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A Nonribosomal Peptide Synthetase-Derived Iron(III) Complex from the Pathogenic Fungus *Aspergillus fumigatus*

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Supporting Information

ABSTRACT: Small molecules (SMs) play central roles as virulence factors of pathogenic fungi and bacteria; however, genomic analyses suggest that the majority of microbial SMs have remained uncharacterized. Based on microarray analysis followed by comparative metabolomics of overexpression/knockout mutants, we identified a tryptophan-derived iron(III)-complex, hexadehydro-astechrome (HAS), as the major product of the cryptic *has* nonribosomal peptide synthetase (NRPS) gene cluster in the human pathogen *Aspergillus fumigatus*. Activation of the *has* cluster created a highly virulent *A. fumigatus* strain that increased mortality of infected mice. Comparative metabolomics of different mutant strains allowed to propose a pathway for HAS biosynthesis and further revealed cross-talk with another NRPS pathway producing the anticancer fumitremorgins.

Aspergillus fumigatus is a ubiquitous filamentous fungus and causative agent of invasive aspergillosis (IA), a life-threatening disease. IA associated mortality ranges from 50% to 90%.[1] One of the few proteins that have been shown to be required for *A. fumigatus*' pathogenicity is LaeA, a conserved virulence factor in filamentous fungi.[2−5] LaeA is a global regulator of secondary metabolism in fungi and effects virulence, in part, by positive regulation of multiple small-molecule gene clusters.[3−6] For example, LaeA controls the biosynthesis of the epidithiodiketopiperazine gliotoxin,[7−10] which contributes to *A. fumigatus*' success as a pathogen.[11]

We reasoned that SMs most likely to have a role in virulence would be positively regulated by both *laeA* and exposure to host environments (host exposure itself or hypoxic environments). By comparing microarrays of genes down-regulated in *laeA* deletants but up-regulated by host exposure/hypoxia,[6,12−14] we identified an eight-gene SM cluster named *has* meeting these criteria. The eight cluster genes encode putative C6 transcription factors (HasA and HasF), one transporter (HasB), one *O*-methyltransferase (HasC), one nonribosomal peptide synthetase (NRPS, HasD, previously denoted as Nrps 5[15] and PesF[16]), one 7-dimethylallyltryptophan synthase (DMATS)[17] (HasE), one putative FAD binding protein (HasG) and one cytochrome P450 (HasH) (Figure 1a and Tables S1−S3). Often SM gene clusters are regulated by C6 transcription factors. Because *hasA* was the third most down-regulated gene in the Δ*laeA* array and strongly induced in conidia during neutrophil exposure,[6,12−14] we chose to overexpress (OE) this gene to facilitate chemical characterization of *has*-associated metabolites.[18,19] Replacement of the *hasA* promoter with the constitutive *gpdA* promoter (OE::*hasA*) resulted in induction of the other seven genes (Figure 1b) and yielded a pink pigmented strain (Figure S1a). Three cluster flanking genes (AFUB_036320, AFUB_036310, and AFUB_036220, Figure 1a,b) were not regulated by *hasA* and thus likely defined the boundaries of the cluster. To probe the functions of the individual *has* genes, we additionally constructed knockout mutants (KO) for *hasB-hasH* in the OE::*hasA* background (Figure S2).

Previous biochemical characterization of the DMATS HasE had proven its capacity to prenylate a variety of indole derivatives in position 7, with L-tryptophan as a preferred substrate;[17] however, no cluster-associated metabolites were identified. We employed HPLC-UV-MS (Figure 1c) and NMR-based comparative metabolomics[8,19,20] to probe for *has*-dependent metabolites in the OE::*hasA* strain. Analysis of the ^{1}H NMR spectrum from the OE::*hasA* extract revealed signals indicative of a 3-substituted indole moiety that were absent in the WT extract spectrum (Figure S3). All signals of this compound suffered from intense line broadening, suggestive of a paramagnetic species such as an iron complex. Comparison of high-resolution (HR)-HPLC-MS data from the OE::*hasA* and WT extracts showed a OE::*hasA*-specific ion cluster eluting at 46.8 min whose *m*/*z* values and isotopic distribution suggested a molecular formula of $C_{60}H_{60}FeN_9NaO_9$ (*m*/*z* calcd. 1129.3761; obsvd. 1129.3751), suggesting an Fe(III) complex. Supporting the requirement of iron for production of this metabolite, OE::*hasA* cultures in iron-deficient medium did not produce the peak at 46.8 min or the pink color (Figure. S1b). Because extreme line-broadening prevented full spectroscopic characterization of the *has*-dependent iron complex, we treated the isolated starkly pink compound with strong base to precipitate iron as the hydroxide. Following iron removal, we obtained the *O*-methylated diketopiperazine derivative **9**, whose structure was determined using a routine set of 2D NMR

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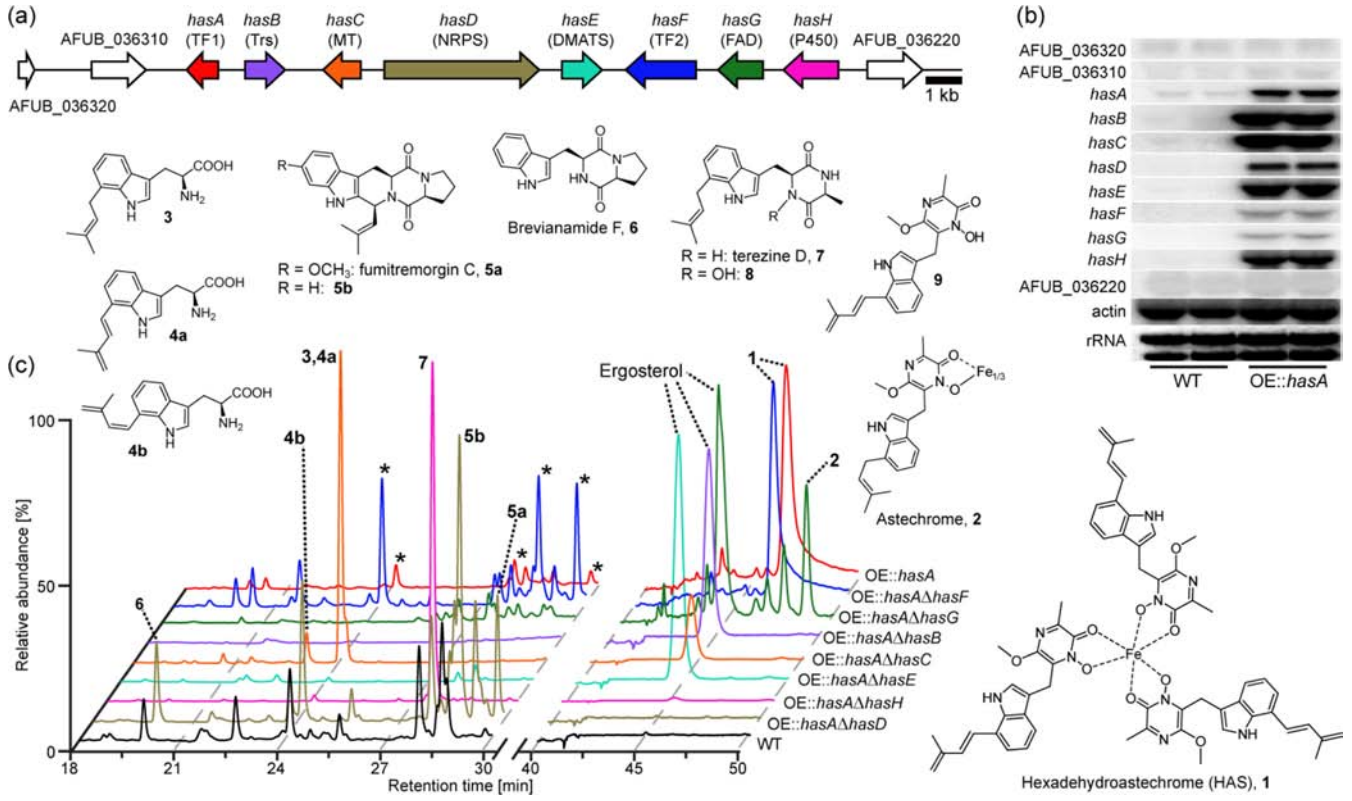

Figure 1. *hasA* controls *has* cluster gene expression and metabolite production. (a) *has* gene cluster and putative assignments. *hasA* and *hasF*, C6 transcription factors; Trs, transporter; MT, methyltransferase; NRPS, nonribosomal peptide synthetase; DMATS, dimethylallyltryptophan synthase; FAD, FAD-binding domain protein; P450, cytochrome P450. Flanking genes are shown in white. (b) Northern analysis of *has* cluster gene expression in the WT and OE::*hasA* strains. (c) HPLC-UV chromatograms for *A. fumigatus* mutant strains and structures of *has*-dependent metabolites; (*) denotes HAS decomposition products. Ergosterol amounts were highly variable between replicates and are not *has*-dependent.

spectra and HR-MS (Figure 1c). On the basis of the combined spectroscopic data, the iron-containing metabolite was identified as a trimeric complex of **9** with Fe(III), similar to the previously described *Aspergillus terreus* metabolite, astechrome (1113.1, $[M+H]^+$).[21] Accordingly, we named the *has*-dependent metabolite we identified from *A. fumigatus* hexadehydroastechrome "HAS" (**1**; Figure 1c).

To gain insight into HAS biosynthesis, we examined the metabolomes of the seven double mutants carrying deletions of the other *has* genes in OE::*hasA* background (Figure S2) by 2D NMR and HPLC-UV-MS for the presence of pathway intermediates or shunt metabolites, focusing on compounds whose UV and NMR spectra suggested incorporation of tryptophan. Putative pathway-related metabolites were characterized by HR-MS and purified as needed to complete NMR-spectroscopic assignments (Tables S4−S9). Loss of the second transcription factor (OE::*hasA*Δ*hasF*) did not result in any detectable metabolomic change compared to the OE::*hasA* strain, indicating that under the growth conditions used in this study HasF does not affect the *has* pathway (Figure 1c and Figure S2). In contrast, deletion of the transporter (HasB) led to complete abolishment of HAS production and no intermediates or shunt metabolites were observed (Figure 1c), which is not unexpected given that deletions of SM cluster transporter genes have previously been shown to inhibit SM production.[22,23]

The metabolite profiles of several other double mutant strains provided important clues for the HAS biosynthetic pathway. Deletion of the cytochrome P450 (OE::*hasA*Δ*hasH*) abolished HAS production and instead yielded large quantities of a single shunt metabolite, the prenylated Trp-Ala diketopiperazine, terezine D (**7**),[24] small amounts of which were also detected in the parent OE::*hasA* strain. In the OE::*hasA* strain, but not the P450 knock out, terezine D was accompanied by its hydroxy derivative, **8**.[24] Deletion of the putative *O*-methyltransferase (OE::*hasA*Δ*hasC*) unexpectedly led to production of large quantities of L-7-dimethylallyltryptophan (**3**), as well as the *trans*- and *cis*-isomers of L-7-(3-methylbutadienyl)tryptophan (**4a** and **4b**, respectively), whereas production of all diketopiperazine derivatives was completely abolished. L-configuration of compounds **3**, and **4a**/**b** was confirmed by converting them to their corresponding Mosher amides (Figure S4).[25] In contrast, the NRPS deletant (OE::*hasA*Δ*hasD*) did not accumulate prenylated tryptophan derivatives such as **3** or **4a**/**b**, suggesting that prenylation of tryptophan may not precede formation of NRPS-tethered tryptophan. Lastly, the FAD deletant (OE::*hasA*Δ*hasG*) yielded the previously described *A. terreus* metabolite, astechrome (**2**) instead of HAS (Figure 2b and Table S4), suggesting that HasG converts the prenyl to a methylbutadienyl side chain. Bioinformatic examination of the *A. terreus* genome revealed a gene cluster with close homology to the *A. fumigatus has* cluster; however, the *A. terreus* cluster lacks a gene encoding a homologue of the FAD HasG (Figure S5), supporting that HasG is required for introducing the diene moiety in the side chain (Figure 2a). We further noted that isolated samples of the *cis*-isomer **4b** slowly isomerized to **4a**, suggesting that HasG produces *cis*-7-(3-methylbutadienyl)indole derivatives, which

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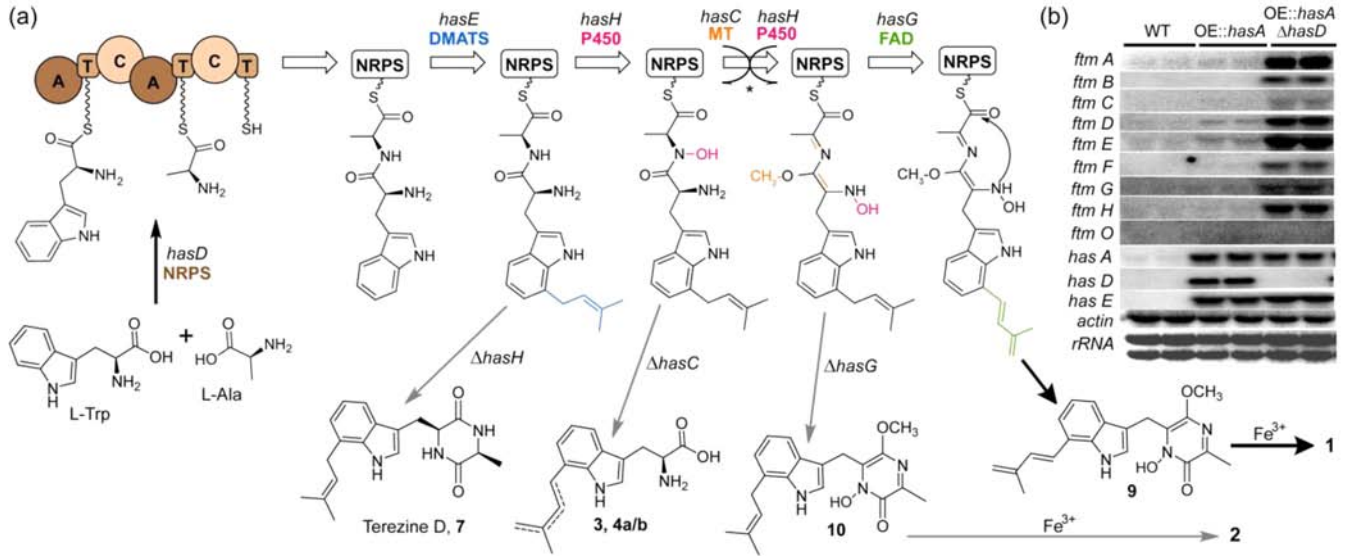

Figure 2. Model for HAS biogenesis and crosstalk with the *ftm* gene cluster. (a) Putative *has* biosynthetic pathway (A = adenylation, T = thiolation, C = condensation domains[16,17]), (*) order of enzyme activity unclear. (b) *ftm* gene cluster expression in two *has* mutants.

then slowly convert to the corresponding *trans*-isomers such as **4a** and **9** during extraction and isolation. Therefore, isolated hexadehydroastechrome (**1**) is shown as *trans* in Figure 1.

These results are consistent with a biogenesis model in which the NRPS is loaded with tryptophan and alanine, followed by prenylation of the tethered tryptophan or the resulting tethered Trp-Ala-dipeptide (Figure 2a). Deletion of the P450 *hasH* appears to stall further processing of the tethered dipeptide, which then undergoes spontaneous cyclization to form the diketopiperazine, terezine D, the only *has* pathway metabolite detected in the OE::*hasA*Δ*hasH* strain. Our observation that the OE::*hasA*Δ*hasC* (MT) mutant strain does not accumulate diketopiperazines, but instead yields large quantities of prenylated tryptophan derivatives, suggests a model in which HAS biosynthesis continues via tethered intermediates as shown in Figure 2a, as opposed to an alternative pathway involving further modification of terezine D-derived diketopiperazines. In this model, the presence of prenylated tryptophan derivatives **3** and **4a**/**b** could result from enzymatic hydrolysis of a tethered, partially oxidized dipeptide, for example by the DMATS HasE, which has previously been reported to hydrolyze linear dipeptides such as of H-L-Trp-L-Ala-OH, but not diketopiperazines.[26] Deletion of the prenyltransferase (DMATS, HasE) led to complete abolishment of HAS production and no intermediates or shunt metabolites were observed.

Surprisingly, we found that the NRPS deletant (OE::*hasA*Δ*hasD*), although producing no HAS intermediates, yielded large amounts of metabolites derived from the partially characterized *ftm* (fumitremorgin) NRPS cluster, including brevianamide F, fumitremorgin C and demethoxy-fumitremorgin C (**5a**, **5b**, **6**, Figure 1c). Fumitremorgin biosynthesis also involves prenylation of tryptophan derivatives;[18] however, the *ftm* cluster does not contain a pathway-specific transcription factor. Examination of *ftm* gene expression in wild type (WT), OE::*hasA*, and OE::*hasA*Δ*hasD* strains showed greatly increased *ftm* cluster gene expression in the latter strain (Figure 2b), consistent with the observation of increased *ftm*-metabolite production in OE::*hasA*Δ*hasD*. These results suggest that tryptophan and prenylation activity is shunted toward fumitremorgin biosynthesis when the *has* pathway is shut down in OE::*hasA* background. Interestingly, fumitremorgin production was also increased in NRPS deletants which abolished fumigaclavine C production,[27] possibly due to a similar mechanism.

To gain initial insight into the possibility that HAS might contribute to virulence, WT, OE::*hasA* and OE::*hasA*Δ*hasD* strains were compared in a neutropenic murine pulmonary model. Infection with the OE::*hasA* strain resulted in significantly higher mortality than infection with WT and OE::*hasA*Δ*hasD* (Figure 3a). Murine lung tissue from all

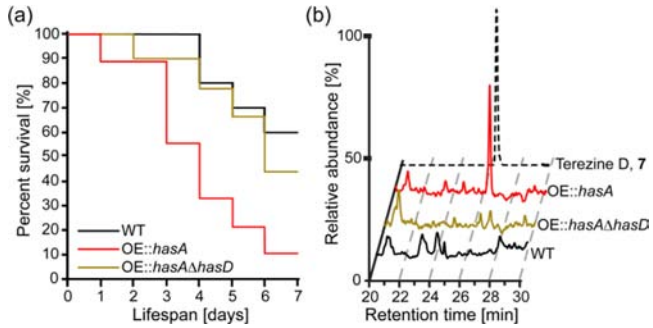

Figure 3. The *has* pathway affects survival in a mouse model of IA. (a) Mantel-Cox survival analysis of mice infected with WT, OE::*hasA*, or OE::*hasA*Δ*hasD* *A. fumigatus* in a neutropenic pulmonary mouse model of IA. OE::*hasA* is significantly more virulent than WT or OE::*hasA*Δ*hasD* ($P < 0.05$, Log-Rank Test). There was no significant difference between WT or OE::*hasA*Δ*hasD* strains. (b) MS ion chromatograms for terezine D (**7**) standard and lung extracts from mice infected with WT, OE::*hasA*, or OE::*hasA*Δ*hasD*.

treatments were examined for the presence of *has* metabolites. Whereas none of the identified *has*-dependent metabolites could be detected in the WT- and OE::*hasA*Δ*hasD*-infected samples, tissue samples from OE::*hasA*-inoculated mice revealed the presence of terezine D (Figure 3b).[24] HAS was not detected in the OE::*hasA* lung extracts; however, based on our experience with isolating the iron(III) complex from fungal cultures, this main product of the *has* pathway is likely too

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unstable to permit isolation from animal tissue. Our observation that in laboratory culture OE::*hasA*Δ*hasD* produces large amounts of fumitremorgins suggests the possibility that crosstalk with other metabolic pathways may in part be responsible for the increased virulence of OE::*hasA* in mice. However, fumitremorgins could not be detected in any of the mouse lung tissue samples, and production of gliotoxin, another metabolite contributing to *A. fumigatus* virulence,[11] was not increased in either the OE::*hasA* or OE::*hasA*Δ*hasD* samples relative to WT (Figure S6).

In conclusion, we show that microarray analysis coupled with comparative metabolomics using knockout and overexpression *A. fumigatus* mutants enables identification of potential virulence factors of a pathogenic fungus and proposing a tentative biosynthetic pathway. Examination of available fungal genomes indicates that *has*-related gene clusters are found only in a few opportunistic human pathogenic Aspergilli and related dermatophytic fungi of the *Trichophyton* and *Arthroderma* genera (Figure S5), providing additional motivation to further investigate the impact of HAS and related metabolites on virulence. Because of its iron-binding properties, we considered the possibility that **9** could be acting as a siderophore, as siderophores are well-known virulence factors in *A. fumigatus* and other fungi.[28] However, for entropic reasons, it seems unlikely that the hydroxamic acid **9** is able to compete with potent multidentate siderophores such as triacetylfusarinine C (TAFC) or ferricrocin.[28] Correspondingly, we found that neither **9** nor **1** was produced by OE::*hasA* cultures grown in iron-deficient medium, whereas TAFC production was strongly upregulated (Figure S7). Nevertheless, the iron requirement for HAS biosynthesis suggests that the *has* pathway and siderophore production may interact. In addition, the highly lipophilic trimer HAS may function as a vehicle for the delivery of its much more polar monomer **9** and/or serve to inflict oxidative damage.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Methods and details regarding enzyme assays and analytical methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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