Natural Products

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Homologous NRPS-like Gene Clusters Mediate Redundant Small-Molecule Biosynthesis in *Aspergillus flavus***

Ry R. Forseth, Saori Amaike, Daniel Schwenk, Katharyn J. Affeldt, Dirk Hoffmeister, Frank C. Schroeder, and Nancy P. Keller**

Fungi are among the most prolific sources of pharmacologically relevant natural products.[1] This large diversity of fungal small molecules serves important functions in fungal ecology, for example as virulence factors and as chemical defense agents. However, only a fraction of the biosynthetic capabilities suggested by genomic analyses has been observed under laboratory conditions, because expression of many, perhaps even most biosynthetic pathways depends strongly on environmental conditions.[2] Here we demonstrate the use of comparative metabolomics[3] for the analysis of knock-out, overexpression, and knock-down strains to identify metabolites derived from two nonribosomal peptide synthetase (NRPS) gene clusters in the aflatoxin-producing ascomycete *Aspergillus flavus,* a crop contaminant[4] and opportunistic pathogen that causes aspergillosis in immunocompromised humans.[5]

Although the *A. flavus* genome encodes at least 25 polyketide synthase (PKS), 18 NRPS, and two hybrid NRPS-PKS gene clusters,[6] assignments have been made for only four metabolites.[7] Many *A. flavus* secondary-metabolite pathways are under the control of the nuclear protein LaeA, a global regulator of morphogenesis and virulence factor in *A. flavus* and other pathogenic fungi.[8] Two LaeA-regulated clusters, which were named *lna* and *lnb*, exhibit a striking level of genetic similarity (Figure 1a, Table S1). The *lna* and *lnb* clusters contain two noncanonical NRPS genes with high sequence homology (58% identical at the amino acid level), *lnaA* and *lnbA*, respectively, which are accompanied by matching sets of genes likely coding for tailoring enzymes. *lna* and *lnb* are orphan clusters that have no known associated metabolites and belong to a family of noncanonical NRPS genes that consist of an adenylation (A) domain, a peptidyl carrier protein (PCP) domain, and a thioester reductase (R) domain, but lack a canonical condensation (C) domain (Figure 1a). The functions of this unusual family of NRPSs, which share homology to reductases participating in the biosynthesis of L-lysine in fungi,[9] have not been explored. We here demonstrate that the *lna* and *lnb* clusters encode sets of enzymes that produce overlapping sets of previously undescribed metabolites, and show that one primary function of the noncanonical NRPSs LnaA and LnbA likely consists in the reduction of L-tyrosine. Furthermore, the *lna* and *lnb* biosynthetic pathways appear to be part of a signaling network that controls the formation of sclerotia, a resilient overwintering structure.

To identify *lna*-associated metabolites by means of comparative metabolomics, we created deletion and overexpression[10] mutants of the NRPS gene *lnaA* (Δ*lnaA* and *OE::lnaA*, respectively) as well as double-mutant strains *ΔlnaA, KD::lnbA* and *OE::lnaA*, *KD::lnaB*, where KD indicates a knock-down of gene expression using RNAi (strains are listed in Table S3; for methods, see the Supporting Information, Section 1). Unexpectedly, we noted that in the Δ*lnaA*, *KD::lnbA* double-mutant strain, in which both the *lna* and *lnb* pathways are disrupted, formation of sclerotia was strongly suppressed relative to the wild-type(WT; see the Supporting Information, Figure S1).

The WT, Δ*lnaA*, and *OE::lnaA* metabolomes were compared using differential analysis by 2D NMR spectroscopy (DANS) as described previously.[3b–d] Comparison of the *OE::lnaA* spectra with either WT or Δ*lnaA* revealed a large number of spin systems present only in the *OE::lnaA* spectra (see Figure S2). Available structure databases indicated that most of the *OE::lnaA*-specific signals did not correspond to

[*] R. R. Forseth,[+] Prof. Dr. F. C. Schroeder
Boyce Thompson Institute and Department of Chemistry and Chemical Biology, Cornell University
1 Tower Road, Ithaca, NY 14850 (USA)
E-mail: fs31@cornell.edu
Homepage: http://www.bti.cornell.edu/schroeder/
K. J. Affeldt, Prof. Dr. N. P. Keller
Department of Medical Microbiology and Immunology
University of Wisconsin-Madison
3476 Microbial Sciences Building, Madison, WI 53706 (USA)
E-mail: npkeller@wisc.edu
Dr. S. Amaike[+]
Department of Plant Pathology
University of Wisconsin-Madison (USA)
Prof. Dr. N. P. Keller
Department of Bacteriology
University of Wisconsin-Madison (USA)
D. Schwenk, Prof. Dr. D. Hoffmeister
Department of Pharmaceutical Biology, Hans Knöll Institute
Friedrich Schiller Universität
Beutenbergstrasse 11a, 07745 Jena (Germany)
[+] These authors contributed equally to this work.

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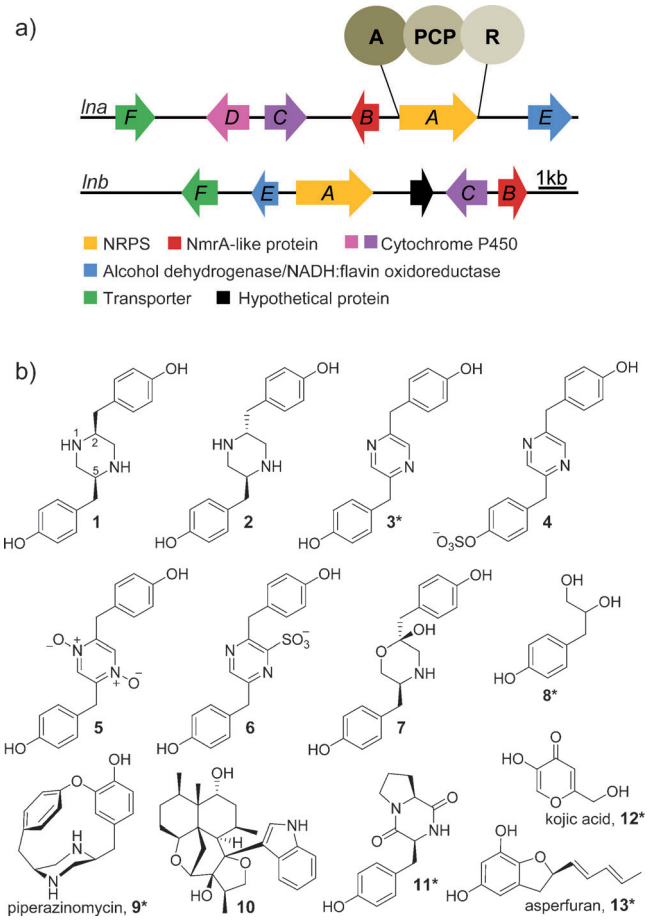

Figure 1. a) The *lna* and *lnb* gene clusters in *A. flavus*.[2c] Both *lna* and *lnb* include noncanonical NRPS genes encoding adenylation (A), carrier protein (PCP), and a short-chain dehydrogenase/reductase domain (R-domain). There is no *lnaD* orthologue in the *lnb* gene cluster. See also Table S1 in the Supporting Information. b) Metabolites identified in this study and the known piperazinomycin (**9**). Compounds marked with an asterisk have been previously described.

any known metabolites from Aspergilli or other fungi. Detailed NMR spectroscopic and high-resolution (HR) MS analysis of purified fractions containing the *OE::lnaA*-specific components suggested a pair of diastereomeric piperazines, **1** and **2**, as major *lnaA*-dependent compounds (Figure 1 b; see Tables S4–S10 for spectroscopic data). Comparison with synthetic samples prepared from cyclo(L-Tyr-L-Tyr) and cyclo(D-Tyr-L-Tyr) derivatives confirmed these structural assignments (Supporting Information, Section 8). The structures of **1** and **2** are reminiscent of piperazinomycin (**9**),[11] a bacterial metabolite first identified from *Streptomyces olivoreticuli*, which may be derived from oxidative macrocyclization of **1**.

The piperazines **1** and **2** are accompanied by a derivative that includes a quaternary carbon ($\delta = 93.1$ ppm), two isolated methylene groups, and an OCH_2-CH(N)-CH_2-phenyl fragment. HRMS indicated a molecular formula of $C_{18}H_{21}NO_4$, which in conjunction with 2D NMR spectroscopic data (Table S9) led to the identification of the hemiacetal-containing morpholine **7**. ROESY correlations between the hydroxy proton with the axial proton at C-6 defined the relative configuration of **7** as (2*R**,5*S**). We further identified a fully deaminated "monomer", 3-(*p*-hydroxyphenyl)-1,2-propanediol (**8**), a previously described fungal metabolite,[12] and four pyrazine derivatives. These include the known actinopolymorphol C (**3**),[13] an *O*-sulfonated derivative (**4**), as well as two other highly polar derivatives. The NMR spectra of one of these suggested *N*-oxidation which was confirmed by HRMS; the molecular formula of $C_{18}H_{16}N_2O_4$ found corresponded to an unusual *N*,*N*-dioxide (**5**; for a related synthetic compound, see Ref. [14]). NMR and HRMS spectra of the second derivative of **3** indicated sulfonylation at C-3 in this metabolite (→**6**). In addition to **1**–**8**, which were consistently produced by *OE::lnaA* but not Δ*lnaA*, we detected occasional production of several other metabolites, whose structures (**10**–**13**) appeared unrelated to those of **1**–**8**, suggesting that they are not derived from the *lna* biosynthetic pathway. Among these, compound **10** is a novel metabolite featuring an intriguing ring system that is likely derived from the known *A. flavus* terpenoid aflavinine (see the Supporting Information, Section 9).[15]

Next we investigated whether production of **1**–**8** can be elicited under specific conditions in wild-type *A. flavus*, and whether these metabolites are in fact strictly *lna*-dependent (see Table 1). Highly sensitive single-ion-monitoring MS (SIMMS) analysis of Δ*lnaA*, WT, and *OE::lnaA* cultures grown using conditions previously shown to support *lnaA* expression[2c] revealed the presence of piperazines **1** and **2** in WT, although at roughly 100-fold lower concentrations than in *OE::lnaA* (see the Supporting Information, Section 11). Pyrazine **3** was found only in some, but not all WT extracts, whereas **4**–**8** were not detected. *OE::lnaA* produced compounds **1**–**3**, **7**, and **8**, whereas **4**–**6** were absent under these culturing conditions. Notably, SIMMS also detected very small quantities of **1** and **2** in Δ*lnaA* extracts, at levels roughly 10 times lower than in WT. This suggested that **1** and **2** are perhaps also produced by enzymes encoded by the homologous *lnb* cluster. To test this hypothesis, extracts derived from two different Δ*lnaA, KD::lnbA* double-mutant strains were

Table 1: *A. flavus* strains (also see Table S3) and occurrence of *lna*/*lnb*-associated metabolites **1**–**8**.[a]

Cmpd	*OE::lnaA* (rich medium)	*OE::lnaA*	*OE::lnaA, KD::lnaB*	Wild type	Δ*lnaA*	Δ*lnaA, KDlnbA*
1	+++	+++	+++	++	+	–
2	+++	+++	+++	++	+	–
3	+++	+++	+++	++*	–	–
4	+++	–	–	–	–	–
5	+++	–	–	–	–	–
6	+++	–	–	–	–	–
7	+++	+++	+++	–	–	–
8	+++	+++	+++	–	–	–

[a] "+++" = abundant, detected by DANS and MS, "++" = detected by MS only ("*" = occasionally detected), and "+" = detected by MS, roughly 10-old less abundant than in WT. Strains were cultured on GMM unless indicated otherwise.

analyzed by HPLC/ESI$^+$-SIMMS, which showed that compounds **1**–**8** are completely absent in the double mutants, whereas WT and Δ*lnaA*, grown as positive controls in parallel, produced **1** and **2** as before (Figure S4). These results indicate that the amounts of **1** and **2** found in Δ*lnaA* are derived from *lnbA* expression and thus the *lna* and *lnb* clusters (Figure 1 a) encode partially redundant biosynthetic pathways.

Since the structures of **1**–**8** appear to be tyrosine (Tyr) derived, the two putative NRPS proteins LnaA and LnbA were tested for their ability to specifically activate Tyr by assaying amino acid dependent ATP-[^{32}P]-pyrophosphate exchange activity.[16] Both LnaA and LnbA specifically activated L-Tyr, although LnaA was somewhat DL-unspecific and also activated D-Tyr (Figure 2 a). However, neither LnaA nor LnbA include a condensation domain, and correspondingly, none of the *lna*- or *lnb*-associated metabolites identified in the WT nor in the *OE::lnaA* background feature a peptide bond. In addition, neither protein activated the L-Tyr-L-Tyr dipeptide (Figure S5). However, both LnaA and LnbA include C-terminal R- (putative short-chain reductase and/or epimerase) domains, with amino acid sequence similarity to other microbial reductase domains.[17] Based on the chemical structures of the identified *lna*- and *lnb*-dependent metabolites, it appeared likely that the R-domains in LnaA and LnbA are involved in the reduction of L-Tyr derivatives. In a biosynthetic model, LnaA- and/or LnbA-derived L-Tyr aldehyde (**15**) may form a dimer such as **17**, perhaps via a tethered intermediate, for example, **16** (Figure 2 b). Formation of the most abundant *lna*- or *lnb*-associated metabolites (**1**, **2**, **7**, **8**) requires additional reduction from aldehydes or imines to the corresponding amines and alcohols. These reductive steps seemed likely to involve putative NmrA-like proteins encoded by *lnaB* and *lnbB* (Figure 1 a), as NmrA-like proteins are believed to serve enzymatic functions as epimerases or reductases,[18] in addition to possible roles in gene regulation.[19]

To examine the putative role of LnaB in the biosynthesis of **1** and **2**, the metabolite profile of an *OE::lnaA, KD::lnaB* double-mutant strain was compared to that of *OE::lnaA* by 2D NMR and HPLC/ESI$^+$-SIMMS studies. These analyses showed that the morpholine **7** is much more abundant in *OE::lnaA*, *KD::lnaB* than in the *OE::lnaA* background, relative to the piperazines **1** and **2** (Figure 2 c). This shift towards a relatively greater production of morpholine **7**, whose biosynthesis requires one reduction step less than production of **1** and **2**, suggests that LnaB participates in the reduction of LnaA-derived intermediates. Knock-down of *lnaB* would result in increased accumulation of **17**, which may partially undergo hydrolysis leading to increased production of **7** (Figure 2 b, Figure S6). Notably, production of piperazines **1** and **2** is not fully abolished in the *OE::lnaA*, *KD::lnaB* strain, and formation of morpholine **7** from L-Tyr aldehyde (**15**) or **17** still requires one reductive step. The residual reductase activity in the *OE::lnaA*, *KD::lnaB* double mutant could result from expression of the second *nmrA*-like gene *lnbB* (or another related gene proximal to *lnbB*, Table S1) or

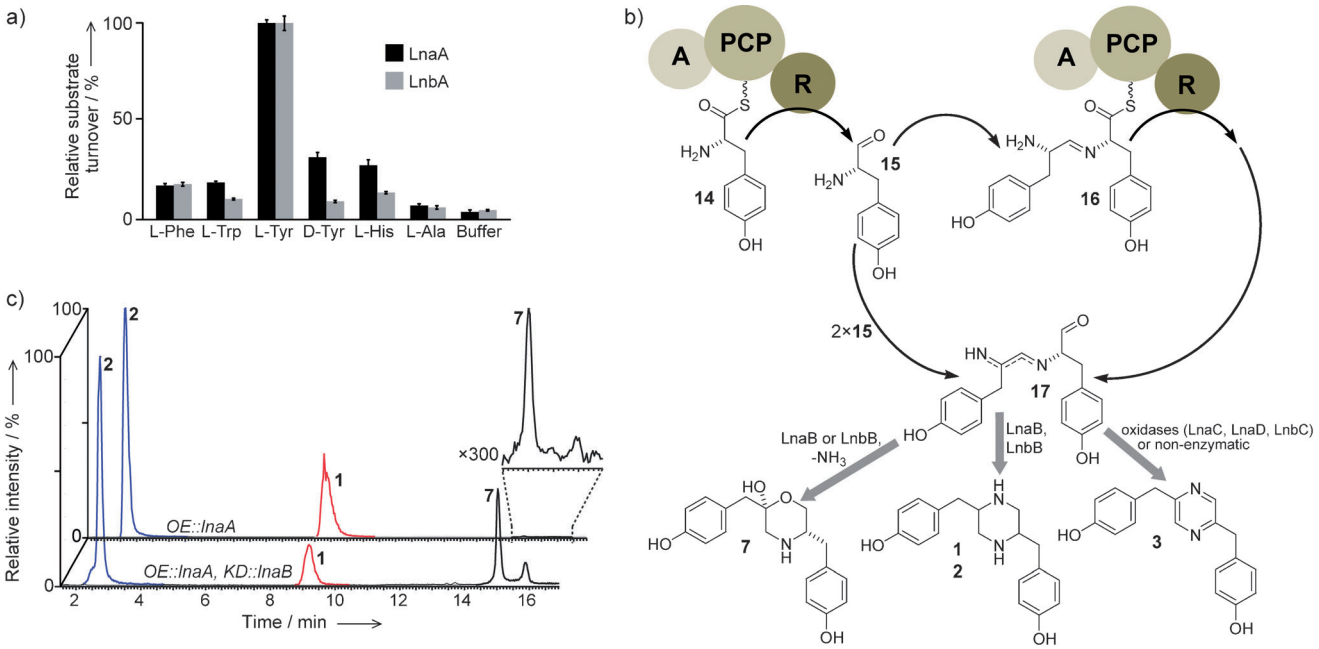

Figure 2. a) Substrate specificity of LnaA (black bars) and LnbA (gray bars). Recombinant LnaA and LnbA were tested for their ability to adenylate various amino acids. Shown are relative activity values normalized to L-Tyr activity. Error bars: standard deviation. b) Simplified model for the biosynthesis of *lnaA*-dependent metabolites by *lna* and *lnb*- cluster genes (see also Figure S6). Tethered L-Tyr (**14**) is reduced to **15** by the R-domains of LnaA or LnbA, resulting in the formation of a dimeric imine (**17**) potentially via a tethered intermediate (**16**). Cyclization and reduction by LnaB (and perhaps LnbB) leads to formation of **1** or **2**, whereas oxidation yields **3**. In the absence of LnaB, isomerization of **17** (or a related intermediate) to the enamine followed by loss of ammonia leads to increased formation of **7**. c) Single-ion chromatograms from HPLC/ESI$^+$-SIMMS analysis corresponding to **1**, **2**, and **7** for the *OE::lnaA* and *OE::lnaA*, *KD::lnaB* strains. The *OE::lnaA*, *KD::lnaB* strain shows a dramatic increase in production of **7** relative to **1** and **2**, compared to the *OE::lnaA* strain.

incomplete silencing of *lnaB*. Lastly, whereas cyclization of **17** to a corresponding diimine followed by LnaB- (or LnbB-) mediated reduction leads to piperazines **1** and **2**, oxidation, non-enzymatically or catalyzed by putative oxidases LnaC, LnaD, or LnbC, would explain formation of pyrazine **3**.

HPLC/ESI$^+$-SIMMS analysis further showed that the ratio of the two diastereomers **1** and **2** is significantly greater in WT than in Δ*lnaA* (Figure 3a and Figure S7), suggesting that the *lnb* pathway produces relatively larger amounts of the

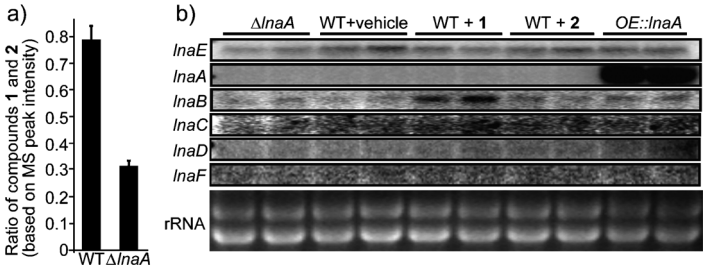

Figure 3. a) Comparison of the diastereomeric ratios of compounds **1** and **2** in WT and Δ*lnaA* metabolite extracts. Error bars: standard deviation. b) Effect of supplementation with synthetic **1** or **2** on *lna* gene cluster expression. Northern analysis showed that addition of **1**, but not **2** increased *lnaB* expression. rRNA served as loading control.

(2*R**,5*S**) isomer, **2**, than the *lna* pathway. Given that LnbA activates L-Tyr with high selectivity, it seems unlikely that this greater relative abundance of **2** results from incorporation of both D-Tyr and L-Tyr. Instead, these stereochemical differences probably originate from different degrees of epimerization at the aldehyde or imine stage. Next, we asked whether the ratio of diastereomers is actively regulated at the gene expression level. Northern analysis of WT cultures incubated with synthetic **1** or **2** showed that treatment with **2** did not lead to any significant changes in *lna* gene expression, whereas addition of **1** to WT cultures greatly increased expression of *lnaB* (Figure 3b). This result strongly suggests metabolite-mediated crosstalk between the *lna* and *lnb* pathways.

In conclusion, comparative metabolomics revealed eight *lna*-associated metabolites, **1**–**8**, of which **1**–**3** could be detected in WT *A. flavus*. The two most consistently produced *lna* metabolites, **1** and **2**, are also produced under participation of *lnb* genes, providing a first example for partially redundant biosynthesis involving an NRPS-like pathway. LnaA and LnbA deviate from canonical NRPS domain structures in that they lack condensation domains, and thus, as the structures of identified metabolites suggest, do not act as peptide synthetases. Instead, LnaA and LnbA follow the domain layout of Lys2 and related fungal enzymes that serve as α-aminoadipate aldehyde reductases in fungal L-lysine biosynthesis (24% and 25% identity of LnaA and LnaB to *S. cerevisiae* Lys2, respectively[9]). Examination of fungal genomes showed that LnaA-like proteins are primarily confined to the Ascomycete taxon Plectomycetes, with between one and five different LnaA-like proteins in different Aspergilli. Recently published genomes (e.g., of *Serpula lacrymans* and *Heterobasidion annosum*[20]) show that LnaA/LnbA-type enzymes are also encoded in basidiomycete genomes. The piperazinomycin-producing bacteria (*Streptomyces* sp.) have not been sequenced; however, available *Streptomyces* genomes contain a number of putative genes encoding proteins with high (up to 38%) amino acid sequence similarity to LnaA. The close similarity of some bacterial NRPS-like proteins to LnaA/LnaB despite bacteria not synthesizing lysine by the Lys2 pathway suggests a complex evolution of these proteins.[21]

Our work provides the first evidence that Lys2-type enzymes also function in secondary metabolism and additionally suggests that NRPS-like proteins may serve as amino acid reductases, indicating that a reaction hitherto considered as an NRP-offloading mode can stand by itself. This study also demonstrates the consequences of *lnaA*/*lnbA* gene disruption/silencing and the concomitant loss of piperazine/pyrazine/morpholine secondary products on fungal development, linking sclerotia formation in *A. flavus* and the *lna*/*lnb* metabolic pathways. This observation adds a unique role to previously known functions of NRPS-like genes in fungi. The occurrence of a functionally duplicated biosynthetic pathway can be interpreted as a safeguard to ensure timely sclerotial production and hence, persistence during unfavorable environmental conditions. Our results are suggestive of a complex signaling network regulating the biosynthesis of the *lna*- and *lnb*-pathway metabolites, which may include cross-pathway interactions mediated by sensing of **1** and **2** or shared biosynthetic intermediates. Detailed analysis of the metabolomes and associated phenotypes of *lna*/*lnb* single and double knock-out strains will be required to clarify the extent of interactions between the *lna*/*lnb* clusters and determine the roles of the identified metabolites for sclerotia formation and other aspects of *A. flavus* biology.

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