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A New Protein Factor in the Product Formation of Non-Reducing Fungal Polyketide Synthase with a *C*-Terminus Reductive Domain S

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Azaphilone polyketides are synthesized by iterative non-reducing fungal polyketide synthases (NR-fPKSs) with a *C*-terminus reductive domain (-R). Several azaphilone biosynthetic gene clusters contain a putative serine hydrolase gene; the *Monascus* azaphilone pigment (MAzP) gene cluster harbors *mppD*. The MAzP productivity was significantly reduced by a knockout of *mppD*, and the MAzP NR-fPKS-R gene (*MpPKS5*) generated its product in yeast only when co-expressed with *mppD*. Site-directed mutations of *mppD* for conserved Ser/Asp/His residues abolished the product formation from the *MpPKS5*/*mppD* co-expression. MppD and its homologs are thus proposed as a new protein factor involved in the product formation of NR-fPKS-R.

Keywords: *Monascus* pigment, azaphilone polyketide, non-reducing fungal polyketide synthase, MppD, Ser/Asp/His catalytic triad

Polyketide pathways, categorized by their common biochemical strategy for *C-C* bond formation, generate structurally diverse natural products with diverse biological activities, including antibacterial, antifungal, antitumor, and immunomodulating properties. A polyketide backbone is generated by the assembly of acetate and its derivatives through decarboxylative Claisen condensation of their α-carboxylated thioester forms. This condensation is carried out between the acyl-thioester that is attached to the catalytic Cys residue of β-ketoacyl-thioester synthase and an incoming malonyl-thioester, generally malonyl-acyl carrier protein (-ACP) or methylmalonyl-ACP. In this basic theme, the biosynthetic mechanism of a polyketide is comparable to that of a fatty acid [14]. Polyketide biosynthesis differs from that of a fatty acid because reductive modification of the β-carbonyl moiety is optional [19]. One of the options is a total lack of reductive modifications, and the cognate enzyme is called a non-reducing polyketide synthase (NR-PKS), which is the topic of this study. Hypothetical poly-β-carbonyl chains that are generated by NR-PKS are regiospecifically cyclized to form aromatic ring structures [10]. The NR-PKS of fungal origin (NR-fPKS) operates iteratively and belongs to the type I PKS that is defined as a multidomain polypeptide. Together with the cyclization pattern and the final number of iterations, a selection of starting acyl units and α-methyl decorations are the basic origins for the structural diversity of NR-fPKS products [9, 19].

Azaphilones are a family of fungal polyketides possessing a highly oxygenated pyranoquinone bicyclic core. Azaphilone polyketides display diverse biological activities and are known to be potent in interfering with specific protein-protein interactions [12]. The name azaphilone represents an affinity for nitrogen and originates from the tendency of the 4*H*-pyran moiety to form a vinylogous 4-pyridone structure. Some azaphilones that have reduced

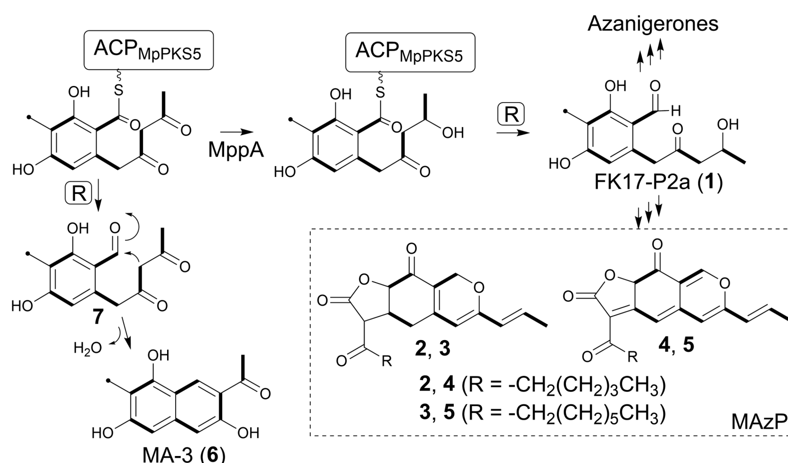

Fig. 1. Biosynthetic pathway of MAzP.

Bold lines and black circles denote acetate units and *S*-adenosylmethionine-derived carbons.

4*H*-pyran moieties are not azaphilic, however. The biosynthesis of the pyranoquinone core was first proposed for AsPKS1, an *Acremonium strictum* NR-fPKS that bears a *C*-terminal reductive (R) domain and was used for 3-methylorcinaldehyde synthesis [1, 11]. This R domain was proposed to perform reductive release of a PKS-tethered thioester intermediate, generating free 3-methylorcinaldehyde. The R domain has been regarded as one among the four known product release catalysts for NR-fPKS; the three others are the thioesterase (TE) domain, TE/Claisen cyclase, and *trans*-acting TE [7].

The correlation between NR-fPKS-R and azaphilone biosynthesis was first demonstrated in azanigerone biosynthesis [22]. This study suggested that FK17-P2a (**1**) is synthesized by an NR-fPKS-R and an undefined ketoreductase (Fig. 1). A study on *Monascus* azaphilone pigment (MAzP, **2**-**5**) biosynthesis in *Monascus purpureus* substantiated that a NR-fPKS-R (MpPKS5) and a ketoreductase (MppA) generate **1** [5] (Fig. 1). In the absence of *mppA*, *M. purpureus* accumulated four C_{10}-bicyclic compounds, among which MA-3 (**6**) was suggested to be the direct product of MpPKS5 [5]. Another prominent azaphilone compound from *M. purpureus* is a mycotoxin citrinin, which is generally regarded as a member of azaphilone but it bears a 2,3-dihydro 4*H*-pyran moiety.

In an effort to delineate MAzP biosynthesis, several targeted gene inactivation mutants have been generated in the MAzP biosynthetic gene cluster and their product profiles were examined [2-5]. Among those mutants, a Δ*mppD* mutant displayed a low productivity of **2**-**5**, approximately 10% of that of the wild-type strain (WT) (Fig. 2). The gene inactivation scheme is shown in Fig. S1 and the supplementary data also contain other experimental details. HPLC-MS analysis supported the identities of **2**-**5** in each extract by detecting the peaks with relevant *m/z* values for these compounds (Fig. S2). The production levels of citrinin were comparable between WT and the Δ*mppD* mutant (Fig. 2 and S2). The protein ID of MppD is

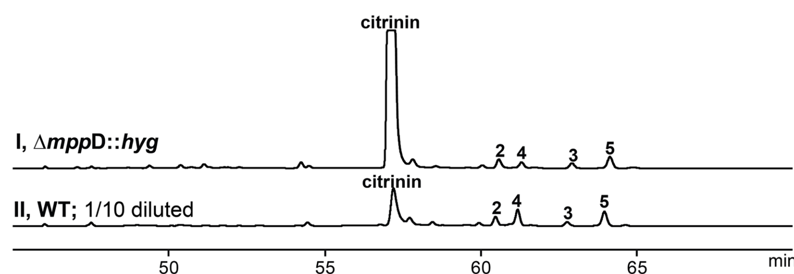

Fig. 2. HPLC analysis of the culture media extracts of *M. purpureus* WT and a Δ*mppD* mutant.

HPLC traces of the Δ*mppD* mutant (I) and WT (II), monitored at 330 nm, are drawn to the same scale. Both extracts were prepared with the same method and the WT extract was injected after being diluted 10 times.

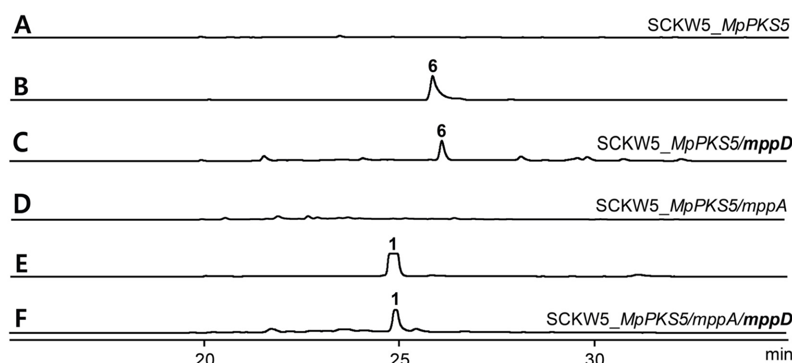

Fig. 3. HPLC chromatograms of the extracts from SCKW5 transformants of *MpPKS5* (A; cell), *MpPKS5/mppD* (C; cell), *MpPKS5/mppA* (D; medium), and *MpPKS5/mppD/mppA* (F; medium), compared with those of authentic **6** (B) and **1** (E) [5].
The traces are drawn to the same scale.

486313 in Monpu1_GeneCatalog_proteins_20130806.aa of the webpage of the Joint Genome Institute, Department of Energy, USA. A conserved domain search indicated that *mppD* belongs to the serine hydrolase family (CD domain accession pfam03959) [20]. Homologs of *mppD* exist in other azaphilone biosynthetic gene clusters: *ctnB* for citrinin [18], *afoC* for asperfuranone [6], and *azaC* for azanigerones [22]. It had been previously shown that neither *afoC* nor *ctnB* (in *Monascus aurantiacus*) is essential for final product formation, while the productivities were significantly impaired in each knockout mutant [6, 17]. It was thus envisioned that MppD homologs would play a pivotal role in the product formation from its genetically paired NR-fPKS-R.

In order to substantiate that MppD is involved in the production formation of MpPKS5, we conducted ectopic expression of *MpPKS5* in conjunction with *mppD*, by using the *Saccharomyces cerevisiae* SCKW5 strain (*matB*/*npgA*) [15]. It was predicted that *MpPKS5* expression would result in the production of **6** only when *mppD* is co-expressed (Fig. 1). A putative benzaldehyde intermediate **7** was proposed to undergo non-enzymatic Knoevenagel aldol condensation, generating **6**, a C_{10}-bicyclic compound [5]. As predicted, *MpPKS5/mppD* expression produced **6**, which was not found upon *MpPKS5* expression (Fig. 3, traces A–C). The product of *mppA* belongs to the Rossmanfold $NAD(P)^+$-binding protein family (cd05233, COG1028) and was proposed to mediate ω-2 ketoreduction of a polyketide intermediate tethered on the ACP-domain of MpPKS5 [5] (Fig. 1). Thus, co-expression of *mppA* with *MpPKS5/mppD* was expected to generate **1** instead of **6**. When *mppA* was co-expressed with *MpPKS5/mppD*, **1** was produced (Fig. 3, traces D–F), supporting the proposed biosynthetic pathway of **1** (Fig. 1). Hence MppD seems to be an obligate accessory protein involved in the product formation of MpPKS5.

The serine hydrolase family encompassing the MppD homologs has a catalytic Ser/Asp/His triad (S122/D215/H243 for MppD) [20]. To assert a catalytic role for MppD,

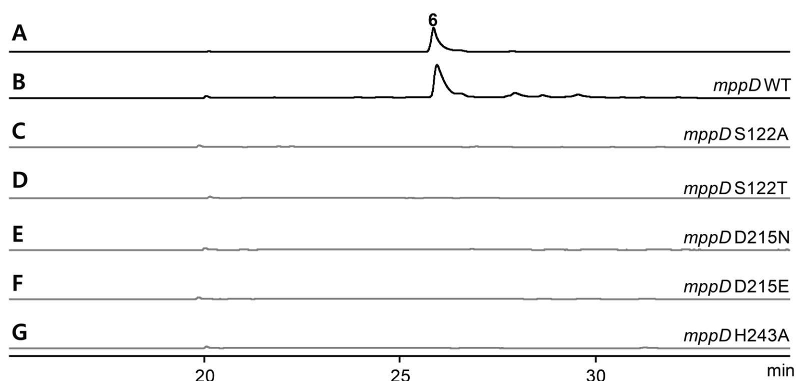

Fig. 4. Mutagenesis study of *mppD* in the SCKW5 transformant of *MpPKS5/mppD*. The identity of each sample is shown on the right edge of each lane. The traces are drawn to the same scale.

site-directed mutation experiments were performed. Contrary to *mppD*, the co-expression of mutated copies of *mppD* (S122A, S122T, D215N, D215E, and H243A) with *MpPKS5* was incapable of producing **6** (Fig. 4).

We examined co-localization patterns of *mppD* homologs and NR-fPKS-R genes in other filamentous fungal genomes. In the *Aspergillus terreus* genome, at least two MppD homologs are predicted (the sequence identity higher than 40%). The two hits are ATEG_03437 and ATEG_07663. The ATEG_03437 gene is neighbored by ATEG_03432, whose product is predicted to be identical to MpPKS5 [5, 8]. ATEG_07663 (*afoC*) belongs to the *afo* gene cluster (the locus tag of the NR-fPKS-R gene *afoE* is ATEG_07661) [8]. Another NR-fPKS-R gene (ATEG_08662) is not neighbored by an *mppD* homolog, however. Based on the genome information, *Aspergillus nidulans* is predicted to harbor six NR-fPKS-R genes, four of which are clustered with an *mppD* homolog but two of which (*AN3230.2* and *AN3386.2*) are not. This genome analysis indicates that NR-fPKS-R genes are classified into two types depending on co-localization with an *mppD* homolog. Sequence comparison analysis of NR-fPKS-Rs failed in drawing a recognizable clade to discriminate between the suggested two types, however. An azaphilone biosynthetic gene cluster, which lacks an *mppD* homolog, was previously characterized for chaetoviridin/chaetomuglin biosynthesis in *Chaetomium globosum* [21]. Genome search indicates that the *C. globosum* genome contains no *mppD* homolog, but at least three NR-fPKS-R genes can be identified (CHGG_07645 to 07647 for *cazM*, CHGG_10027, and CHGG_09586).

In summary, the present study shows that MppD is an obligate accessory protein involved in the product formation of MpPKS5. This finding is significant because the *mppD* homologs are widespread in NR-fPKS-R gene clusters. For the case of the NR-fPKS-R gene neighbored with an *mppD* homolog, an ectopic expression of the NR-fPKS-R gene needs to include the neighboring *mppD*-like gene for a proper yield of polyketide product. However, the catalytic function of MppD is yet veiled and demands further biochemical characterizations. MppD may act as an editing hydrolase, like type II TE proteins in modular type I PKSs, promoting product formation through eliminating aberrant intermediates tethered on ACP [13, 16]. It is widely accepted that the terminal R-domain converts the thioester moiety on ACP into thiohemiacetal form and this unstable intermediate is readily cleaved into a benzaldehyde derivative such as **7** [11]. We could not exclude a possibility that MppD acts on this release process. A hypothesis is that a thiohemiacetal-ACP linkage is stabilized inside a pocket of MpPKS5, and MppD catalyzes the release of the aldehydic product(s).

Acknowledgments

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