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Application of an Efficient Gene Targeting System Linking Secondary Metabolites to their Biosynthetic Genes in *Aspergillus terreus*

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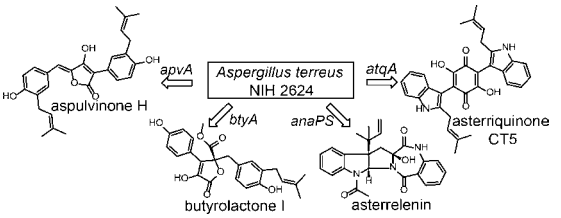
ABSTRACT



Nonribosomal peptides (NRPs) are natural products biosynthesized by NRP synthetases. A *kusA*-, *pyrG*- mutant strain of *Aspergillus terreus* NIH 2624 was developed that greatly facilitated the gene targeting efficiency in this organism. Application of this tool allowed us to link four major types of NRP-related secondary metabolites to their responsible genes in *A. terreus*. In addition, an NRP affecting melanin synthesis was also identified in this species.

Aspergillus terreus, the producer of lovastatin, biosynthesizes a number of nonribosomal peptide (NRP)-related natural products with diverse biological activities. Examples include the antitumor metabolites asterriquinones and butyrolactones.[1−3] Analysis by Secondary Metabolite Unique Regions Finder (SMURF)[4] showed that the genome sequenced *A. terreus* NIH 2624 strain contains 38 NRPS genes and one hybrid PKS (polyketide synthase)-NRPS gene. Little is known about the secondary metabolites produced by these NRPSs in *A. terreus*, due in large part to the lack of available tools for efficient genetic manipulation of this fungal species. A previous study successfully linked the hybrid gene ATEG_00325.1 (*ftmA*) to its secondary metabolite, the root toxin flavipucine.[5] Recently, a cluster of nine genes including one NRPS gene *ataP* was shown to be responsible for acetylaranotin biosynthesis in *A. terreus*.[6]

In this study, several laboratory culture media including glucose minimal medium (GMM), yeast agar glucose (YAG) medium and lactose dextrose minimal medium (LCMM) were tested to identify secondary metabolites

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biosynthesized by wild type *A. terreus*. HPLC-DAD-MS analyses allowed us to identify five major types of UV-active natural products including indole alkaloids (**1** and **4**), asterriquinone CT5 (**8**), aspulvinones (**7** and **9**), butyrolactones (**2** and **6**) and terretonins (**3** and **5**) from the ethyl acetate extract of *A. terreus* (Figure 1). Previous studies have reported the identification of the polyketide synthase (PKS) gene in *A. terreus* involved in terretonin biosynthesis.[7,8] Here we focused on identifying the gene clusters that are responsible for the biosynthesis of additional natural products that are synthesized by *A. terreus* on various growth conditions. As detailed below, their structures suggest that they are biosynthesized via an NRP or NRP-like synthesis pathway.[1,3,9]

To facilitate the rapid generation of a large number of gene deletions we created a *kusA*-, *pyrG*- double mutant strain of *A. terreus* NIH 2624. We used this strain to link the NRPS and NRPS-like genes in *A. terreus* to their corresponding secondary metabolites. The *kusA* gene deletion improves gene targeting efficiency due to high homologous recombination rates.[5,10] To create the *kusA* deletion strain, we replaced the *kusA* gene in the wild type strain with the hygromycin resistance marker (*hph*) (Figure 2A). Next we created a *pyrG* auxotrophic mutant in the *kusA*- background using a previous described approach.[11] In brief, we first amplified sequences from the *pyrG* coding region that were 30 base pairs apart. These two fragments were joined using fusion PCR and the resulting amplicon generates a small deletion within *pyrG* when integrated through homologous recombination (Figure 2B). Transformants that contain this construct integrated at the *pyrG* locus were selected on media supplemented with uracil and 5-fluoroorotic acid (FOA). The FOA is toxic to cells that contain a functional *pyrG* gene and therefore only the *pyrG* auxotrophic mutants can grow on this media.

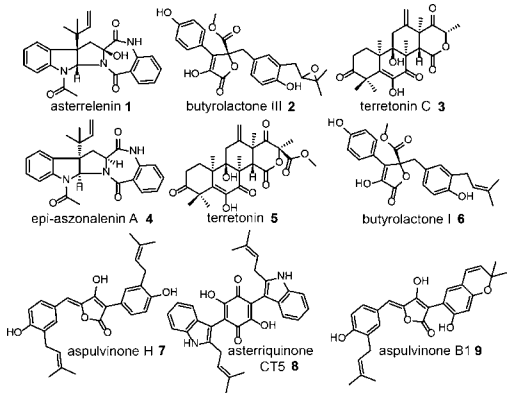

Figure 1. Natural products isolated in this study.

Identification of the NRPS and NRPS-like genes responsible for the dihydroxybenzoquinone core of **8** and the indole alkaloid core of **1** and **4** was facilitated by the fact that the biosynthesis pathways of structurally similar compounds have been identified in *A. nidulans* and *Neosartorya fischeri*, respectively. The structural similarity between terrequinone A and **8** suggests that there is a cluster in *A. terreus* that is orthologous to the terrequinone

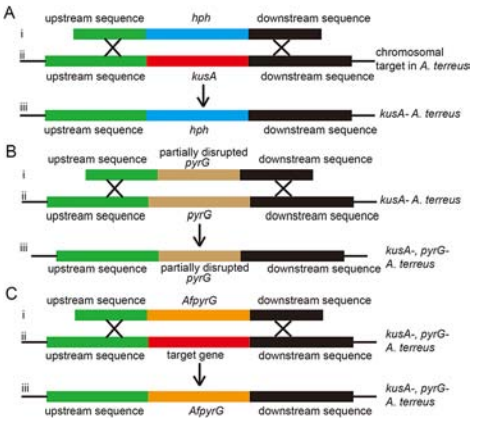

Figure 2. Genetic manipulation of *A. terreus* strains.

A cluster (*tdi* cluster) in *A. nidulans*.[1] A blast search of the NRPS-like gene (*tdiA*) enabled us to locate one of its homologues, encoded by the gene ATEG_00700.1 (*atqA*), in *A. terreus*. As for **1** and **4**, we used the acetylaszonalenin NRPS in *Neosartorya fischeri*[9] for our blast search and we identified ATEG_10305.1 (*anaPS*) as the most likely candidate. We created an *atqA* deletion strain and an *anaPS* deletion strain by replacing the targeted gene with the *A. fumigatus pyrG* (*AfpyrG*) marker in a *kusA*-, *pyrG*- background (Figure 2C). The deletants were verified by diagnostic PCR (Table S2 and Figure S3, Supporting Information). Examination of the secondary metabolite profiles of the *atqA* deletion strain in comparison with the wild type strain clearly showed that compound **8** is missing in the deletion strain and is still produced in the wild type strain (Figure 3). A similar comparison of the *anaPS* deletion strain with the wild type strain showed the disappearance of compounds **1** and **4** in the deletant strain (Figure 3).

The identification of genes responsible for the biosynthesis of the core structures of butyrolactones and aspulvinones was more difficult because genes for the biosynthesis of these types of fungal natural products were unknown prior to this study. Early biosynthesis studies were limited to use of labeled precursors which showed that phenylalanine and tyrosine

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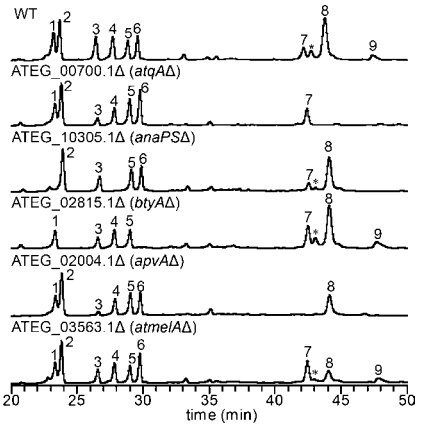

Figure 3. HPLC traces of the wildtype and the mutants at total spectrum scan by DAD detector. *This metabolite is related with aspulvinones according to its UV absorption spectrum but not isolated due to low production yield. Numbering on peaks corresponds to Figure 1.

could be incorporated into butyrolactones.[3,12] The use of amino acids in the pathway but lack of peptide bonds in their structures (copmounds **2**, **6**, **7**, and **9**) suggests that NRPS-like genes with A-T-TE domain structures are likely responsible for the biosynthesis of these two families of natural products.[1] The TE domain could catalyze release of the final product by hydrolysis to the free acid or macrocyclization to an amide or ester.[13] A recent study also shows that the TE domain of TdiA could catalyze a double Claisen condensation to generate two carbon−carbon bonds followed by the release of the dihydroxybenzoquinone core.[1] Another study reveals a TE domain of CurM, the PKS involved in curacin A biosynthesis, could direct a hydrolysis coupled with a decarboxylative elimination to yield a terminal olefin moiety.[14]

To locate the genes for the biosynthesis of butyrolactones and aspulvinones biosynthesis we targeted 19 NRPS-like genes in *A. terreus* with the A-T-TE domain architecture or similar for deletion. The targeted genes were individually replaced by the *AfpyrG* marker and verified by diagnostic PCR (Figure 2C). It should be noted that using a *kusA*-, *pyrG*- strain resulted in a significant improvement in gene targeting efficiency (Table S2, Supporting Information).

Comparison of the secondary metabolite profiles of each of the 19 NRPS-like deletion strains to the control strain allowed us to identify the NRPS-like gene *btyA* ATEG_02815.1 as the gene responsible for the biosynthesis of the butyrolactone core of **2** and **6**. The same comparative analysis identified the NRPS-like gene ATEG_02004.1 which we named *apvA* as the gene required for the production of the aspulvinone core of **7** and **9**. Recently the NRPS-like genes responsible for the biosynthesis of ralfuranone and microperfuranone, natural products that shares structural similarity with butyrolactones and aspulvinones were reported.[15,16] Our data suggest that the biosynthetic route of butyrolactones and aspulvinones are similar to that of ralfuranone and microperfuranone.

BLAST alignment analysis of the *btyA* and *apvA* genes reveals strong similarity between the proteins in which they encode. The structures of the two final products, butyrolactones and aspulvinones, suggest that both BtyA and ApvA utilize the same *p*-hydroxyl PPA (HPPA) substrates but undergo different cyclization mechanisms. For butyrolactone biosynthesis we propose that HPPA is activated by the BtyA A domain to AMP-HPPA (Figure 4A). The activated HPPA is then loaded to the T domain and transferred to the TE domain. Upon loading of another HPPA unit to the T domain, the TE domain promotes the enolate formation on the unit attached. Then aldol condensation establishes the carbon−carbon bond between the two units, followed by ester cyclization, and keto−enol tautomerization to yield the γ-butyrolactone core. Hydrolysis, and finally esterification of the exposed carboxylic acid group yields butyrolactone II. Two additional enzymes, a prenyltransferase and an epoxidase, may be involved in the tailoring modifications of butyrolactone II to give compounds **2** and **6** (Figure 4A).

For aspulvinone biosynthesis, we propose that HPPA is the substrate of ApvA and the mechanism of activating and loading HPPA onto the TE domain closely parallels that of BtyA. The TE domain promotes the enolate formation on the unit attached. The next step involves head to tail Claisen condensation, followed by the keto−enol tautermerization and a nucleophilic attack on the carbonyl carbon to yield the furanone partial structure. A spontaneous oxidation at the β-carbon of the thioester might occur in aerobic condition. The TE domain then catalyzes the hydrolysis of the thioester, followed by spontaneous decarboxylation, dehydroxylation and keto−enol tautermerization to give the aspulvinone core (Figure 4B). The structural diversity of the aspulvinones[17] suggests that other tailoring enzymes are involved. Since secondary metabolite genes are usually clustered,[18] we examined the genes proximal to *apvA*. The presumed enzyme products of these genes are predicted to be essential for fungal survival (Table S3, Supporting Information) and might not be involved in the tailoring modifications of aspulvinone core, suggesting that these tailoring genes for the aspulvinones biosynthesis may reside in a different locus in the *A. terreus* genome.

One albino mutant was identified after deleting the gene ATEG_03563.1 (*atmelA*) (Figure S4, Supporting Information).

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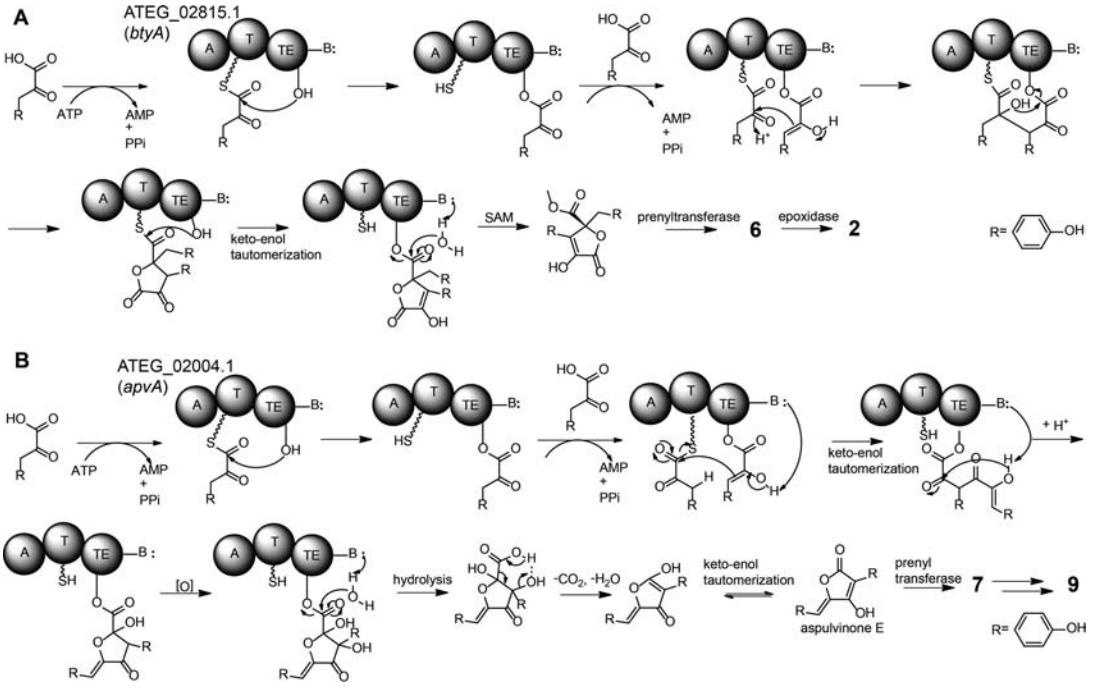

Figure 4. Proposed biosynthesis pathway for (A) butyrolactones and (B) aspulvinones.

The albino phenotype was not identified in the aforementioned mutant strains, indicating that the morphological change was not due to changes in any secondary metabolites we had identified. This suggests that the NRPS-like gene ATEG_03563.1 is involved in conidial pigment synthesis in *A. terreus*. Due to melanins' large molecular size and insolubility in any aqueous or organic solvent, the structure of natural melanin remains to be clarified.[19] The majority of melanins belong to two types, dihydroxyphenylalanin (DOPA)-melanin and dihydroxynaphthalene (DHN)-melanin.[19] *A. fumigatus* can also produce an alternative pyomelanin via an L-tyrosine degradation pathway.[20] The LC-MS profile of the *atmelA* deletant strain was almost the same as that of wild type (Figure 3). However, it is possible that a different extraction condition or chromatographic method might reveal the monomer substrate, which could be the first intermediate in the melanin biosynthesis, produced by AtmelA. An alternative possibility is that this NRPS-like gene *atmelA* is involved, but not directly related, to the melanin biosynthesis in *A. terreus* and thus would not produce the first substrate to be incorporated and condensed to form melanin. The product of AtmelA might regulate the production of melanin via an unknown mechanism that needs to be further explored.

In conclusion, we reported here the development of an effective gene targeting system in *A. terreus*. Application of this tool allowed us to quickly and efficiently construct an NRPS gene deletion library containing 21 mutants. Analyses of the secondary metabolite profiles of these mutants enabled us to link four NRPS genes in *A. terreus* to their natural products. In our study, three families of natural products asterriquinone CT5 (**8**), butyrolactones (**2** and **6**) and aspulvinones (**7** and **9**) were associated with genes encoding NRPS-like enzymes terminating with a TE domain and missing the canonical C domain found in NRPS enzymes. Our experiments provided evidence that fungi are able to utilize similar enzymes to produce a diversity of secondary metabolites.

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Supporting Information Available. General methods, compounds characterization with spectral data, and diagnostic PCR results. This material is available free of charge via the Internet at http://pubs.acs.org.

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The authors declare no competing financial interest.