP synthetases SidC, D and E potentially biosynthesize siderophores responsible for iron acquisition by *A. fumigatus* – although direct proof of this remains outstanding.

Thus, although advances in NRPS are emerging, further work is urgently required to fully elucidate the nature of this important process, in particular, adenylation domain specificity and NRP product identification and function.

Biopharmaceutical and diagnostic potential of NRPS

Exploitation of fungal metabolites as biotherapeutics forms the basis of a major component of the modern pharmaceutical industry. Indeed, the production of pharmacologically active biochemicals by micro-organisms is a well-established and valid approach for the treatment of human and animal diseases. Cost-effective and appropriate chemistries do not exist for the synthesis of complex organic molecules, many of which are synthesized non-ribosomally in fungi, such as penicillin and cephalosporin antibiotics and immunosuppressive therapies such as cyclosporin (Keller *et al.*, 2005). Cholesterol-lowering statins (polyketides) are produced by a related process of polyketide synthesis (Keller *et al.*, 2005). In fact, the synthesis of penicillins and cyclosporin intimately involves the use of micro-organisms (fungi) for the biosynthesis of critical reaction intermediates which can be subsequently modified to 'active' components (Grunewald & Marahiel, 2006). Gliotoxin may have potential as an immunosuppressive agent; however, toxicity problems could limit its application in clinical medicine (Gardiner *et al.*, 2005). Additionally, gliotoxin has also been shown to exhibit significant inhibitory activity against rat mammary carcinoma, *in vivo*, in the absence of measurable toxicity (Vigushin *et al.*, 2004). Thus, further work is essential so that our present limited understanding of fungal NRPS will not impede therapeutic exploitation of this process and resultant NRP peptides.

The *in vivo* detection of fungal-specific, 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 -aminoacidate 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 aryllic 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/PP_i 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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