Pentaketide Melanin Biosynthesis in *Aspergillus fumigatus*
Requires Chain-length Shortening of a Heptaketide Precursor*

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**Abstract**

Chain lengths and cyclization patterns of microbial polyketides are generally determined by polyketide synthases alone. Fungal polyketide melanins are often derived from a pentaketide 1,8-dihydroxynaphthalene, and pentaketide synthases are used for synthesis of the upstream pentaketide precursor, 1,3,6,8-tetrahydroxynaphthalene (1,3,6,8-THN). However, *Aspergillus fumigatus*, a human fungal pathogen, uses a heptaketide synthase (Alb1p) to synthesize its conidial pigment through a pentaketide pathway similar to that which produces 1,8-dihydroxynaphthalene-melanin. In this study we demonstrate that a novel protein, Ayg1p, is involved in the formation of 1,3,6,8-THN by chain-length shortening of a heptaketide precursor in *A. fumigatus*. Deletion of the ayg1 gene prevented the accumulation of 1,3,6,8-THN suggesting the involvement of ayg1 in 1,3,6,8-THN production. Genetic analyses of double-gene deletants suggested that Ayg1p catalyzes a novel biosynthetic step downstream of Alb1p and upstream of Arp2p (1,3,6,8-THN reductase). Further genetic and biochemical analyses of the reconstituted strains carrying alb1, ayg1, or alb1 + ayg1 indicated that Ayg1p is essential for synthesis of 1,3,6,8-THN in addition to Alb1p. Cell-free enzyme assays, using the crude Ayg1p protein extract, revealed that Ayg1p enzymatically shortened the heptaketide product of Alb1p to 1,3,6,8-THN. Thus, the protein Ayg1p facilitates the participation of a heptaketide synthase in a pentaketide pathway via a novel polyketide-shortening mechanism in *A. fumigatus*.

Polypeptides are important natural products that include numerous toxins, antibiotics, a variety of therapeutic compounds, fungal melanins, and other pigments. Polypeptides have attracted great attention because of their biosynthetic complexity and importance in the pharmaceutical industry. Extensive molecular genetic studies of polypeptide biosynthesis have been carried out in actinomycetes and Gram-positive bacteria. Microbial polyketides are generally assembled by three types of polyketide synthase (PKS)1 (1). Type I modular PKSs are large multifunctional polypeptides that consist of a number of modular units (modules), each of which is responsible for single β-ketoacyl condensation and the following reduction steps. Because modules are used sequentially and nonrepetitively, the number of modules determines the length of the carbon backbone of reduced complex-type polyketides. On the other hand, type II PKSs consist of several single-function enzymes that are used iteratively for bacterial aromatic polyketides, and the determinants for polyketide skeletons are still unclear. Type III are small plant chalcone synthase-type PKSs, and RppA was identified as the first microbial PKS of this class (2). Fungal PKSs fall into type I, consisting of a single large polypeptide with a set of active site domains similar to the modular type I PKSs, but they work iteratively to produce their specific products including both aromatic and reduced complex-type compounds. Thus they might be classified as an independent group of PKSs (3). Although several PKS genes have been identified from various fungal species (4–15), exactly how a fungal PKS synthesizes a specific polyketide remains unclear. In general, PKS is the sole determinant of the chain length and cyclization pattern of a polypeptide. However, a recent report showed that an accessory protein (LovC) is needed to enable the PKS (LovB) of *Aspergillus terreus* to synthesize a full-length polypeptide precursor, dihydromonacolin L, for lovastatin biosynthesis. This suggests that fungal PKS might be of a more complex nature than the modular PKSs (9).

Pentaketide melanins have been shown to be important virulence factors in fungal species pathogenic to plants or humans (13, 16–18). It is generally believed that acetyl-CoA and malonyl-CoA are the starter and extender of polypeptide synthases involved in the fungal 1,8-dihydroxynaphthalene (DHN)-melanin pathway. However, recently malonyl-CoA was demonstrated as the sole starter of *Colletotrichum lagenarium* PKS1 for production of the precursor 1,3,6,8-tetrahydroxynaphthalene (1,3,6,8-THN) (see Fig. IA) (19). The pentaketide, 1,3,6,8-THN, is then reduced by 1,3,6,8-THN reductase to scytalone, which is subsequently converted to DHN following the dehydration and reduction steps. Finally, DHN is polymerized to form DHN-melanin. Tricyclazole, a fungicide, specifically inhibits both THN reductase reactions involved in the DHN-melanin pathway (see Fig. IA) (20). To date, the genes encoding

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1 The abbreviations used are: PKS, polyketide synthase; DHN, 1,8-dihydroxynaphthalene; 1,3,6,8-THN, 1,3,6,8-tetrahydroxynaphthalene; YWA1, heptaketide naphthopyrone; ASA, asparagine-sucrose agar; HPLC, high-performance liquid chromatography; LC-APCIMS, liquid chromatography-atmospheric pressure chemical ionization mass spectrometry; kb, kilobase.

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PKS, THN reductases, and scytalone dehydratases have been characterized in several fungal species (21–24).

Aspergillus fumigatus, a ubiquitous fungus, causes allergy, noninvasive colonization, or life-threatening invasive pulmonary aspergillosis. A. fumigatus synthesizes its bluish green conidial pigment through a pentaketide pathway similar to the DHN-melanin pathway (24). Genetic and biochemical investigations have shown that biosynthesis of the conidial pentaketide melanin in A. fumigatus requires a six-gene cluster that includes the genes alb1, arp2, and arp1 coding for PKS, 1,3,6,8-THN reductase, and scytalone dehydratase, respectively (24). These enzymes also are referred to hereafter as the gene products Alb1p, Arp2p, and Arp1p. The amino acid sequence of Alb1p PKS has a significantly higher similarity to the heptaketide synthase WA of Aspergillus nidulans (67% identity, 80% similarity) than to the pentaketide synthase PKS1 of C. lagennarium (43% identity, 60% similarity). Therefore, Alb1p is likely a heptaketide synthase, which has been demonstrated by the heterologous expression of alb1 in Aspergillus oryzae (25). A. nidulans uses WA to synthesize a heptaketide naphthopyrone, YWA1 (see Fig. 1B), as a precursor for its green conidial pigment, but it does not use the DHN pentaketide pathway (26). On the other hand, C. lagennarium uses PKS1 to synthesize the pentaketide precursor 1,3,6,8-THN directly for DHN melanin (19, 27). To understand how A. fumigatus uses a pentaketide synthase to initiate the biosynthesis of a pentaketide melanin, we explored the possible involvement of accessory protein(s) in the biosynthetic pathway. We discovered a novel protein, Ayg1p, which is required for synthesis of the pentaketide 1,3,6,8-THN via a novel polyketide-shortening mechanism.

EXPERIMENTAL PROCEDURES

Strains and Media—The A. fumigatus strains used in this study are listed in Table I and Fig. 2, A and B. Strain B-5233 is a clinical isolate that produces blue-green conidia. Deletion strains of A. fumigatus were constructed by targeted gene disruption as described previously (28). Correct gene replacement events were confirmed by Southern blot analyses. For gene reconstitution in the six-gene cluster-deleted strain, RGD15, alb1 and/or ayg1 was reintroduced and confirmed by Southern blot analyses. A. oryzae strain M-2–3 harboring an argB auxotroph was used as a host for overexpression.

Aspergillus minimal medium contained 1% glucose, 10 mM NaNO₃, and trace elements (29). Malt extract medium contained 2% glucose, 2% malt extract, and 0.1% peptone. Cultures were grown at 37 °C. Asparagine-sucrose agar (ASA) medium is identical to the alkaline medium described previously (30). For tricyclazole inhibition assays, ASA was modified to contain 1% EtOH with or without 30 μg/ml tricyclazole (Lilly) (13). Controls with 1% EtOH alone or no EtOH were compared to exclude the possibility that 1% EtOH may affect morphology or conidial pigment production. A. fumigatus cultures used for thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) analyses were grown on ASA and potato-dextrose agar (Difco), respectively. For overexpression of ayg1, A. oryzae transformants were grown in Czapek-Dox medium containing starch (31).

Preparation and Analysis of Nucleic Acids—Isolation of total DNA from Aspergillus cultures was performed as described previously (32). A GeneCleave II kit (Bio101, Vista, CA) was used to purify recovered DNA fragments. DNA cloning and Southern blot analyses were performed according to standard protocols (33). Hybond™-N nylon membrane (Amersham Pharmacia Biotech) was used for blot analysis. DNA probes were labeled with [α-32P]dCTP (Amersham Pharmacia Biotech) using the Prime-It kit (Stratagene, La Jolla, CA).

Plasmids—Cosmid pG1–1, containing the vector pCosHX (Dr. J. Hamer, Purdue University) and a 42.5-kilobase (kb) genomic DNA fragment of A. fumigatus, was obtained via plasmid rescue from a complemented conidial color mutant, RP3/G1–1 (28). It carries a six-
gene cluster of 19 kb (6-, 11-, and 2-kb HindIII fragment) involved in conidial pigment biosynthesis (see Fig. 3A). Vector pBC-phleo was a gift from Dr. P. Silar (Centre de Génétique Moléculaire du Centre National de la Recherche Scientifique).

The six-gene cluster disruption construct, pRGD19, is a pBC KS+ (Stratagene)-based plasmid in which the 13.8-kb EcoRV-AvrII fragment (nucleotides 2006–15796, Fig. 3A) was replaced with a 2.8-kb hygromycin B resistance gene-selective marker (hph). The abi1 disruption construct, pRGD18, was made by replacing the 2.6-kb EcoRV-AvrII fragment (nucleotides 13197–15796, Fig. 3A) of the 6-kb AvrII-HindIII DNA fragment (nucleotides 10971–17029, Fig. 3A) with a 3-kb phleomycin resistance-selective marker (ble). The ayg1 disruption plasmid, RGD16, was constructed by replacing the 1-kb SmaI-BamHI fragment (nucleotides 6718–7725, Fig. 3A) of the 2.6-kb HindIII-SacI DNA fragment (nucleotides 6090–8765, Fig. 3A) containing ayg1 with a 3-kb phleomycin resistance selection marker.

The abi1 gene reconstitution construct, pALB1, contains the 9.2-kb SspI-HindIII DNA fragment (nucleotides 9756–18937, Fig. 3A) that carries the entire abi1 gene in pBCC-phleo. The ayg1 reconstitution construct, pAYG1, is a pBC-phleo-based plasmid and contains a 4.2-kb MluI-SacI DNA fragment (nucleotides 4580–8765, Fig. 3A) that carries the entire ayg1 gene. The abi1 + ayg1 reconstitution plasmid, pALBAYG51, was constructed by cloning the 3.5-kb blunt-ended NotI-SacI fragment (nucleotides 5250–8765, Fig. 3A) carrying the ayg1 gene into the blunt-ended NotI site of pALB1.

For overexpression of ayg1 in A. oryzae, the ayg1 gene was cloned into the pTAex3 expression plasmid to yield pTA-ayg1. It uses an α-amylase promoter of A. oryzae to express ayg1 and contains argB of A. nidulans as an auxotrophic selection marker (34).

Transformation of A. fumigatus and A. oryzae—A. fumigatus protoplasts were prepared with Mureinase (Amersham Pharmacia Biotech) and transformed using the polyethylene glycol method described by Yelton et al. (35). Transformants were selected on Aspergillus minimal medium containing hygromycin B (200 μg/ml) for hph-based constructs or phleomycin (30 μg/ml) for ble-based constructs. Transformation of A. oryzae protoplasts using argB as a selection marker was described previously (36).

Identification of Polyketide Products—The inhibitory effects of tricyclazole on the metabolism of 1,3,6,8-THN by B-5233 and RGD15 were visually compared using TLC analysis. The culture and TLC analysis conditions were as described previously (13, 37).

For HPLC analysis of polyketide products, A. fumigatus spore suspensions prepared from 40-h-old sporulating potato-dextrose agar cultures were acidified by HCl and then extracted with ethyl acetate. Extracts were dried with N2 gas and redissolved in acetonitrile (CH3CN) (27). HPLC analysis was carried out using a Tosoh 8020 with a photodiode array detector. A reverse phase column (Tosoh ODS-80Ts, 4.6 × 150 mm) was maintained at 40 °C with a solvent flow rate of 0.8 ml/min. A linear solvent gradient of 5–40% CH3CN in 2% acetic acid was used for the first 30 min; then 40–100% was used for the next 10 min. Liquid chromatography-atmospheric pressure chemical ionization
mass spectrometry (LC-APCIMS) was performed on an ion trap mass spectrometer (LCQ, ThermoQuest).

Cell-free Extract Preparation and in Vitro Enzyme Assay—For in vitro assay of Ayg1p activity, Ayg1p crude protein extract was obtained from 3-day-old cultures of A. oryzae transformed with pTA-ayg1 and grown in Czapek-Dox medium containing starch for induction of expression. Harvested mycelia were blended with 20 mM Tris-HCl buffer, pH 7.5, in a Waring blender. Polyclar AT was added (4.6% w/v) to absorb phenolic compounds. The sample was then gently stirred on ice for 30 min. The mixture was subsequently filtered through four layers of gauze and centrifuged at 17,400 × g for 20 min. The supernatant was used as the crude extract for cell-free enzyme assays. Enzyme activity was assayed at 30 °C in a 50 mM potassium phosphate buffer, pH 6.5, containing 50 μM YWA1 as substrate. The reaction mixture was then analyzed directly by HPLC. The conditions for HPLC analysis were as described above for polyketides except for the solvent gradient; the linear solvent gradient was 10–50% CH3CN in 2% acetic acid for 20 min.

RESULTS

Accumulation of 1,3,6,8-THN Is Absent in the ayg-1 Deletant—The Aspergillus fumigatus ayg1 deletant (RGD15) and the wild-type strain B-5233 responded differently to the presence of tricyclazole in the ASA medium. The wild-type strain produced blue green conidia under normal culture conditions; however, when grown on medium containing 30 μg/ml tricyclazole, it produced reddish pink conidia (24). Alteration of the conidial color was due to blockage of the 1,3,6,8-THN reduction...

FIG. 4. Phenotypes and HPLC analysis of the alb1 and/or ayg1 reconstituted strains. A, sporulated cultures of the six-gene cluster deletant and the reconstituted strains. B-5233, the wild-type strain; RGD19, the six-gene cluster deletant; BC-phleo, strain RGD19 transformed with the vector pBC-phleo; ALB6, the alb1 complemented strain; ALBAYG5, the alb1 and ayg1 complemented strain; and AYG1, the ayg1 complemented strain. B, HPLC analysis of the conidial pigment extracts. I, B-5233; II, ALB6; III, ALBAYG5; IV, AYG1; V, BC-phleo.
TABLE I
A. fumigatus strains

<table>
<thead>
<tr>
<th>Strains</th>
<th>Genotype</th>
<th>References</th>
</tr>
</thead>
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<tr>
<td>B-5233</td>
<td>Wild type</td>
<td>Clinical isolate</td>
</tr>
<tr>
<td>RGD12</td>
<td>alb1::hph</td>
<td>B-5233/GRD12–8 (13)</td>
</tr>
<tr>
<td>RGD10</td>
<td>arp2::hph</td>
<td>B-5233/GRD10–1 (24)</td>
</tr>
<tr>
<td>RGD15</td>
<td>ayg1::hph</td>
<td></td>
</tr>
<tr>
<td>RGD16</td>
<td>arp2::hph ayg1::ble</td>
<td>This study</td>
</tr>
<tr>
<td>RGD18</td>
<td>ayg1::hph alb1::ble</td>
<td>This study</td>
</tr>
<tr>
<td>RGD19</td>
<td>(alb1 arp1 arp2 ayg1 alb1::hph)</td>
<td></td>
</tr>
<tr>
<td>ALB6</td>
<td>RGD19 + pALB1</td>
<td>This study</td>
</tr>
<tr>
<td>ALBAYG5</td>
<td>RGD19 + pALBAYG51</td>
<td>This study</td>
</tr>
<tr>
<td>AYG1</td>
<td>RGD19 + pAYG1</td>
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<tr>
<td>BC-phleo</td>
<td>RGD19 + pBC-phleo</td>
<td>This study</td>
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A. fumigatus Polyketide Shortening

Polyketide Shortening in A. fumigatus

Because deletion of ayg1 prevented the accumulation of 1,3,6,8-THN and flaviolin, Ayg1p is likely to be involved in the biosynthetic step prior to 1,3,6,8-THN reduction (Fig. 1A). Previous studies showed that deletion of alb1 also blocked the production of 1,3,6,8-THN and flaviolin and deletion of arp2 (1,3,6,8-THN reductase) led to the accumulation of flaviolin (13, 24). To understand the function of ayg1, it is essential to find out the biosynthetic step catalyzed by Ayg1p. Because A. fumigatus is an assexual fungus, and standard genetic crosses are not feasible, double gene disruptions were carried out to determine the epistatic order of alb1, arp2, and ayg1. Conidial color was used as an indicator for the epistasis analysis of the genes because disruption of each individual gene resulted in a distinct conidial color (24). Disruption of both alb1 and ayg1 (RGD18) resulted in an albino conidial phenotype similar to the single alb1 deletant (RGD12) (Fig. 2A). This suggests that alb1 is epistatic to ayg1. On the other hand, the mature conidia produced by the arp2 and ayg1 double deletant (RGD16) had a yellow green color similar to that of the single ayg1 deletant suggesting that ayg1 is epistatic to arp2 (Fig. 2B, see colony centers). Additionally, alb1 but not arp2 is essential for 1,3,6,8-THN production, indicating that alb1 is epistatic to arp2 (24). These results imply that the order of the three enzymes in the pathway is Alb1p-Ayg1p-Arp2p and that there is an unknown biosynthetic step requiring Ayg1p in the production of 1,3,6,8-THN in A. fumigatus.

Reconstitution of alb1 and ayg1 in A. fumigatus

Restored 1,3,6,8-THN Production—The role of ayg1 in the pentaketide synthesis of A. fumigatus was further demonstrated by biochemical analyses of the alb1 and/or ayg1 reconstituted strains. To prevent the polyketides produced by Alb1p and Ayg1p from being metabolized by other enzymes involved in the pathway, the whole six-gene cluster was deleted by targeted gene replacement as illustrated in Fig. 3A. The gene disruption construct pRGD19 was used to transform B-5233. Transformants producing albino conidia were further analyzed by Southern analysis to determine whether these albino transformants had the expected deletion. Genomic DNA of B-5233 and an albino transformant, RGD19, were hybridized with the 13.8-kb EcoRV-AvrII DNA fragments that were replaced with the hygromycin-B resistance gene in the disruption cassette. B-5233 gave two hybridizing signals of 6.0 and 11 kb, whereas RGD19 did not reveal any hybridizing signal (Fig. 3B, panel I). The blot was stripped and rehybridized with the entire disruption cassette, pRGD19 (Fig. 3A). B-5233 gave two hybridizing signals of 6.0 kb and 11.0 kb, whereas RGD19 showed one hybridizing fragment of 6.0 kb (Fig. 3B, panel II). These results indicate that the whole gene cluster was disrupted in RGD19 via a double-crossover event, and the albino phenotype of RGD19 was the result of six-gene cluster disruption.

Strain RGD19 was then used as a recipient strain for alb1 and ayg1 reconstitution. RGD19 transformed with ayg1 (strain AYG1) retained albino whereas the alb1 transformant (ALB6) produced yellow conidia (Fig. 4A). HPLC analysis of the conid-

step by tricyclazole, which also caused the accumulation of flaviolin, an autoxidation product of 1,3,6,8-THN (Fig. 1A) (24). Under normal culture conditions, RGD15 produced yellow green conidia, which appeared yellow at an early stage and gradually became a greener color as the cultures aged (24). Unlike B-5233, the ayg1 deletant produced conidia of the same color on ASA medium with or without tricyclazole (data not shown). TLC analysis of culture extracts of the ayg1 deletant revealed that neither 1,3,6,8-THN nor its autoxidation product flaviolin had accumulated regardless of the presence of tricyclazole (data not shown). Therefore, deletion of ayg1 apparently prevented the synthesis of 1,3,6,8-THN and flaviolin.

Epistatic Order Is Determined as alb1-ayg1-arp2—Because deletion of ayg1 prevented the accumulation of 1,3,6,8-THN and flaviolin, Ayg1p is likely to be involved in the biosynthetic step prior to 1,3,6,8-THN reduction (Fig. 1A). Previous studies showed that deletion of alb1 also blocked the production of 1,3,6,8-THN and flaviolin and deletion of arp2 (1,3,6,8-THN reductase) led to the accumulation of flaviolin (13, 24). To understand the function of ayg1, it is essential to find out the biosynthetic step catalyzed by Ayg1p. Because A. fumigatus is an assexual fungus, and standard genetic crosses are not feasible, double gene disruptions were carried out to determine the epistatic order of alb1, arp2, and ayg1. Conidial color was used as an indicator for the epistasis analysis of the genes because disruption of each individual gene resulted in a distinct conidial color (24). Disruption of both alb1 and ayg1 (RGD18) resulted in an albino conidial phenotype similar to the single alb1 deletant (RGD12) (Fig. 2A). This suggests that alb1 is epistatic to ayg1. On the other hand, the mature conidia produced by the arp2 and ayg1 double deletant (RGD16) had a yellow green color similar to that of the single ayg1 deletant suggesting that ayg1 is epistatic to arp2 (Fig. 2B, see colony centers). Additionally, alb1 but not arp2 is essential for 1,3,6,8-THN production, indicating that alb1 is epistatic to arp2 (24). These results imply that the order of the three enzymes in the pathway is Alb1p-Ayg1p-Arp2p and that there is an unknown biosynthetic step requiring Ayg1p in the production of 1,3,6,8-THN in A. fumigatus.

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Dissertation pigment extracts showed that a yellow compound produced by the \textit{alb1} transformant (ALB6) had the same retention time of 30 min as the heptaketide naphthopyrone, YWA1 (Fig. 4B, panel II). LC-APCIMS analysis of the yellow pigment gave [M+H]$^+$ and [M-H]$^-$ values of 277 and 275, identical with the authentic YWA1. Neither YWA1 nor 1,3,6,8-THN was detected in the transformants carrying either \textit{ayg1} or the vector alone (Fig. 4B, panels IV and V). Thus without the presence of polyketide-metabolizing enzymes, Alb1p produces the heptaketide YWA1 in \textit{A. fumigatus}. This agreed with the recent finding that heterologous expression of \textit{alb1} in \textit{A. oryzae} resulted in the production of YWA1 (25). Importantly, reintroduction of both \textit{alb1} and \textit{ayg1} into the strain RDG19 resulted in transformants with brown conidia (ALBAYG5) (Table I), a hallmark for the presence of 1,3,6,8-THN (Fig. 4A). HPLC analysis indicated that the \textit{alb1} + \textit{ayg1} transformant produced compounds with retention times of 16 and 22 min, identical to those indicated that the compound has not been confirmed.

**Ayg1p Enzymatically Converted the Heptaketide Naphthopyrone to the Pentaketide 1,3,6,8-THN**—Cell-free enzyme assays were carried out to determine whether Ayg1p converts the heptaketide naphthopyrone YWA1 to 1,3,6,8-THN enzymatically. The expression plasmid pTA-ayg1, which uses an \textit{o-amylase} promoter for expression of \textit{ayg1}, was introduced into \textit{A. oryzae}. The crude Ayg1p protein extract was obtained from the strain, \textit{A. oryzae/pTA-ayg1}, for \textit{in vitro} assay. To test conversion of the heptaketide YWA1 to the pentaketide 1,3,6,8-THN by Ayg1p, YWA1 was used as the substrate, and the reaction mixtures were analyzed by HPLC. At incubation time 0, only input substrate YWA1 was detected in the reaction mixture with a peak at a retention time of 17 min (Fig. 5A). However, after 5 min of incubation, 1,3,6,8-THN and flavolin were observed with the appearance of two new peaks at the retention times of 9 and 13.5 min, respectively (Fig. 5B). During the 5-min incubation period, the amount of YWA1 decreased with time as evidenced by absorption at 406 nm, the absorption maximum of YWA1 (data not shown). In contrast, neither 1,3,6,8-THN nor flavolin was detected in the reaction mixture using the crude extract of \textit{A. oryzae} transformed with the vector pHtx3 after 5 min of incubation (Fig. 5C). Therefore, the cell-free enzyme assay demonstrated that Ayg1p enzymatically converts the heptaketide YWA1 to the pentaketide 1,3,6,8-THN via a post-PKS polyketide shortening mechanism.

**DISCUSSION**

A cluster of six genes, \textit{alb1}, \textit{arp2}, \textit{arp1}, \textit{abr1}, \textit{abr2}, and \textit{ayg1}, involved in conidial pigment biosynthesis in \textit{A. fumigatus} was previously identified and characterized (24). Genetic and biochemical analyses indicated that \textit{A. fumigatus} synthesizes its conidial pigment through a pentaketide pathway similar to the DHN-melanin pathway found in many brown-to-black fungi. In this study, we showed that unlike the known DHN-melanin pathway, \textit{A. fumigatus} uses a heptaketide synthase, Alb1p, instead of a pentaketide synthase for the production of the pentaketide precursor 1,3,6,8-THN. In addition, a novel protein, Ayg1p, is required for Alb1p to produce 1,3,6,8-THN. We demonstrated that Ayg1p converted the heptaketide product of Alb1p to the pentaketide 1,3,6,8-THN through a novel polyketide-shortening mechanism.

Disruption of \textit{ayg1} prevented the accumulation of the pentaketide 1,3,6,8-THN and its autoxidation product flavolin suggesting that Ayg1p is involved in synthesis of 1,3,6,8-THN in \textit{A. fumigatus}. Reconstitution of both \textit{alb1} and \textit{ayg1} in the cluster deletant, RDG19, resulted in the production of brown conidia suggesting that Ayg1p altered the polyketide product of Alb1p. In fact, the \textit{(alb1 + ayg1)}-reconstituted strain did produce 1,3,6,8-THN and flavolin, whereas YWA1, the product of Alb1p, was undetectable. This is fundamentally different from the known pentaketide melanin pathway in which PKS alone directly synthesizes the pentaketide 1,3,6,8-THN (19, 27). Identification of this novel shortening step offers mechanistic insights into how the polyketide shortening is achieved. Our data show that Ayg1p could enzymatically convert the heptaketide YWA1 to the pentaketide 1,3,6,8-THN. The cell-free enzyme studies support the view that Ayg1p is capable of shortening the carbon backbone of the heptaketide YWA1. A data base-homology search with the Ayg1p amino acid sequence did not identify any homologous protein (24). However, a motif search showed the presence of a hydrolytic (lipase, peptidase-type) enzyme motif around the Ser$^{257}$ residue, which is located at the possible active site of Ayg1p (kmVvGLS$^{257}$AGGYyA motif). As shown in Fig. 6, the acyl side-chain open form of YWA1 is likely decylated by Ayg1p hydrolytically, producing 1,3,6,8-THN and the diketide acetocacetate. The demonstration that Ayg1p shortened YWA1 to 1,3,6,8-THN in the absence of Alb1p also indicates that the polyketide shortening is a post-PKS modification.

The carbon skeletons of polyketides are generally determined by polyketide synthases alone, although exceptions have been reported previously (9). In \textit{A. terreus}, for example, an accessory protein is required for the polyketide synthase
to produce the full-length polyketide for lovastatin biosynthesis (9). Lack of the accessory protein led to the synthesis of polyketides with shortened polyketide chains. However, in contrast to lovastatin biosynthesis in \textit{A. terreus} through polyketide extension, the novel Ayg1p protein in \textit{A. fumigatus} shortens a polyketide carbon chain through a post-PKS modification. Discovery of the post-PKS modification implies that the polyketide backbone is not solely determined by the polyketide synthase reaction itself. Our study showed that post-PKS enzymatic steps can greatly influence the carbon skeleton of polyketides and are not restricted to modifications of functional groups. To date, the engineering of polyketide synthases has been the main focus of combinatorial biosynthesis aimed at creating new compounds through genetic manipulation of the microbial genes and enzymes. The identification of a protein capable of modifying the polyketide carbon backbone may have a significant impact on the application of post-PKS modifications to the combinatorial synthesis of polyketides.

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Pentaketide Melanin Biosynthesis in *Aspergillus fumigatus* Requires Chain-length Shortening of a Heptaketide Precursor

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