A nonribosomal peptide synthetase (Pes1) confers protection against oxidative stress in *Aspergillus fumigatus*

Emer P. Reeves¹, Kathrin Reiber¹, Claire Neville¹, Olaf Scheibner², Kevin Kavanagh¹ and Sean Doyle¹

1 National Institute for Cellular Biotechnology, Department of Biology, National University of Ireland Maynooth, Co. Kildare, Ireland
2 Leibniz-Institute for Natural Product Research and Infection Biology, Hans-Knoll-Institute, Jena, Germany

*Aspergillus fumigatus* is a filamentous fungus that is responsible for approximately 4% of all tertiary hospital deaths in Europe [1]. *A. fumigatus* has emerged as a significant human pulmonary pathogen capable of inducing disease in patients undergoing immunosuppressive therapy or those with pre-existing pulmonary malfunction [2,3]. Invasive aspergillosis is the most serious form of the disease, involving the invasion of viable tissue and resulting in a mortality rate of 80–95% [4,5]. Circumvention of the host immune response facilitates *in vivo* fungal dissemination, and recent work has demonstrated that 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 [6]. In addition, the release of hydroxamate-type siderophores, to facilitate iron acquisition by the organism, is also essential for fungal virulence [7].

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 organism growth and pathogenicity. Indeed, interest in these compounds is considerable, as many natural products are of medical or economic importance [8,9]. One mechanism that has been shown to be responsible for the biosynthesis of bioactive metabolites is nonribosomal peptide synthesis [10]. Most bioactive metabolites exhibit a peptidyl and/or polyketide composition, along with elaborate architecture including cyclic or branched-cyclic structures and modified proteogenic or nonproteogenic amino acids. Nonribosomal peptide synthetases (NRPS synthetases) generally possess a colinear modular structure, with each module responsible for the activation, thiolation and condensation of one specific amino acid substrate [11]. In linear NRPS synthetases, the three core domains are organized in the order condensation, adenylation and thiolation (CAT)_n to form an elongation module that adds one amino acid to the growing chain. Variations on this structure include the iterative NRPS synthetases characteristic of siderophore synthetases [10] or nonlinear NRPS synthetases that deviate in their domain organization from the standard (CAT)_n architecture. NRPS synthetases that fall into this group include a peptide synthetase involved in biosynthesis of the siderophore yersiniabactin from Yersinia species [12] and the NRPS synthetase Pes1 of A. fumigatus [13].

It is now clear that 14 NRPS genes are present in the genomes of A. fumigatus and Aspergillus nidulans, respectively [14,15]. Given that few functional NRPS synthetase genes or proteins have been identified to date in fungi, the possibility that NRPS synthetase pseudogenes may undergo transcription due to the presence of functional promoters [16,17], and the difficulties associated with predicting metabolites synthesized by cognate NRPS synthetases, both gene and protein expression analysis of pes1 was undertaken in A. fumigatus, coupled with the disruption of pes1 to facilitate the assessment of the role played by pes1 in mediating the virulence of A. fumigatus.

Results

Gene expression analysis

Growth curves for the three Aspergillus isolates, ATCC 26933, 16424 and 13073, showed that the exponential growth phase began at 12 h and extended until 48 h. Idiophase, the period when logarithmic growth had ceased, was reached at approximately 72 h, with similar biomass obtained for all three isolates (data not shown).

RT-PCR analysis was performed to investigate the relationship between fungal growth and pes1 expression. Owing to the large size of the pes1 transcript, different regions spanning the gene were selected for RT-PCR analysis (Fig. 1A). Primers employed were specific for adenylation domain 2 or 4 (pes1A2, pes1A4), the epimerase-condensation domains (pes1E1-C1) and, for A. fumigatus AF293, epimerase domain 2 (pes1E2). The presence of genomic DNA was excluded by analysis of the size difference between the genomic (617 bp) and cDNA (348 bp) amplicons of calm (5) (Fig. 1B).

A time-dependent difference in the expression level of pes1 for the four Aspergillus isolates was evident. Amplicon presence corresponding to pes1A2, pes1A4 and pes1E1-C1 confirmed that pes1 of A. fumigatus ATCC 26933 was expressed at all time points (Fig. 1C–E). At the time corresponding to idiophase (72 h), the highest expression was apparent. Semiquantitative analysis of pes1 expression was undertaken (amplicon pes1A2; Fig. 1H) and was confirmed to be significantly increased by 38% (P < 0.005) over the culture period (24–72 h). Analysis of the pes1 expression of A. fumigatus ATCC 13073 (Fig. 1C–E) showed very low levels of expression at 24 h. Pes1 expression by isolate ATCC 13073 demonstrated an increase in transcript level from 24 h to 48 h and a further significant (2.5-fold; pes1A2) increase after 72 h (P < 0.04) (Fig. 1H). In contrast, upregulation of the pes1 gene expression was not observed for Aspergillus isolate ATCC 16424 (Fig. 1C–E). Expression was evident at all time points during growth from 24 to 72 h; however, basal levels of expression were maintained as the culture ceased logarithmic growth, with relative expression for pes1A2 calculated as 61%, 57% and 66% for 24, 48 and 72 h, respectively (Fig. 1H).

Simultaneous expression analysis of A. fumigatus sidD was undertaken using precisely the same culturing conditions as used for pes1 analysis, for comparative expression analysis. The results are illustrated in Fig. 1F. Expression of sidD is evident at all time points (24, 48 and 72 h) and for three Aspergillus isolates investigated and appears to be reduced under prolonged culturing, with at least a five-fold decrease at the 72 h time point for isolates ATCC 26933 and 13073, in contrast to the observed pes1 expression profile in both isolates.

An amplicon corresponding to pes1E2 confirmed the presence and expression of pes1 in the transformation recipient pyrG auxotrophic strain AF293.1 (Fig. 1G). In accordance with results obtained for A. fumigatus ATCC 26933 and 13073, pes1 was expressed in A. fumigatus 293.1 at all time points, with the highest expression apparent at 72 h, thereby validating the use
of this strain in subsequent gene-disruption experiments.

In order to find whether pes1 was expressed during fungal infection in G. mellonella, A. fumigatus ATCC 26933 conidia were injected into larvae and total RNA was isolated between \(T = 24\) and 96 h. It is clear from Fig. 2 that pes1 was expressed during fungal growth in G. mellonella, as the pes1A2 cDNA was detected at 72 and 96 h postinoculation (confirmed by DNA sequence analysis; data not shown). Moreover, pes1 expression appeared to increase relative to the actin cDNA control, which indicates elevated pes1 expression as opposed to an increase in total fungal RNA concomitant with increased fungal mass. No pes1A2 cDNA was detected in uninfected larval controls.

Purification and immunological detection of Pes1

A recombinant protein corresponding to the second epimerase domain of pes1 (pes1E2) was expressed (Fig. 3A, lane 1) (34 kDa) and verified by MALDI-TOF MS; 54.5% of peptides (28% sequence coverage) obtained corresponded to the theoretical amino acid sequence of Pes1E2 (data not shown). Polyclonal antiserum was generated, and western blot characterization of the anti-Pes1E2 reactivity was evident (Fig. 3A, lane 2). Immunoreactivity was also evident against baculovirus-expressed recombinant Pes1TEA [13] (Fig. 3A, lanes 3 and 4). Immunoaffinity-purified Pes1E2 antibodies (IgG-Pes1) were used in western blot analysis to detect recombinant Pes1TEA, resulting in an immuno-reactive band of the correct size (120 kDa), thereby...
confirming that immunoaffinity-purified antibodies to Pes1E2 successfully recognized this domain within the larger Pes1TEA protein.

Purification of native Pes1 from mycelial lysates (250 mg protein) of *A. fumigatus* ATCC 26933 was undertaken using IgG-Pes1 to detect the presence of the

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**Fig. 3.** Purification of the Pes1 protein from *Aspergillus fumigatus*. (A) Immunoblotting of recombinant proteins with antibodies directed to condensation domain 5 of Pes1. Lane 1, Coomassie Blue-stained SDS/PAGE gel (12.5%) of purified recombinant Pes1E2 (34 kDa). Molecular mass markers are indicated. Lane 2, immunodetection of Pes1E2 using Pes1E2 antiserum (1:2500 dilution). Lane 3, SDS/PAGE analysis of Pes1TEA (120 kDa). Lane 4, western analysis of Pes1TEA probed with affinity-purified IgG-Pes1 (1:1000 dilution); this confirmed that immunoaffinity-purified antiserum was functional. (B) Anion-exchange chromatography of native Pes1 from *A. fumigatus*. All fractions were subject to western analysis using IgG-Pes1, and fractions 28–32, which were found to contain the highest amounts of Pes1, were pooled. The protein profile was also visualized by Coomassie Blue-stained SDS/PAGE gels (5%). (C) Gel filtration (Superose 6) chromatography of the nonribosomal (NRP) synthetase Pes1. The protein elution profile with molecular mass markers is illustrated. The start material for the gel filtration chromatography consisted of pooled fractions from the Q-Sepharose separation step. Fractions 12–16 were found to contain immunoreactive proteins when probed with IgG-Pes1. Coomassie Blue-stained gel of the eluted fractions. Arrows indicate proteins subjected to MALDI-TOF and LIFT-TOF/TOF MS analyses. (D) SDS/PAGE and immunological analysis of the final protein preparation. Lane 1, Coomassie Blue-stained SDS/PAGE analysis illustrating the peak fraction from the Superose 6 column, which chromatographed around 500 kDa. Lane 2, western analysis of this fraction probed with IgG-Pes1. Lane 3, phosphoserine antiserum (rabbit) reactivity towards Pes1.
protein. Pes1 was retained on a Q-Sepharose ion exchanger and eluted between 250 and 300 mM NaCl (Fig. 3B). Western blot analysis (Fig. 3B) consistently detected a single band in fractions 28–32 that migrated at 210–220 kDa. The predicted molecular mass of Pes1 is 698 kDa but no immunoreactive band within this range was visible. Analysis (5% SDS/PAGE) revealed a number of proteins of similar molecular mass (210–240 kDa) (Fig. 3C), indicative of partial proteolytic fragmentation of the NRP synthetase. Fractions containing Pes1 eluted from Q-Sepharose media (fractions 28–34; 14 mL total) were pooled, concentrated (5 mg in 500 μL) and loaded on a Superose 6 gel filtration column (Fig. 3C). Pes1 eluted from the column at an apparent molecular mass of about 500 kDa. As no protein of this approximate mass was observed by SDS/PAGE (Fig. 3C), it was possible that breakdown of the NRP synthetase occurred during SDS/PAGE sample preparation. However, it cannot be excluded the intact Pes1 did not enter the 5% SDS/PAGE gels used for these analyses. Overall, Pes1 was purified to approximately 50% purity (250 μg total protein), and a typical final protein profile is shown in Fig. 3D. A dominant protein band was obvious at approximately 220 kDa (indicated by arrow) that was associated with an immunoreactive band of the identical size using IgG-Pes1 (Fig. 3D). The observed protein was approximately 35% of the predicted mass of Pes1 and may represent the C-terminal proteolytic fragment that contained the second epimerase domain to which antibodies had been raised. Interestingly, an immunoreactive band was also detected at an identical molecular mass using phosphoserine antisera and may result from detection of the phosphoserine moiety of the 4’-phosphopantetheine cofactor bound to the NRP synthetase (Fig. 3D).

**MS analysis of high molecular mass proteins**

High molecular mass proteins were excised from SDS/PAGE gels and subjected to peptide mass fingerprinting by MALDI-TOF or LIFT-TOF/TOF analysis. From the MALDI-TOF spectrum of band 1 (Fig. 3C) (approximately 220 kDa), 195 out of 266 peptides were observed with identical monoisotopic values (m/z tolerance < 1 Da) to the theoretical digest of Pes1, thereby providing 35.9% sequence coverage of the NRP synthetase. The LIFT-TOF/TOF post-source decay fragmentation of the selected peptides with monoisotopic masses of 1262.633 and 1323.275 Da revealed the amino acid sequences TVARVKDLR and SIRELATRVK, respectively. As the predicted and calculated molecular mass of Pes1 is estimated to be 698 kDa (observed 440–550 kDa), it would appear that Pes1 fragmented into at least two breakdown products (Fig. 3C, protein bands 1 and 2; 220 and 240 kDa, respectively), although it is possible that further differential proteolysis had occurred.

**Disruption of pes1 in A. fumigatus**

A Δpes1 mutant was generated by homologous transformation of *A. fumigatus* strain 293.1 with an 8.4 kb fragment containing the pes1A2 domain (Fig. 1) disrupted by a zeocin-pyrG-encoding region plus 3 kb of 5’ and 3’ flanking regions, respectively (Fig. 4A). This construct was generated by double-joint PCR [18] and characterized by KpnI restriction, and DNA sequence analysis confirmed the replacement of the pes1A2 domain by the zeocin-pyrG region surrounded by intact 5’ and 3’ flanking regions of the target gene (Fig. 4B). Following protoplast transformation, PCR screening for pes1A2 (negative) and zeocin (positive) colonies identified two transformants (out of 53 in total), one of which was confirmed by Southern analysis (using identical DNA loading (Fig. 4C) to lack the pes1A2 domain, while containing an adjacent ABC multidrug transporter (GenBank accession number EAL90367) (Fig. 4C). Subsequent RT-PCR analysis confirmed that pes1 expression in day 3 cultures was absent in the Δpes1 mutant, compared to *A. fumigatus* 293.1. ABC multidrug transporter expression was intact in both *A. fumigatus* 293.1 and the Δpes1 mutant (Fig. 4D).

Importantly, western analysis, using immunoaffinity-purified Pes1-IgG, showed that the Pes1 protein was completely absent from the Δpes1 mutant. Interestingly, Pes1 was primarily located in the cytosolic fraction (C) of *A. fumigatus* 293.1 protoplast lysates, and to a lesser extent in the microsomal (M) fraction (Fig. 4E).

**The pes1 mutant displays reduced virulence**

Altered growth rates have the potential to affect pathogenesis during comparison of the virulence of
wild-type (parental) and mutant strains, and so the growth rate of *A. fumigatus* 293.1 was compared with that of the Δ*pes1* mutant. Growth curves (Fig. 5A) showed that the exponential growth phase began at 24 h and extended until 72 h for both, and that the stationary phase was reached at 96 h, with similar biomass obtained for both 293.1 and the Δ*pes1* mutant (379 and 359 mg \%/100 mL culture, respectively). In order to determine whether human neutrophils killed *A. fumigatus* 293.1 and Δ*pes1* similarly, the fungicidal activity of purified human neutrophils was determined in vitro. The kinetics of fungal killing are shown in Fig. 5B for a ratio of neutrophils to *A. fumigatus* conidia of 4 : 1. Killing of *A. fumigatus* 293.1 conidia occurred slowly, and only 23% of the conidia were killed after 40 min. There was a difference in the pattern of killing of conidia of *A. fumigatus* Δ*pes1*. After 40 min, 56% of the conidia were killed, and only 4% remained viable after 80 min. To further test the reduced virulence of *A. fumigatus* Δ*pes1*, we investigated the pathogenicity of the mutant using the G. mellonella virulence model. Figure 5C shows the mortality of larvae following infection with *Aspergillus* conidia. Avirulence of *A. fumigatus* 293.1 (pyrG mutant) was observed, as larvae were fully protected against infection with 1 × 10⁶ viable conidia, as previ-
ously described [19]. After 2 days, 25% of the larvae infected with wild-type 293 spores had died, in contrast to the attenuated virulence seen when conidia from \( \text{D} \)pes1 were used (\( P < 0.045 \)). Extending this study, larvae were infected with a higher conidial dose (\( 1 \times 10^7 \)) (Fig. 5D). Conidia of the wild-type 293 strain caused the death of virtually all larvae within 2 days, while the virulence of conidia of \( \text{D} \)pes1 was significantly reduced to 40%, as shown by the death of 12 of 30 larvae (\( P < 0.001 \)). Taken together, these data establish the critical role of \( \text{pes}1 \) in the success of \( \text{A. fumigatus} \) infection \( \text{in vivo} \).

**Effect of \( \text{pes}1 \) disruption on conidial phenotype**

Conidia of the parental \( \text{A. fumigatus} \) 293.1 and of the \( \text{Δ} \)pes1 mutant were point inoculated on AMM agar plates containing 5 mM uracil and uridine (293.1 only) and 5 mM glucose at 37 °C. As shown in Fig. 6A, B, disruption of \( \text{pes}1 \) resulted in a rough surface covered with ornamentation; in contrast, conidia of the \( \text{Δ} \)pes1 mutant possessed a smoother surface with a lower degree of ornamentation on the conidial wall. In concurrence with the altered conidial phenotype, a hydrophobicity assay (Fig. 6C) of conidia from both wild-type and mutant \( \text{Aspergillus} \) strains revealed the \( \text{Δ} \)pes1 mutant to be 51% more hydrophobic than the 293.1 strain (\( P = 0.003 \)).

In order to investigate whether the altered conidial morphology affects the sensitivity to \( \text{H}_2\text{O}_2 \), conidia of the \( \text{Δ} \)pes1 mutant or \( \text{A. fumigatus} \) 293.1 (as a control) were exposed to different \( \text{H}_2\text{O}_2 \) concentrations in plate diffusion assays. The inhibition zones obtained with the two different conidia were compared and are shown in Fig. 6D. Both \( \text{A. fumigatus} \) 293.1 and \( \text{Δ} \)pes1 strains showed an increase in the diameter of the inhibition zone as the dose of \( \text{H}_2\text{O}_2 \) increased, but the effect was stronger in the case of the \( \text{Δ} \)pes1 mutant (for 8 \( \mu \)L of 3% \( \text{H}_2\text{O}_2 \) (v/v), \( P = 0.002 \)).

Investigation of the fungicidal effectiveness of reactive oxygen species (ROS) against the parental strain and \( \text{Δ} \)pes1 mutant was extended to the effects of
HOCl. HOCl is a strong nonradical oxidant and is the most fungicidal agent thought to be produced by neutrophils [20]. Data for incubation of \( A. fumigatus \) 293.1 and \( \Delta pes1 \) mutant in 1 or 2.5 \( \mu \)M HOCl are shown in Fig. 6E. Killing by 2.5 \( \mu \)M HOCl occurred quickly, and over 90% of both strains were killed after just 4 min. Interestingly, there was a difference in the pattern of killing by 1 \( \mu \)M HOCl, and after 8 min of exposure, 51% of parental 293.1 were still viable compared to only 17% of the \( \Delta pes1 \) mutant (\( P = 0.005 \)). These results imply that conidial morphology is closely linked to resistance against ROS and thus provide an explanation for the reduced virulence levels observed for \( A. fumigatus \) \( \Delta pes1 \) in \textit{in vitro} and \textit{in vivo} pathogenesis assays (Fig. 5).

**Discussion**

Here we present data that demonstrate the differential expression of a nonribosomal peptide synthetase, Pes1, in four strains of \( A. fumigatus \). Native Pes1 protein was partially purified from \( A. fumigatus \) ATCC 26933 and found to exhibit a molecular mass of approximately 500 kDa upon gel filtration. Pes1 was identified both by immunoreactivity, using immunoaffinity-purified antibodies, and by peptide mass fingerprinting (35.9% and 37.2% sequence coverage of the N-terminal and C-terminal domains, respectively, of Pes1). Furthermore, using MALDI LIFT-TOF/TOF MS, the sequence of four peptides derived from Pes1 was determined. Deletion of \( pes1 \) was confirmed by Southern
analysis and RT-PCR, in addition to western blot analysis, and the mutant was shown to be significantly less virulent in the G. mellonella model system (P < 0.001) and more susceptible to oxidative stress (P = 0.002), both in culture and during neutrophil-mediated phagocytosis. The Δpes1 mutant also exhibited altered conidial morphology and hydrophobicity. Taken together, these results confirm a role for pes1 in protecting A. fumigatus against oxidative stress.

Semiquantitative analysis of pes1 expression has confirmed that the gene is present, and differentially expressed, in four strains of A. fumigatus. Increased levels of pes1 expression were evident in strains ATCC 26933 and 13073 over the culture time course, while expression in ATCC 16424 remained static over the 72 h culture period. Using the well-established G. mellonella model of fungal virulence, we have previously shown that A. fumigatus ATCC 26933 exhibits significantly greater virulence than either ATCC 16424 or ATCC 13073 [21], and we have hypothesized that the Pes1 protein may contribute to this differential virulence (see below). Recent studies on pes1 expression in A. fumigatus ATCC 26933, simultaneously determined by northern and RT-PCR analysis, showed detectable expression [13]. However, only northern analysis confirmed the constitutive nature of pes1 expression at all time points, while RT-PCR analysis failed to detect expression at 24 h. The higher sensitivity of the RT-PCR analysis in the present work most likely accounts for this observation, and is in turn related to the low abundance level of fungal NRP synthetase transcripts – possibly only 2% of actin gene expression [22]. In the present study, we also confirmed that increased A. fumigatus pes1 expression occurred in G. mellonella following larval inoculation. Indeed, the G. mellonella system has recently been used to detect upregulation of Metarhizium anisophilae-derived Pr1 (which encodes a subtilisin-like protease) in infected insect larvae as the mycelia emerge and produce conidia on the surface of the cadaver [23].

It seems unlikely that pes1 encodes a destruxin synthetase [24], as this toxin was not detected in A. fumigatus culture filtrates by RP-HPLC analysis (data not shown). The NRP synthetase gene of Alternaria brassicae has also been suggested to play a role in siderophore biosynthesis, yet upregulation of expression in a low-iron environment was not observed [16]. Direct comparison of pes1 expression with that of sidD in A. fumigatus revealed concomitant upregulation of pes1 and diminution of the latter, possibly implying a difference in functionality and bringing into question the classification of pes1 as a putative siderophore synthetase-encoding gene. Lee et al. [22] have recently identified a number of NRP synthetase genes in the plant pathogen Cochliobolus heterostrophus (NPS1-12). These authors demonstrated that only the NPS6 gene was essential for fungal virulence; however, a distinct NRP synthetase (NPS4; 20 kb) was found to encode four adenylation, six condensation, six thiolation and three epimerase domains. Whole protein-based and adenylation domain-based phylogenetic analysis has now demonstrated that NPS4 clusters with Pes1, in particular with respect to Pes1A4 and NPS4A4 (supplementary Fig. S1 and Table S1). Moreover, Pes1 and NPS4 share 37% amino acid identity (56% similarity). We have also bioinformatically identified a putative Aspergillus oryzae NRP synthetase (GenBank accession number BAE64185.1) that exhibits significant 61% identity and 76% similarity to Pes1, and two A. nidulans NRP synthetases (GenBank accession numbers EAA65335 and EAA65835) that share approximately 50% identity and 67–71% similarity, respectively, with Pes1 (supplementary Fig. S1). Thus, it is now clear that the number of fungal NRP synthetases identified is set to expand as fungal genome sequence data emerge.

Microarray analysis has shown that certain disabled open reading frames are expressed in Saccharomyces cerevisiae [25]. Thus, the possibility that NRP synthetase pseudogenes may undergo transcription due to the presence of functional promoters, allied to the difficulty in confirming the NRP synthetase gene expression [17,22], necessitate that consideration be given to the functional identification of NRP synthetases, at the protein level, by emerging technologies. Here, monospecific, immunoaffinity-purified antibodies have been used to facilitate Pes1 purification, and MALDI LIFT-TOF/TOF MS has been deployed to unambiguously confirm the presence of native Pes1 in A. fumigatus. Interestingly, while the molecular mass of detectable Pes1 was shown to be about 500 kDa by gel filtration analysis, SDS/PAGE analysis demonstrated the existence of two lower molecular mass subunits. To our knowledge, immunodetection of Pes1 using phosphoserine antisera is novel; however, further studies are required to determine whether this reactivity is directed towards the phospho component of the 4’-phosphopantetheine arm or against phosphoserine residues in Pes1.

Specific interruption of pes1 gene expression and confirmation that the cognate protein product is completely absent in A. fumigatus is significant, as it represents one of the first successful attempts to disrupt an NRP synthetase gene in the organism. Historically, gene disruption/deletion in A. fumigatus has been
hampered by low frequencies of homologous recombination of the deletion construct [18]. In our hands, the double joint-PCR approach described by these authors for preparation of deletion constructs worked well and greatly simplified construct generation. Furthermore, although not used during the present study, the demonstration that *A. fumigatus* ΔakuA [26] and ΔakuB [27] mutants can yield up to 80–95% site-specific homologous transformation, following protoplast transformation, is significant, as it should greatly improve the success rate for gene deletion in this organism.

*G. mellonella* is attracting ever-increasing attention as a model organism for the study of microbial virulence in general [23], and *Aspergillus* virulence in particular [26,28]. The *in vitro* generation of ROS has been observed in the self-defence system of *G. mellonella*, with both O₂⁻ [29] and its dismutation product H₂O₂ [30] being found in phagocytic cells. The significantly reduced virulence of the Δpes1 mutant, compared to *A. fumigatus* A293, is evident at conidial loads of both 10⁶ and 10⁷ per larva. These data confirm the suitability of the *G. mellonella* virulence model to detect alterations in the pathogenicity of *A. fumigatus* mutants and complement the recent demonstration that the system can also be used to confirm lack of virulence following gene deletion [26]. Thus, the elucidation of significantly reduced virulence of the *A. fumigatus* Δpes1 mutant further enhances the utility of this model system, which provides an alternative, or complementary, approach to the use of animal model systems.

ROS production following activation of the respiratory burst NADPH oxidase of neutrophils is required for optimal antimicrobial function, and its importance is demonstrated by the syndrome of chronic granulomatous disease (CGD) [31]. CGD is a rare condition and is associated with the absence of the generation of ROS. ROS have widely been thought to be responsible for the killing of phagocytosed microorganisms, either directly (O₂⁻ and H₂O₂) or by acting as substrate for myeloperoxidase-mediated halogenation (HOCl) [20]. In previous studies, inhibitors of the NADPH oxidase that decreased the production of ROS inhibited the killing of *A. fumigatus* [32], and invasive aspergillosis is the primary cause of death in patients suffering from CGD [33]. The primary observations of this study on neutrophil-mediated killing of *A. fumigatus* 293.1 conidia highlight the importance of pes1 as an important contributor to fungal virulence. Killing of conidia demonstrated a clear time-dependent index, with neutrophils exhibiting the ability to kill conidia of *A. fumigatus* Δpes1 at a higher rate than those of 293.1. The fungicidal effects of increasing concentra-

tions of H₂O₂ and HOCl were studied, with greater sensitivity to both ROS being exhibited by *A. fumigatus* Δpes1. Oxidants such as HOCl are known to react with thiol groups, thioesters, and aliphatic or aromatic groups [34]. Most of these reactions lead to a loss in oxidative capacity, resulting in the loss of microbial properties. However, the effect of HOCl is directly related to the presence of protein on the surface or in the surrounding environment [35], and higher amounts of protein will consume the available HOCl. The Δpes1 mutant displayed differences in conidial surface morphology and was shown to be significantly more hydrophobic than the parental 293.1 strain. Previous studies have implicated both pigment and altered conidial protein surface in increased susceptibility to oxidative damage [36,37]; accordingly, the differences in conidial ornamentation observed for *A. fumigatus* Δpes1 may render this mutant more sensitive to applied ROS. Interestingly, upregulation of pes1 expression was not observed following H₂O₂-induced oxidative stress in cultures of *A. fumigatus* 293.1 grown in either S. aureus 9474 or 5% FBS in MEM (data not shown). Moreover, expression of neither of the two *A. nidulans* orthologues of pes1 (GenBank accession numbers EAA65335 and EAA65835; supplementary Fig. S1 and Table S1) was upregulated following exposure to H₂O₂ [38].

Sheppard et al. [39] have recently described the importance of the transcription factor StuA in the acquisition of developmental competence in *A. fumigatus*. These authors showed pes1 expression to be the most significantly altered (downregulated) in an *A. fumigatus* stuA mutant, following whole genome microarray analysis, during the onset of developmental competence. Significantly, the stuA mutant exhibited enhanced sensitivity to H₂O₂-induced oxidative stress, and a small, although not significant, reduction in virulence in a murine model system. This pattern of altered resistance to oxidative stress is similar to that observed in the Δpes1 mutant, so it is possible that the Pes1 peptide product may be involved in mediating the downstream effects of StuA-induced gene expression. Secondary metabolites may play a significant role in fungal development [14]. For example, in *Aspergillus parasiticus* and *A. nidulans*, chemical inhibition of polyamine biosynthesis inhibits sporulation, in addition to aflatoxin and sterigmatocystin production, respectively [40]. As late growth phase expression of pes1 is evident, it is possible that the Pes1 peptide product may be involved in the sporulation process of this fungus.

In summary, our data show that pes1 expression is temporally regulated in *A. fumigatus* both *in vitro*
Table 1. Nucleotide sequence of oligonucleotide primers used to amplify Aspergillus fumigatus genes from A. fumigatus genomic DNA and cDNA.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence (5'- to 3')</th>
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<tbody>
<tr>
<td>pes1A2 forward</td>
<td>GCACGGATCCGACCTGGAATTTGAGC</td>
</tr>
<tr>
<td>pes1A2 reverse</td>
<td>GATCCGCTGGAATTAGTCTGCTGCTGCTAGT</td>
</tr>
<tr>
<td>pes1A4 forward</td>
<td>CTGAGGCGCTGGAATTTGAGC</td>
</tr>
<tr>
<td>pes1A4 reverse</td>
<td>GATCCGCTGGAATTAGTCTGCTGCTGCTAGT</td>
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</tr>
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<tr>
<td>5' flanking forward</td>
<td>GCCTGGGAAAGCTTATATATAGT</td>
</tr>
<tr>
<td>5' flanking reverse</td>
<td>GCCTGGGAAAGCTTATATATAGT</td>
</tr>
<tr>
<td>3' flanking forward</td>
<td>GCCTGGGAAAGCTTATATATAGT</td>
</tr>
<tr>
<td>3' flanking reverse</td>
<td>GCCTGGGAAAGCTTATATATAGT</td>
</tr>
<tr>
<td>zeocin-pyrG forward</td>
<td>GGCGATCCTGACCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG</td>
</tr>
<tr>
<td>zeocin-pyrG reverse</td>
<td>GGCGATCCTGACCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG</td>
</tr>
<tr>
<td>Nested forward</td>
<td>GCCTGGGAAAGCTTATATATAGT</td>
</tr>
<tr>
<td>Nested reverse</td>
<td>GCCTGGGAAAGCTTATATATAGT</td>
</tr>
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</table>

and during infection of G. mellonella, respectively. Pes1 protein was also demonstrated in A. fumigatus, thereby confirming that pes1 is a functional gene. Disruption of pes1 led to decreased fungal virulence, and increased susceptibility to oxidative stress and neutrophil-mediated killing, in addition to altered conidial morphology and hydrophobicity. Taken together, these data strongly suggest that pes1 significantly contributes to the resistance of A. fumigatus to oxidative stress.

**Experimental procedures**

**Chemicals**

All chemicals and reagents were purchased from Sigma-Aldrich (Sigma-Aldrich Chemical Co., Poole, UK), unless stated otherwise.

**Microorganisms and culture conditions**

Clinical isolates of A. fumigatus used in this study included ATCC 26933, ATCC 16424 and ATCC 13073 (obtained from the American Type Culture Collection, MD, USA) with culture conditions and growth curves constructed as previously described [21]. The A. fumigatus strain Af293 and the transformation recipient pyrG auxotrophic strain Af293.1 were obtained from the Fungal Genetics Stock Center, Kansas City, USA [41] and cultured on Aspergillus minimal medium (AMM), supplemented with 5 mM uridine and uracil (auxotrophic strain) and 1% (w/v) glucose. Aspergillus growth curves were obtained as previously described [21].

**Isolation of genomic DNA, RNA and RT-PCR amplification**

Preliminary sequence data were obtained from The Institute for Genomic Research website at http://www.tigr.org. The extraction of genomic DNA was as previously described [42]. Fungal RNA was isolated and purified from crushed hyphae of Aspergillus, employing the Rneasy™ plant mini kit (Qiagen, Crawley, UK). Total RNA was extracted from Aspergillus-infected G. mellonella using TRI REAGENT™ according to the manufacturer’s instructions. Prior to cDNA synthesis, RNA was treated with DNase I. cDNA synthesis from mRNA (1 μg) was performed using the SuperScript™ kit (Invitrogen, Crawley, UK) using oligo(dT) primers. PCR was performed using AccuTaq polymerase with 1–10 ng genomic DNA as template. PCR was performed using the primers summarized in Table 1. PCR conditions were as follows: 95 °C denaturing for 5 min (95 °C denaturing for 30 s, 55 °C annealing for 30 s, 72 °C extension for 6 min) × 28 cycles; and 72 °C extension for 6 min. The gene encoding calmodulin (calm), which is constitutively expressed in Aspergillus...
**Cloning and expression of pes1E2**

The pes1E2 sequence was amplified from *A. fumigatus* ATCC 26933 genomic DNA, using primers incorporating terminal *Hind*III and *Xba*I sites (New England Biolabs, Ipswich, UK). PCR products were cloned directly into the pProEx-Hta™ expression vector (Invitrogen), and the resultant expression vector containing pes1E2 was transformed into *Escherichia coli* strain DH5α. After confirmatory DNA sequence analysis, expression of pes1E2 was induced and recombinant Pes1E2 purified [44]. Recombinant Pes1TEA (Fig. 1) was purified as previously described [13].

**Antiserum production**

Rabbit antiserum was raised against purified Pes1E2 using standard protocols [44]. Pes1E2-specific antibodies were immunoaffinity purified against Pes1E2 immobilized on nitrocellulose, eluted with 0.1 M glycine/HC1, pH 2.9, and immediately neutralized with 0.5 M NaOH. Immunoaffinity-purified antibodies (termed IgG-Pes1) were used (1:1000) for 1 h in western blot analyses. Phosphoserine antiserum (Abcam, Cambridge, UK) was used at a dilution of 1:250 and incubated for 16 h at 4°C. Horseradish peroxidase-conjugated donkey anti-rabbit IgG (1:5000 dilution) (Amersham Biosciences, Freiburg, Germany) was used to detect reactive bands by the enhanced chemiluminescence (ECL) system (Pierce Biotechnology, Cramlington, UK).

**Protein purification**

Hyphae were harvested from 4 L of cultured *A. fumigatus* ATCC 26933. All protein isolation and purification steps were performed at 4°C. Protein concentrations were determined using the Bradford method with BSA as a standard. Hyphae were washed twice in NaCl/Pi and ground to a fine powder under liquid N2. The ground hyphae were resuspended in Break Buffer [45], in the presence of protease inhibitors [46], and sonicated (Bandelin Sonopuls, Progen Scientific Ltd., Mexborough, UK) for 3 x 5 s at maximum power. After centrifugation for 10 min at 40 000 g using a Sorvall Instruments RCSC centrifuge (GSA rotor) (Thermo Electron Corp., Asheville, NC, USA), the supernatant (approximately 250 mg of protein) was chromatographed successively as follows. Starting material was loaded onto Q-Sepharose (1.5 x 8 cm, 1 mL·min⁻¹, 2 mL fractions collected, eluted with a 100 mL linear gradient of 0–1 M NaCl in Break Buffer). Peak fractions containing native Pes1 were identified by immunoreactivity (IgG-Pes1), pooled (14 mL) and concentrated to 0.5 mL using a Centricron 30 (Millipore, Cork, Ireland). The concentrated material (approximately 850 μg of total protein) was further purified by gel filtration using an ÄKTA Purifier 100 system (Amersham Biosciences), whereby a Superose 6 column (10 x 300 mm) was equilibrated in Break Buffer supplemented with 500 mM NaCl at a flow rate of 0.4 mL·min⁻¹. The concentrated material from Q-Sepharose was loaded on the column and 0.5 mL fractions were collected. As molecular mass markers, thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa) and aldolase (158 kDa) were used separately. Protease inhibitors were included in all buffers used for chromatography [46]. Electrophoretic analysis was carried out using 5% SDS/PAGE to facilitate detection of high molecular mass proteins.

**MS**

Peptide mass fingerprinting and LIFT-TOF/TOF MS analysis of trypsin-digested Pes1 were carried out using a Bruker ultraflex LIFT-TOF/TOF (Bruker, Rheinsteetten, Germany), as previously described [46]. Only peptides with high signal intensity were subject to LIFT-TOF/TOF analysis [47] and resultant spectra processed using FLEXANALYSIS software (Bruker). Database searches and sequence comparisons were carried out via MASCOT inhouse server (Matrix Science, London, UK) and BIOTOOLS (Bruker), respectively.

**Disruption of *A. fumigatus* pes1**

Disruption of pes1 was performed using the double-joint PCR method as previously described [18]. The first-round PCR generated amplicons containing 5’ and 3’ flanking regions of pes1E2 and carried 25 bp of homologous sequence overlapping with the ends of the pyrG selection marker. The sequences of primers used to amplify the flanking regions (5’ and 3’ flankong forward and reverse) are given in Table 1. The pyrG selection marker was amplified from the pCD21 plasmid (a gift from AA Brakhage, Leibnitz-Institute for Natural Product Research and Infection Biology) using primers pyrG forward and pyrG reverse (Table 1). Conditions for the first-round PCR were as follows: 93°C for 5 min; four cycles of 93°C for 30 s, 58°C for 2 min and 72°C for 3 min; 24 cycles of 93°C for 30 s, 60°C for 2 min and 72°C for 3 min; and finally 72°C for 10 min. PCR products were gel purified (gel extraction kit, Qiagen), and for the second-round PCR, 1 μL of both the 5’ flanking and 3’ flanking amplicons were mixed with 3 μL of the purified pyrG amplicon. The second-round PCR...
conditions (using Long Expand polymerase; Roche Diagnostics GmbH, Mannheim, Germany) were: 94 °C for 2 min; 15 cycles of 94 °C for 45 s, 62 °C for 2 min, 68 °C for 12 min; and finally 15 min postpolymerization. Nested primers for the third-round PCR were designed (Table 1) including a 5'-AvrII (New England Biolabs) restriction site on the forward primer and a 3'-SacII (New England Biolabs) restriction site on the reverse primer. Conditions for the third-round PCR were as previously described [18]. Prior to cloning into the pCR 2.1-TOPO expression vector, PCR products were confirmed on the basis of size, sequencing (Lark Technologies, Takeley, UK) and KpnI (New England Biolabs) restriction enzyme digestion.

Aspergillus transformation

A. fumigatus protoplasts were prepared from conidia of A. fumigatus 293.1 grown for 7 h at 37 °C in AMM supplemented with 5 mM uracil and uridine. Hyphal cells were harvested by centrifugation at 200 g for 15 min (IEC Centra CL3R, swingout rotor, Biosciences, Dublin, Ireland) and resuspended in 40 mL of Protoplasting Buffer (0.4 M (NH₄)₂SO₄, 50 mM potassium citrate, 10 mM MgSO₄, 0.5% (w/v) surose, pH 6.2) containing Zymolase (120 mg), Driselase (400 mg), Glucanase (200 mg), BSA (400 mg) and 10 mM 2-mercaptoethanol. The suspension was incubated at 37 °C for 1.5–2 h, and filtered through Miracloth (Calbiochem, Bad Soden, Germany), and protoplasts were pelleted by gentle centrifugation (200 g, 5 min). Protoplasts (1 × 10⁷) were resuspended in 200 μL of Transformation Buffer (TM) (0.6 M KCl, 50 mM CaCl₂, 10 mM methanesulfonic acid, pH 6.0) containing 10–20 μg of transformation DNA, and 100 μL of polyethylene glycol (PEG) solution (25% (w/v) PEG 6000, 50 mM CaCl₂, 0.6 M KCl, 10 mM Tris/HC1, pH 7.5). The suspension was chilled to 4 °C for 15 min, and a further 1 mL of PEG solution added at room temperature for 15 min. TM (10 mL) was added to the mixture, and the transformed protoplasts were pelleted by centrifugation (200 g, 5 min). Protoplasts were resuspended in 500 μL of TM, and 50 μL aliquots were mixed with 10 mL of AMM (minus uracil and uridine) containing 1 M sorbitol as osmotic stabilizer plus 2% (w/v) molten agar, and then poured onto minimal medium agar plates. Putative transformants became visible after 2 days of incubation at 37 °C and were subcultured onto AMM. Southern blot analysis was carried out as previously described [48].

Subcellular fractionation and localization of Pes1

To localize Pes1, protoplasts were prepared as described above and homogenized in Breuk Buffer containing 10% (v/v) glycerol. A postnuclear supernatant (PNS) was centrifuged (40 000 g for 3 h at 4 °C in a Beckman SW40 T1) to yield microsomal (M) pellet and soluble cytosol (C) fractions as previously described [49]. Fractions were analysed by SDS/PAGE and immunodetection using immunoaffinity-purified antibodies, IgG-Pes1.

In vitro killing of conidia by human neutrophils

Neutrophils were purified from fresh human blood by dextran sedimentation and centrifugation through Ficoll/ Hypaque as previously described [50]. Cells (5 × 10⁸) were incubated at 37 °C in 1 mL NaCl/Pi in a rapidly stirred chamber. IgG opsonized conidia were added (1.25 × 10⁹) and killing measured as described by Segal et al. [51], omitting lysostaphin. Results were calculated as the mean (± se) from three experiments with colony counts performed in triplicate for each sample and expressed as a percentage of the original numbers at time zero.

In vivo testing of virulence

A. fumigatus strains were grown on AMM for 14 days at 37 °C. Conidia were harvested [21] and infection studies carried out in the insect model G. mellonella, according to standard protocols [29,52]. A group of 30 larvae were infected with the A. fumigatus 293.1, 293 or Δpes1 by injecting 20 μL of an inoculum suspension (per larvae) containing 1 × 10⁹ or 1 × 10⁸ conidia into the hemocoel/body cavity via the last proleg. Larvae were observed for mortality, twice daily, over a period of 7 days.

Scanning electron microscopy (SEM)

Conidia were fixed in 5% (v/v) formaldehyde and 2% (v/v) glutaraldehyde in cacodylate buffer (0.1 M cacodylate, 0.01 M CaCl₂, 0.01 M MgCl₂, 0.09 M surose, pH 6.9) and washed with cacodylate buffer and then with TE buffer (10 mM Tris/HCl, 2 mM EDTA, pH 6.9). Conidia were placed onto poly(L-lysine) coated glass slides and SEM carried out as previously described [28].

In vitro test for H₂O₂ and HOCl sensitivity

Conidia of A. fumigatus 293.1 and Δpes1 were harvested from AMM plates [21] and resuspended in NaCl/Pi at a final concentration of 1 × 10⁸ conidia/mL. AMM agar (100 mL) with added uracil and uridine (293.1 only) was cooled to 38 °C and 1 mL of conidia added before pouring into a Petri dish (240 × 240 mm). Nine holes with a diameter of 1 cm were punched into each agar plate and different amounts of 3% (v/v) H₂O₂ solution applied. Plates were incubated for 16 h at 37 °C and inhibition zones determined as an average of three specimens each.

Conidia (1 × 10⁷/mL) were suspended in 1 mL of NaCl/Pi and exposed to two different concentrations of HOCl (1 and 2.5 μM) at 37 °C. After mixing for 1, 2, 4 and
8 min, aliquots were removed and diluted 1 : 10 in ice-cold AMM. Serial 10-fold dilutions were then made, and plated in triplicate for each specimen; results were calculated as the mean (± se) from three separate experiments. The pH remained stable during assays to within 0.15 pH units of the starting pH.

**Hydrophobicity assay**

Conidia were harvested [21], washed twice and suspended in 0.05 M sodium phosphate buffer (pH 7.4) containing 0.15 M NaCl to D$_{540}$ nm = 0.4. The conidial suspension was treated with xylene (2.5 : 1, v/v), vigorously mixed for 2 min, and allowed to settle for 20 min. The absorbance of the aqueous phase was then determined at 540 nm and the relative hydrophilicity determined [53].

**Acknowledgements**

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**References**


**Supplementary material**

The following supplementary material is available online:

**Fig. S1.** Phylogenetic analysis of adenylase (A) domains from a range of fungal nonribosomal peptide synthetases (NRPS). GenBank accession numbers for all NRPS are given in supplementary Table 1. The location of the four *Aspergillus fumigatus* Pes1-derived A domains is shown (*). Pes1A4 clusters with *C. heterostrrophus* NPS4 A4 and *A. brassicae* NRPS1 A4, respectively. The A3 domains for all three proteins also exhibit evolutionary relatedness, but to a lesser extent.

**Table S1.** Genbank accession numbers of all fungal nonribosomal peptide synthetases used to construct the data in supplementary Fig. S1.

This material is available as part of the online article from http://www.blackwell-synergy.com