A 4′-Phosphopantetheinyl Transferase Mediates Non-Ribosomal Peptide Synthetase Activation in *Aspergillus fumigatus*

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Aspergillus fumigatus *is a significant human pathogen. Non-ribosomal peptide (NRP) synthesis is thought to be responsible for a significant proportion of toxin and siderophore production in the organism. Furthermore, it has been shown that 4′-phosphopantetheinylation is required for the activation of key enzymes involved in non-ribosomal peptide synthesis in other species. Here we report the cloning, recombinant expression and functional characterisation of a 4′-phosphopantetheinyl transferase from A. fumigatus and the identification of an atypical NRP synthetase (Afpes1), spanning 14.3 kb. Phylogenetic analysis has shown that the NRP synthetase exhibits greatest identity to NRP synthetases from* Metarhizium anisolpiae *(PesA) and* Alternaria brassicae *(AbrePsy1). Northern hybridisation and RT-PCR analysis have confirmed that both genes are expressed in* A. fumigatus*. A 120 kDa fragment of the* A. fumigatus *NRP synthetase, containing a putative thiolation domain, was cloned and expressed in the baculovirus expression system. Detection of a 4′-phosphopantetheinylated peptide (SFSAMK) from this protein, by MALDI-TOF mass spectrometric analysis after coincubation of the 4′-phosphopantetheinyl transferase with the recombinant NRP synthetase fragment and acetyl CoA, confirms that it is competent to play a role in NRP synthetase activation in* A. fumigatus*. The 4′-phosphopantetheinyl transferase also activates, by 4′-phosphopantetheinylation, recombinant α-aminoadipate reductase (Lys2p) from* Candida albicans*, a key enzyme involved in lysine biosynthesis.*

Introduction

Aspergillus fumigatus is a significant human pathogen, particularly in immunocompromised individuals.[1] Three forms of aspergillosis are recognised clinically: saprophytic, allergic and invasive. Invasive aspergillosis (IA) is the most serious form of disease as it involves the invasion of viable tissue and may produce a mortality rate of approximately 90%.[2] IA has emerged as an important disease in recent decades due to the use of aggressive immunosuppressive therapy causing prolonged neutropenia in the treatment of cancer and leukaemia. Despite aggressive antifungal chemotherapy, death due to IA usually results 7–14 days after diagnosis.[3]

Amphotericin B, azoles, such as itraconazole, and an emerging array of inhibitors of cell-wall biosynthesis play a key role in combating fungal infection. However, such treatment is not always effective, although expensive, and there is a clear requirement for the identification of new fungal targets and antifungal therapies.[4] The near availability of the complete *A. fumigatus* genome sequence[5] (http://www.tigr.org) has both stimulated significant interest in, and facilitated the search for, such novel fungal targets.

Non-ribosomal peptide (NRP), polyketide (PK), fatty acid and lysine biosyntheses in many bacteria and fungi require post-translational phosphopantetheinylation of key apo-enzymes to facilitate metabolite production. 4′-phosphopantetheinyl transferases (4′-PPTase) catalyse the transfer of 4′-phosphopantetheine from coenzyme A (CoA) to a conserved serine residue within the cognate apo-enzyme (either apo-NRP or PK synthetase or α-aminoadipate reductase (AAR)). These 4′-phosphopantetheinyl prosthetic groups anchor activated intermediates and facilitate their movement between active sites within the multienzyme complex.[6] In lysine biosynthesis, the 4′-PPTases serve to activate AAR, which in turn can convert α-aminoadipate to α-aminoadipic semialdehyde, which is an essential step in lysine biosynthesis in *S. cerevisiae*.[7]

Sfp-type 4′-PPTases are those primarily responsible for NRPS and PKS phosphopantetheinylation, in addition to playing a key role in AAR activation. Recent work[8,9] suggested the existence of an Sfp-type 4′-PPTase gene in *A. fumigatus*, which may encode a protein of 39.6 kDa (359 amino acids) and contain identical conserved sequence motifs to the *npgA* gene in *A. nidulans*. Oberegger et al.[10] have subsequently shown that *npgA* gene expression is essential for the production of the siderophores ferricrocin and triacetylfusarinine, and may also be required for a number of other biosynthetic pathways, including lysine and fungal-pigment biosynthesis.

Although *A. fumigatus* is known to produce a range of low-molecular-weight metabolites (e.g., siderophores and toxins), many of which are likely to be formed by either polyketide or NRP synthesis, little effort has been directed towards the investigation of NRP synthesis. Haas[11] has indicated the presence of an NRPS gene in *A. fumigatus*, but revealed no further details regarding gene sequence or organisation. It is well known from studies in other prokaryotic and fungal species that NRP synthesis occurs in an ATP-dependent manner through the ac-

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tivity of multifunctional enzymes known as non-ribosomal peptide synthetases (NRPS) and that the organisation of modules within these NRPS directly influences the order of the amino acids in the resultant peptide products.[12] Modules within NRP synthetases are composed of domains including adenylation, thiolation (peptidyl carrier protein) and condensation domains.[12] (Other domain types can also be present and are reviewed elsewhere.[13]) Each domain has a specific function: the adenylation domain recognises and adenylates, by ATP hydrolysis, a substrate amino acid, which is in turn transferred to the 4′-phosphopantetheine prosthetic group of the thiolation domain by covalent attachment to an available SH group on the 4′-phosphopantetheine moiety. Ultimately, the covalent attachment of the amino acid to a proximal amino acid occurs through the condensation domain followed by either sequential transfer within the NRPS modular system or release through thioesterase activity to yield functional, non-ribosomally synthesised peptides. To date, no detailed analysis of either NRPS genes or gene expression from *A. fumigatus* has been forthcoming, despite the emergence of the organism as a significant human pathogen.

In an effort to investigate the role of the putative 4′-PPTase gene in, and elucidate the non-ribosomal biosynthetic capacity of, *A. fumigatus*, we have undertaken the isolation of a 4′-PPTase gene, carried out evaluation of 4′-PPTase gene expression and expressed the 4′-PPTase gene in a eukaryotic expression system. Secondly, we have identified and evaluated the expression of an NRPS open reading frame within the *A. fumigatus* genome. Finally, we present data that support the hypothesis that the *A. fumigatus* 4′-PPTase is responsible for the functional activation of an NRPS apo-enzyme through 4′-phosphopantetheinylation.

A partial orf corresponding to a 3.76 kb region of a putative *A. fumigatus* NRP synthetase (termed $Afpes1_{TCA}$) was also identified, sequenced to confirm identity (Genbank Acc No AY607101 and AY607102) and cloned. This 3.76 kb orf (termed $Afpes1_{TCA}$) was found to encode a putative thiolation, AMP-binding and condensation domain, similar to those of other non-ribosomal peptide synthetases. Further in silico analysis of the near-complete *A. fumigatus* genome suggested that $Afpes1_{TCA}$ was located within a larger NRP synthetase gene (14.3 kb; termed Afpes1). Afpes1 clusters to a clade that also contains known NRP synthetases possibly involved in destruxin and siderophore formation in other fungi,[14,15] and the protein is shown to be homologous to the NRP synthetase (Abrepsy1) of *Alternaria brassicae* (58% similarity and 38% identity), which is proposed to be involved in the pathogenicity of that organism. Similarity of Afpes1 to other known NRP synthetases ranges from 40–56% and identity ranges from 24–37% (data not shown). Conserved domain analysis of Afpes1 indicates the presence of four adenylation (A), three condensation (C) and two thiolation (T) domains within the gene '(Figure 1). Furthermore, the domain architecture of Afpes1 indicates that it is a type of nonlinear (type C) NRP synthetase, as defined by at least one unusual arrangement of core A, C or T domains.[13] The double condensation domains may allow the formation of cyclic peptides or, if present at the end of the sequence, may be involved in chain termination.

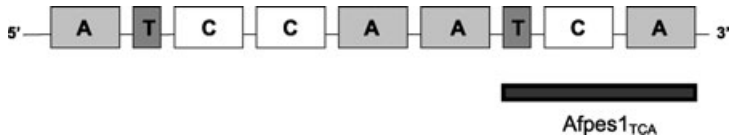

Figure 1. *Schematic diagram showing the domain architecture of* A. fumigatus *pes1 (Afpes1), a 14312 bp putative non-ribosomal peptide synthetase region. A: adenylation domains, C: condensation domains and T: thiolation domains. The adenylation domains occur between nucleotides (amino acids (aa)) 1–912 (1–304), 4326–5505 (1442–1835), 7437–8664 (2479–2888) and 10710–11919 (3570–3973). The condensation domains occur between nucleotides (aa) 1485–2376 (495–792), 2919–3783 (973–1261) and 9336–10161 (3112–3387). The thiolation domains encompass nucleotides (aa) 1251–1446 (417–482) and 9018–9213 (3006–3071). The 3760 bp region (termed $Afpes1_{TCA}$) was cloned, sequenced and expressed in the baculovirus expression system.*

Results

Phylogenetic analysis of 4′-PPTase and NRP synthetase genes

Data mining of the near-complete *A. fumigatus* genome with known sequences enabled the identification of two open reading frames (orf) potentially involved in non-ribosomal peptide synthesis.

The entire coding region (1.12 kb) of a 4′-phosphopantetheinyl transferase (4′-PPTase) was identified, cloned and sequenced (Genbank accession number AY607103). Phylogenetic analysis shows that the *A. fumigatus* 4′-PPTase is an orthologue of the *A. nidulans npgA/cfwA* gene (Genbank accession number AF198117). The *A. fumigatus* 4′-PPTase was also used to BLAST (basic local alignment search tool) the National Center for Biotechnological Information (NCBI) database (http://www.ncbi.nlm.nih.gov) in order to determine the similarity and identity of this sequence to other known 4′-PPTases. Highest similarity levels are seen to *A. nidulans* npgA gene which is known to be involved in penicillin biosynthesis (55% identity; 67% similarity) while the 4′-PPTase displays lower levels of identity (25–32%) and similarity (37–49%) to other known 4′-PPTases (data not shown).

The AMP-binding domains of NRP synthetases are highly conserved and may therefore represent the specificity code of the particular NRP synthetase, as it is this domain that recognises and binds the substrate amino acid.[16] In general, each adenylation domain contains an eight-residue, nonlinear sequence motif that is responsible for binding the substrate amino acid. The four adenylation domains of Afpes1 were aligned with the phenylalanine-activating adenylation domain of GrsA[17] to identify each eight-residue motif. The eight-residue motif for each domain was then used to interrogate a database of domains with assigned specificity, available at http://raynam.chm.jhu.edu/~nrps/ by using BLAST analysis. From this alignment, the putative amino acid substrate of each adenyla-

tion domain was determined. This analysis indicates that adenylation-domain motifs 1 (DAMVAYLS), 2 (DVKSVGAV), 3 (DAMFAGGI) and 4 (DVKSVGAV) encode C, T, V/I and T/D, respectively.

Expression analysis of 4′-PPTase and Afpes1

4′-PPTase and Afpes1 gene expression were both assessed by Northern analysis. In addition, Afpes1 expression was evaluated by RT-PCR. 4′-PPTase expression is evident at all time points during *A. fumigatus* growth from $t = 24$ to 96 h (Figure 2). The observed band is approximately 2 kb; this suggests that the 4′-PPTase transcript is significantly longer than the gene itself. Afpes1 expression was only detectable at 24, 48 and 72 h by Northern analysis (mRNA size: ca. 15 kb). Afpes1 expression at 48 (very weak) and 72 h was evident on RT-PCR analysis, and, despite repeated attempts, no Afpes1 expression could be detected at 96 h; this suggests that Afpes1 gene expression is downregulated under the *A. fumigatus* culture conditions employed for these analyses. The lack of detection of Afpes1 expression by RT-PCR at 24 h is suggestive of a gradual up-regulation in Afpes1 gene expression. Alternatively, this observation could be due to the differential sensitivity of RT-PCR and Northern analysis for the detection Afpes1 expression at low levels of transcript. rRNA loading was used as a control for the amounts of RNA used for Northern analysis and cDNA synthesis. In addition, 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 a *gstA* gene[18] (Genbank accession number AF425746) from which introns have been excised (data not shown).

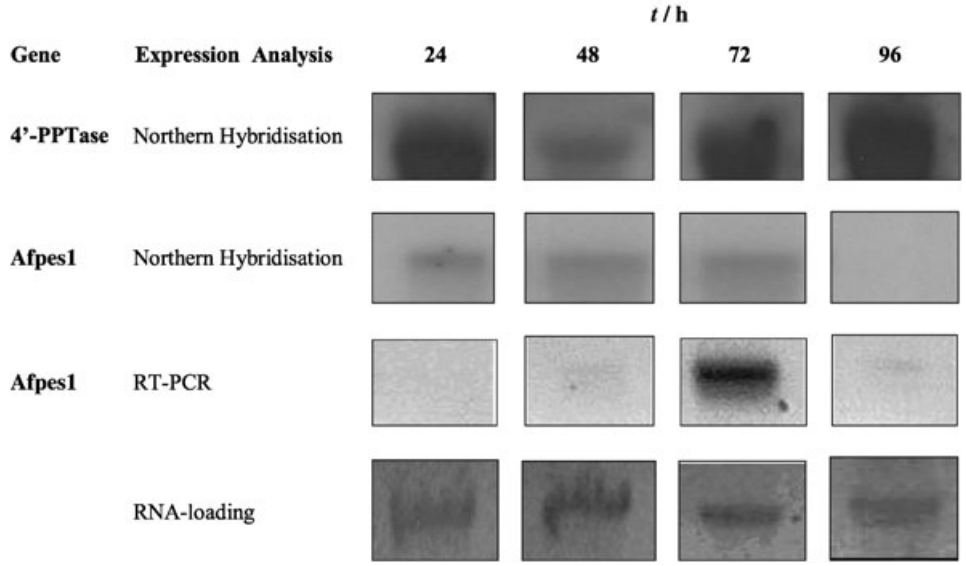

Figure 2. *Gene-expression analysis of 4′-PPTase and Afpes1 in* A. fumigatus. *Northern hybridisation (60 μg total RNA per well) was used to assess 4′-PPTase and Afpes1 expression in fungal cultures ranging from 24–96 h post-inoculation. In addition, Afpes1 expression was evaluated by RT-PCR (1 μg total RNA per cDNA synthesis reaction) over an identical time period. Culture stationary phase was reached at 72 h. The 4′-PPTase is constitutively expressed at all time points evaluated. AfPes1 expression, as judged by Northern hybridisation is observed at 24, 48 and 72 h only. RT-PCR analysis of Afpes1 expression also detects transcripts at 48 and 72 h. RNA from each isolation time point was applied equally for Northern hybridisation by using ribosomal RNA as loading control.*

Baculovirus expression of recombinant 4′-PPTase and partial NRPS (Afpes1$_{TCA}$)

Both 4′-PPTase- and Afpes1$_{TCA}$-encoding regions were independently transferred into a baculovirus transfer vector (pBlueBac4.5) in order to generate recombinant baculoviruses, each encoding 4′-PPTase or Afpes1$_{TCA}$, by cotransfection with linearised baculovirus. *A. fumigatus* 4′-PPTase was purified from recombinant baculovirus-infected insect cells by single-step Ni-NTA agarose affinity chromatography to yield a soluble protein of 42 kDa as judged by SDS-PAGE analysis (Figure 3). The yield of released and soluble 4′-PPTase was calculated to be 0.4 mg per 10^8 cells, although it was clear from subsequent analysis that significant enzyme remained within infected *Spodoptera frugiperda 9* (Sf_9) insect cells (data not shown). MALDI-TOF mass spectrometry was used to verify the identity of the recombinant 4′-PPTase (actual/theoretical peptides detected: 8:106 (7.5%), which represents 16% sequence coverage). *A. fumigatus* pes1$_{TCA}$ was then purified from recombinant baculovirus-infected insect cells by differential extraction and extensive washing to yield a protein of 120 kDa as judged by SDS-PAGE analysis (Figure 3). Lower M_r protein bands present in purified Afpes1$_{TCA}$ at approximately 38 and 62 kDa, (Figure 3, lane 3) were reactive with rabbit antisera previously raised against an *E. coli*-expressed condensation domain of Afpes1$_{TCA}$ and represent proteolytic fragments of the parental protein (data not shown). The yield of recombinant Afpes1$_{TCA}$ was calculated to be 2–3 mg per 10^8 cells. Although a His_6 affinity tag had been engineered into the DNA construct, it was not possible to detect Afpes1$_{TCA}$ on Western blots with murine monoclonal anti-His_6 antibody (data not shown). Consequently, expression of recombinant Afpes1$_{TCA}$ was confirmed by both MALDI-TOF mass spectrometry (actual/theoretical peptides detected: 23:287 (8%) representing 25% sequence coverage) and rabbit antisera previously raised against an *E. coli*-expressed condensation domain also encoded by Afpes1$_{TCA}$ expressed in insect cells (data not shown).

Functional analysis of 4′-phosphopantetheinyl transferase

Functional 4′-PPTase activity was initially investigated by using an α-AAR-activation assay previously described for the assessment of *C. albicans* Lys5p, which ex-

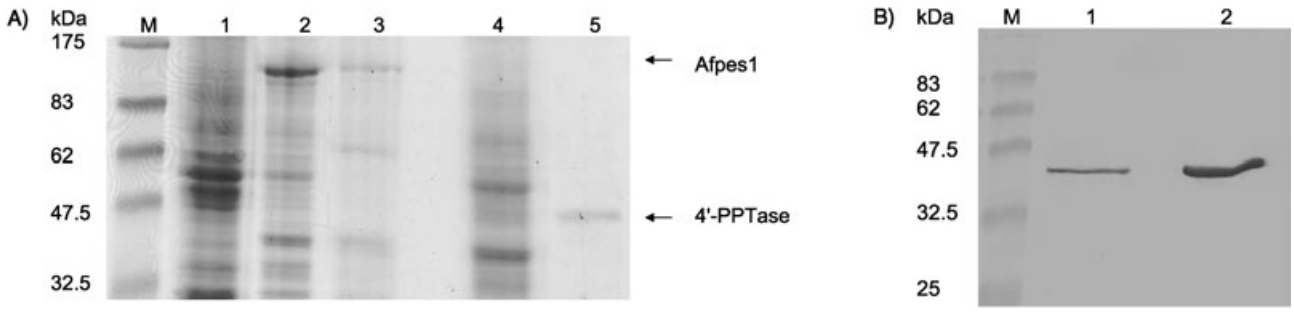

Figure 3. *A) SDS-PAGE analysis of recombinant 4′-PPTase and Afpes1$_{TCA}$ from* A. fumigatus. *Lane M: molecular-weight markers, lane 1: uninfected insect cell lysate (2.5×10^5 cell equivalents), lane 2: Afpes1$_{TCA}$ recombinant baculovirus-infected Sf_9 cells at an MOI 15, lane 3: purified Afpes1$_{TCA}$, lane 4: 4′-PPTase recombinant baculovirus-infected Sf_9 cells at an MOI 15 and lane 5: affinity-purified 4′-PPTase. In lane 3, the protein bands evident at 38 and 62 kDa were reactive with antisera raised against the condensation domain in Afpes1$_{TCA}$ (data not shown). B) Western blot analysis of recombinant 4′-PPTase probed with anti-$(His)_6$ monoclonal antibody. Lane M: molecular-weight markers, lane 1: 4′-PPTase recombinant baculovirus-infected Sf_9 cells at an MOI 15 and lane 2: affinity-purified 4′-PPTase.*

hibits 4′-PPTase activity.[7] Figure 4 clearly shows that AAR activity is detectable in the presence of recombinant Lys2p and *C. albicans* CLD2 (Lys2p mutant) extract, which contains a functional 4′-PPTase. When AAR activity is measured after prior incubation of the recombinant *C. albicans* Lys2p with the putative *A. fumigatus* 4′-PPTase, a significant 4.5-fold enhancement of AAR activity is evident (21.3% of positive control CLD2 activity). This observation confirms that the recombinant 4′-PPTase can activate *C. albicans* Lys2p in the presence of CoA and suggests a possible role for 4′-PPTase in lysine biosynthesis in *A. fumigatus*. Appropriate compensation for background absorbance (due to CLD2, 4′-PPTase and Lys2p) has been made in each case, as it was observed that protein addition contributed to background signals.

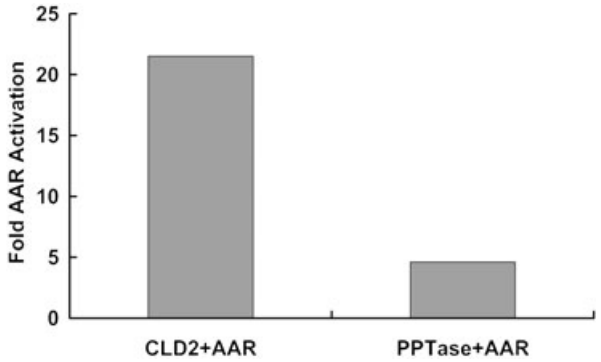

Figure 4. *α-aminoadipate reductase (AAR) activation by recombinant 4′-PPTase from* A. fumigatus. *Activation of* C. albicans *Lys2p (apo-AAR), by a control extract (CLD2) containing a* C. albicans *4′-PPTase, resulted in a 21-fold increase in observed AAR activity. Enhancement of AAR activity (4.5-fold) is evident after prior incubation of the* C. albicans *Lys2p with the* A. fumigatus *4′-PPTase and represents 21.3% of positive control CLD2-induced activity. All reactions were carried out in duplicate and in the presence of 200 μM acetyl CoA.*

NRPS 4′-phosphopantetheinylation by 4′-PPTase

The capacity of recombinant 4′-PPTase to activate non-ribosomal peptide synthetases was investigated by coincubation of 4′-PPTase, CoA and recombinant Afpes1$_{TCA}$, which encodes a putative thiolation domain (Afpes1 aa residues: 3006–3071), followed by MALDI-TOF mass spectrometric analysis of the reaction mixture to detect evidence of 4′-phosphopantetheinylation. Codigestion of the reaction mixture with both trypsin and V8 protease was used as trypsin-only digestion generated peptides from the Afpes1$_{TCA}$ thiolation domain that were too large for reliable analysis (data not shown). In addition, solution-phase enzymatic digestion was employed to optimise peptide recovery prior to MALDI-TOF mass spectrometry. It can be seen from Figure 5 that a peptide with a monoisotopic *m/z* value of 1009.1, representing 4′-phosphopantetheinylated SFSAMK (theoretical $m/z = 1009.3$), is detected by this analysis, thereby confirming that recombinant *A. fumigatus* 4′-PPTase both recognises and 4′-phosphopantetheinylates the thiolation domain present in Afpes1. In the absence of either CoA or recombinant Afpes1$_{TCA}$, no modified peptide is detected at the predicted *m/z* ratio.

Discussion

Here we report the cloning and functional expression of the full-length open reading frame encoding an *Aspergillus fumigatus* 4′-phosphopantetheinyl transferase in the baculovirus-expression system. In addition, an NRP synthetase in *A. fumigatus* (Afpes1), which exhibits unusual domain architecture showing characteristics of a nonlinear NRP synthetase, has been identified from *A. fumigatus* contig 4944. A region of this NRP synthetase (3.7 kb; Afpes1$_{TCA}$) was expressed and found to undergo phosphopantetheinylation to the holo-enzyme form following activation by 4′-PPTase in the presence of CoA; this supports the hypothesis that the 4′-PPTase functions to activate an NRP synthetase by 4′-phosphopantetheinylation.

The *A. fumigatus* 4′-PPTase that has been investigated is identical to that previously noted by Mootz et al. and Keszenman-Pereyra et al.[8,9] Although both previous reports speculated on the function of the 4′-PPTase gene, by comparison to the *A. nidulans* orthologue (*npgA*), definite confirmation that the *A. fumigatus* 4′-PPTase was expressed in vivo and functioned as hypothesised, in vitro, has not been available until now. From phylogenetic analysis, the *A. fumigatus* 4′-PPTase was confirmed to be an orthologue of the *A. nidulans* 4′-

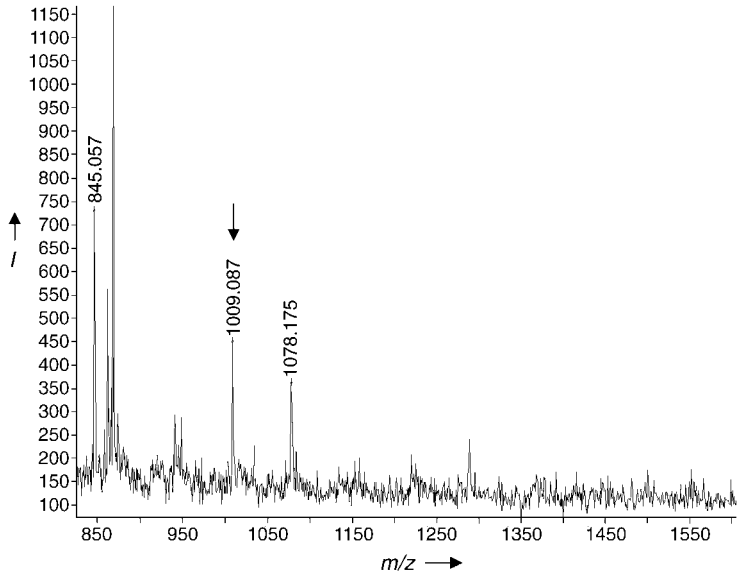

Figure 5. *MALDI TOF MS analysis of in vitro phosphopantetheinylation by* A. fumigatus *4′-PPTase. Mass spectrum of Afpes1$_{TCA}$-derived peptide mixture (following codigestion with trypsin and V8 protease). A specific peptide with a monoisotopic* m/z *value of 1009.1 is evident and represents phosphopantetheinylated-SFSAMK. In the absence of either 4′-PPTase or CoA , during the enzymatic reaction, no modified peptide is subsequently detected at the expected* m/z *ratio.*

PPTase; this suggested common ancestry, and it also showed similarity to a *Bacillus subtilis* 4′-PPTase. Moreover, *A. fumigatus* 4′-PPTase exhibits 25% identity (37% similarity) to the human 4′-PPTase (Genbank accession number BC015470) and shares some significant regions of homology (WxLKExxxK) as previously noted.[9]

In most organisms there is an individual 4′-PPTase for each function, however the possible presence of only one 4′-PPTase in *A. fumigatus* is not unique, since recent work has identified and characterised a human 4′-PPTase that appears to exhibit a broad specificity for all 4′-phosphopantetheinylation reactions including human (apo-ACP domain for cytosolic fatty acid synthetase (FAS), mitochondrial ACP, α-aminoadipate semialdehyde dehydrogenase activation (lysine catabolism)) and non-human (*B. subtilis* ACP-A (involved in fatty acid synthesis) and *B. brevis* tyrocidine synthetase) apo-enzymes[19,20] A new type of 4′-PPTase was also identified in *Pseudomonas aeruginosa* that showed association with fatty acid synthesis and siderophore metabolism.[19]

Northern analysis of *A. fumigatus* 4′-PPTase expression indicates that the gene is constitutively expressed; this strongly suggests that protein 4′-phosphopantetheinylation is required for both primary- and secondary-metabolite production in *A. fumigatus*. Indeed, the putative role of 4′-PPTase in lysine biosynthesis (see below), and its role in NRP-synthetase activation is compatible with this observation.

To date, characterisation of yeast and fungal 4′-PPTases has primarily focussed on genetic complementation studies in either *S. cerevisiae*[8] or *A. nidulans*[9] as opposed to direct biochemical studies. Guo and Bhattacharjee have developed an AAR-activation assay to assess 4′-PPTase activity in vitro.[7] Thus, the potential role of *A. fumigatus* 4′-PPTase in lysine biosynthesis was investigated. The in vitro activation of *C. albicans* Lys2p (Apo-AAR) by *A. fumigatus* 4′-PPTase suggests that the enzyme may play a role in lysine biosynthesis. Although equivalent AAR activity (4.5-fold activation) in comparison to the positive control (22-fold activation) was not evident, this could be accounted for by differences between core 1, 2 and 3 conserved motifs[7] in *C. albicans* Lys5p (4′-PPTase) and those present in the *A. fumigatus* 4′-PPTase. Moreover, phylogenetic analysis confirms that the 4′-PPTase encoded by *C. albicans* clusters to a separate clade to that of the *A. fumigatus* 4′-PPTase.

A number of groups have reported the application of protein mass spectrometry for the determination of apo-enzyme 4′-phosphopantetheinylation.[20,22] In these reports, the substrate proteins exhibited molecular masses in the order of 11–38 kDa; however, in the case of baculovirus-expressed Afpes1$_{TCA}$ (120 kDa), there was concern that protein mass spectrometry would not exhibit the required resolution to confirm 4′-phosphopantetheinylation. Consequently, the post-phosphopantetheinylation reaction mixture was enzymatically digested (with trypsin and V8 protease) prior to peptide mass fingerprinting. As the expected peptide containing the conserved serine (S) within the thiolation domain (SFSAMK) had $m/z = 670.3$ Da, post-translational modification by the 4′-PPTase was expected to add an additional 339 Da and result in a peptide of $m/z = 1009.3$ Da. Our data confirm the presence of the 1009.3 Da peptide following coincubation of CoA, 4′-PPTase and Afpes1$_{TCA}$ under optimal conditions. The confirmation that 4′-PPTase can 4′-phosphopantetheinylate a thiolation domain of an NRP synthetase is highly significant as it strongly indicates that at least one NRP synthetase encoded by the *A. fumigatus* genome can be activated, and thereby participate in non-ribosomal peptide synthesis in the organism. Until now, no mechanistic evidence of NRP synthesis in *A. fumigatus* has been forthcoming, despite the existence of multiple siderophores and other low-molecular-weight metabolites. Although it is unlikely that Afpes1 encodes a siderophore (see below), we hypothesise that 4′-PPTase expression and functionality will also be essential for siderophore production in *A. fumigatus* and so may represent a key drug target in controlling siderophore production with a view to minimising organism virulence.[10,11] Moreover, the identification of only one 4′-PPTase in *A. fumigatus*, to date, that may be involved in multiple 4′-phosphopantetheinylation reactions, makes selective inhibition in the absence of concomitant inhibition of human 4′-PPTase even more attractive with a view to anti-*A. fumigatus* drug therapy in humans. However, the identification of similar enzymes in *A. fumigatus* cannot be completely ruled out at present.

The Afpes1 open reading frame, which extends over 14300 bp, encodes the first NRP synthetase (predicted protein relative molecular mass: 460 kDa) to be definitively identified in *A. fumigatus*. Furthermore, Northern analysis confirms that the Afpes1 gene is differentially expressed (transcript size: ca.

15 kb) at 24, 48 and 72 h post-inoculation when *A. fumigatus* is grown in liquid culture (5%, *v/v* FCS in minimal essential medium (MEM)). Upon phylogenetic analysis, Afpes1 clusters with the NRP synthetase (AbrePsy1) from *Alternaria brassicae*,[15] thus suggesting orthology. Also grouped in the same clade is the *pesA* gene of *Metarhizium anisopliae*.[14] Unfortunately the functions of the NRP synthetase genes of both *A. brassicae* and *M. anisopliae* have yet to be identified, but are proposed to be involved in destruxin biosynthesis, which is thought to play a role in the progression of plant disease mediated by both species.[14,15] Although it has been proposed that the NRP synthetase gene of *A. brassicae* may play a role in siderophore biosynthesis,[15] particularly since the first adenylation domain of AbrePsy1 is predicted to encode 5-hydroxyornithine, the clustering of Afpes1 away from a putative *Aspergillus* NRP synthetase (*A. oryzae* sid2; Genbank accession number AB087617) suggests that this may not, in fact, be the case. A further difference between both *A. fumigatus* and *Alternaria* genes is that while the Afpes1 gene represents a complete exonic region, the AbrePsy1 gene contains seven introns. Thus, while it is possible that Afpes1 may have a similar function to that of AbrePsy1, orthology does not guarantee similarity of function, which can change over time.[23]

One striking observation with respect to the structure of Afpes1 is the arrangement of the individual domains within the protein: ATCCAATCA. Such NRPSs are referred to as non-linear (type C), as recently described.[13] The presence of an adenylation domain at the C terminus of an NRP synthetase is somewhat unusual and suggests that Afpes1 may be involved in a multisubunit NRPS complex with another synthetase. Interestingly, the production of ergot alkaloids containing D-lysergic acid in the fungus *Claviceps purpurea* occurs through a cluster of synthetases, namely LPS1 (370 kDa) and LPS2 (140 kDa). These synthetases are encoded by two genes, *cpps2* and *cpps1*, present in a gene cluster. Mechanistically, it has been shown that D-lysergic acid is initially bound to LPS2 through a thioester bond and that this thioester-bound D-lysergic acid is then transferred to LPS1. Following this, three condensation reactions into the D-lysergyl mono-, di- and, finally, tripeptide thioester occur to result in the formation of D-lysergyl tripeptide lactam. This system of ergot-peptide formation was the first identified fungal-NRP-synthesis system consisting of different NRP synthesis subunits.[24] Other examples of type C NRP synthetases include PesA of *Metarhizium anisolpiae* and AbrePsy1 of *Alternaria brassicae*.[14,15]

The functional assessment of 4′-PPTase activity and the identification of a novel NRP synthetase provide new insights into non-ribosomal peptide synthesis in *A. fumigatus.* In addition, we postulate that the 4′-PPTase may also play a role in lysine production within *A. fumigatus* by activation of key biosynthetic enzymes. Although no definitive role for the NRP synthetase, Afpes1, has been identified, it may be responsible for toxin biosynthesis, and work is ongoing in our laboratory to identify gene function within the ascomycete. Modulation of 4′-PPTase expression or functionality in *A. fumigatus* may represent a novel antifungal target.

Experimental Section

Microorganisms and culture media: *Aspergillus fumigatus* strain ATCC 26933 (obtained from the American Type Culture Collection, Manasas, VA (USA)) was used for this work and was grown at 37 °C in MEM supplemented with foetal calf serum (FCS; 5%, *v/v*) for 48 h in order to facilitate DNA isolation. For gene-expression studies, in which culture was extended to 96 h, it was observed that stationary phase was reached after 72 h post-inoculation. *Escherichia coli* strains Top10′, XL1Blue (Invitrogen, Dublin (Ireland)) and BL21 (Novagen, Nottingham (UK)) were grown on LB agar (Sigma–Aldrich, Dorset (UK)) and LB agar containing ampicillin (100 $\mu g\,mL^{-1}$—chloramphenicol (34 $\mu g\,mL^{-1}$) was also included for BL21 culture), where appropriate, at 37 °C overnight.

Bioinformatic analyses, genomic DNA isolation and PCR cloning: Preliminary *A. fumigatus* sequence data were obtained from The Institute for Genomic Research website at http://www.tigr.org. By using the sequence search facility at http://www.tigr.org, the near-complete *A. fumigatus* genome was interrogated by using enniatin synthetase (Genbank accession number Z18755), with a BLAST program. Genomic DNA was isolated by crushing *A. fumigatus* in liquid N_2 followed by phenol/chloroform extraction, washing with ethanol (70%, *v/v*) and final DNA resuspension in Tris-HCl (10 mM), EDTA (1 mM; pH 7.4, 300 μL). Genomic DNA (5 μL) was used in all PCR reactions containing Tris-HCl (10 mM; pH 9.0), KCl (50 mM), Triton X-100 (0.1%, *v/v*), $MgCl_2$ (1.5 mM), dNTP (200 μM of each), forward and reverse primer (1.0 μM of each; Afpes1-F: 5′-GAGAGGTACCATGTCGGAAGCAACACG-3′ and Afpes1-R: 5′-GAGAGGTACCTTACCAGTCAGCCTC-3′; or 4′-PPTase-F: 5′-GAGAGGATCCATGGGCTCTGCACAAAACG-3′ and 4′-PPTase-R: 5′-GAGAAAGCTTGGGCTGTTTTTTTATACAC-3′) and Taq polymerase (1 unit) (Promega, Southampton (UK)) in a total volume of 49.5 μL. AccuTaq LA DNA polymerase (Sigma–Aldrich) was used to amplify a partial region from Afpes1 only. After incubation at 95 °C for 5 min, all 4′-PPTase and Afpes1 PCR reactions were continued with 35 cycles consisting of 60 s denaturation (90 s for Afpes1) at 95 °C, primer annealing at 55 °C for 60 s, extension at 72 °C for 240 s and finally extension for 360 s at 72 °C. PCR-amplified DNA was analysed by agarose gel electrophoresis whereby product (10 μL) was electrophoresed on agarose (1%, *w/v*) containing ethidium bromide (0.5 $\mu g\,mL^{-1}$) for 30 min at 100 V. Visualisation of the 4′-PPTase- and Afpes1-derived amplicons was performed by using an "Eagle-Eye II" digital still video system. Once PCR had been successfully carried out, both amplicons were individually cloned into the TOPO cloning vector and subsequently transferred into separate pBlueBac4.5 vectors (Clontech, Palo Alto, CA) by using standard molecular biology techniques, for expression in Sf_9 cells.

Northern hybridisation and RT-PCR analysis of 4′-PPTase and Afpes1 gene expression *A. fumigatus* cultures were harvested at defined time points (24, 48, 72 and 96 h), mycelia collected, washed with ice-cold phosphate-buffered saline and total-RNA extracted by using TRI reagent (Sigma–Aldrich). Northern analyses (60 μg total RNA per well) were carried out by using ^{32}P-labelled regions of Afpes1 (NT: 9716–10535) and 4′-PPTase (NT: 1–1123) to detect relevant transcripts according to standard protocols.[25] RT-PCR was performed by following cDNA synthesis with Superscript (Promega; 1 μg RNA per reaction) and relevant PCR primers (see above).

Construction of recombinant baculovirus encoding 4′-PPTase and Afpes1$_{TCA}$: Sf_9 insect cells were co-transfected with recombinant transfer vectors and *Bsu*36 I-linearised BacPAK-6 baculovirus by using Bacfectin liposomal preparation (Clontech, Palo Alto,

CA).[24] Well plates containing Sf_9 cells were infected with the co-transfection mixtures, and the TC-100 supernatant was harvested after 5 days' incubation at 27 °C. Pure clones of either 4′-PPTase- or $Afpes1_{TCA}$-encoding recombinant baculoviruses were identified by plaque assay.[26]

Antigen expression and purification: Recombinant baculoviruses encoding the 4′-PPTase and $Afpes1_{TCA}$ genes were individually used to infect Sf_9 cells in monolayer culture at a multiplicity of infection of 15. Infected cells (5×10^6) were harvested 4 days post-infection. In each case, SDS-PAGE, Western Blotting and MALDI-TOF mass spectrometry were used to confirm the presence of 4′-PPTase and $Afpes1_{TCA}$. It was observed that while the 4′-PPTase was soluble and could be isolated from cell pellets by washing with PBS, followed by Ni chelate affinity chromatography by elution with imidazole (200 mM), a more extensive extraction procedure was required for $Afpes1_{TCA}$ purification. For $Afpes1_{TCA}$ isolation, cells were lysed in the presence of protease inhibitors (phenylmethylsulfonyl fluoride (0.1 mM), pepstatin (2 $\mu g\,mL^{-1}$) and leupeptin (2 $\mu g\,mL^{-1}$)) by the addition of phosphate-buffered saline/sodium deoxycholate (0.5%, *w/v*) and subjected to DNAse (Sigma, Poole (UK)) treatment (final concentration: 10 $\mu g\,mL^{-1}$), and the insoluble pellet was washed extensively to remove contaminating proteins.[26] Insoluble $Afpes1_{TCA}$ was resuspended in guanidinium thiocyanate (0.5–1.0 mL, 6 M), containing dithiothreitol (5 mM), at a concentration of 3 $mg\,mL^{-1}$ and sequentially dialysed against sodium carbonate (50 mM; pH 9.4) containing Urea (3 M) and sodium carbonate (50 mM; pH 9.4) to produce soluble protein.

Enzyme assays: AAR activation by recombinant 4′-PPTase was determined as described by Guo et al. and Guo and Bhattacharjee.[5,25] Briefly, restoration of AAR activity by 4′-phosphopantetheinylation in apo-Lys2p from *C. albicans* is indicative of 4′-PPTase activity. Therefore, recombinant *A. fumigatus* 4′-PPTase activity was assessed by incubating the enzyme (50 μg per reaction) with recombinant Lys2p (100 μg; final volume 500 μL) followed by detection of AAR activity by determination of ΔA460nm due to the conversion of DL-α-aminoadipate to α-aminoadipate-δ-semialdehyde. *C. albicans* CLD2 lysate (a Lys2p mutant expressing *C. albicans* 4′-PPTase) containing 1 mg total protein was used as a positive control for the reaction. NRPS 4′-phosphopantetheinylation was determined by incubating $Afpes1_{TCA}$ (100 μg) and 4′-PPTase (10 μg) in the presence of CoA (100 μM; reaction volume 100 μL)[26,27] followed by urea denaturation (5 M final) and in-solution double digestion with trypsin and *S. aureus* V8 protease prior to MALDI-TOF peptide mass fingerprinting to detect phosphopantetheinylated peptide species.

MALDI-TOF mass spectrometry: Mass spectrometry was carried out by using an Ettan MALDI-TOF mass spectrometer (Amersham Biosciences (Europe), Freiburg (Germany)). Protein samples for peptide mass determination were either i) separated by SDS-PAGE, digested with trypsin or ii) obtained following in-solution enzymatic digestion and deposited (1 μL) with α-cyano-4-hydroxycinnaminic acid (1 μL; acetonitrile in aqueous trifluoroacetic acid 5 mg/200 μL 50%, *v/v*) onto mass spectrometry slides and allowed to dry prior to delayed extraction and reflectron TOF analysis at 20 kV.

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