Published in final edited form as:

Fungal Genet Biol. 2011 April ; 48(4): 430–437. doi:10.1016/j.fgb.2010.12.001.

Characterization of a polyketide synthase in *Aspergillus niger* whose product is a precursor for both dihydroxynaphthalene (DHN) melanin and naphtho- γ -pyrone

Yi-Ming Chiang^{a,b}, Kristen M. Meyer^c, Michael Praseuth^b, Scott E. Baker^c, Kenneth S. Bruno^c, and Clay C. C. Wang^{b,d}

^aGraduate Institute of Pharmaceutical Science, Chia Nan University of Pharmacy and Science, Tainan 71710, Taiwan, ROC

^bDepartment of Pharmacology and Pharmaceutical Sciences, University of Southern California, School of Pharmacy, 1985 Zonal Avenue, Los Angeles, California 90089, USA

^cChemical and Biological Process Development Group, Energy and Environment Directorate, Pacific Northwest National Laboratory, Richland, Washington, 99352, USA

^dDepartment of Chemistry, University of Southern California, College of Letters, Arts, and Sciences, Los Angeles, California 90089, USA

Abstract

The genome sequencing of the fungus *Aspergillus niger* uncovered a large cache of genes encoding enzymes thought to be involved in the production of secondary metabolites yet to be identified. Identification and structural characterization of many of these predicted secondary metabolites are hampered by their low concentration relative to the known *A. niger* metabolites such as the naphtho- γ -pyrone family of polyketides. We deleted a nonreducing PKS gene in *A. niger* strain ATCC 11414, a daughter strain of *A. niger* ATCC strain 1015 whose genome was sequenced by the DOE Joint Genome Institute. This PKS encoding gene we name *alba* is a predicted ortholog of *alb1* from *Aspergillus fumigatus* which is responsible for production of the naphtho- γ -pyrone precursor for the 1,8-dihydroxynaphthalene (DHN) melanin/spore pigment. Our results show that the *A. niger* *alba* PKS is responsible for both the production of the spore pigment precursor and a family of naphtho- γ -pyrones commonly found in significant quantity in *A. niger* culture extracts. The generation of an *A. niger* strain devoid of naphtho- γ -pyrones will greatly facilitate the elucidation of cryptic biosynthetic pathways in this organism.

Keywords

Secondary Metabolism; *Aspergillus niger*; Natural Products; Genomics; Naphtho- γ -pyrone; Polyketides

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Correspondence should be addressed to K.B. (bruno@pnl.gov) and C.W. (clayw@usc.edu).

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INTRODUCTION

Fungi live in complex ecosystems and must compete with other organisms such as bacteria, algae, other fungi, protozoans and small metazoans. Successful competition is often due to the production of metabolites that kill or inhibit growth of other organisms. A number of these fungal metabolites have enjoyed great commercial success as pharmaceuticals (e.g. penicillin, cyclosporine, and lovastatin) (Keller et al., 2005). Recent genome sequencing of fungi in the genus *Aspergillus* has revealed that genes involved in secondary metabolite biosynthesis are more abundant than the known secondary metabolites produced by these organisms (Galagan et al., 2005; Pel et al., 2007).

Aspergillus niger is most widely used industrially for citric acid production and is also a tool for production of enzymes such as α -amylase, cellulase, and pectinases. Although around 60 secondary metabolites have been identified from *A. niger* (Nielsen et al., 2009), they all belong to only a small subset of secondary metabolite families such as fumonisins, naphtho- γ -pyrones, bicoumarins, and malformins. The metabolites with similar chemical skeletons are therefore suspected to be biosynthesized from the same pathways. Analysis of the *A. niger* strain ATCC 1015 genome revealed 32 polyketide synthase genes (PKS), 15 nonribosomal peptide synthetase genes (NRPS) and 9 hybrid NRPS/PKS genes, suggesting that there are still many “cryptic pathways” awaiting to be discovered (Fisch et al., 2009). It seems that many genes responsible for secondary metabolite biosynthesis are either silent or expressed at very low levels under standard laboratory or industrial culture conditions and this explains why so many secondary metabolites are still unknown. Moreover, to the best of our knowledge, none of metabolites isolated from *A. niger* has been associated with a particular gene until now except the fumonisin gene cluster where a putative *A. niger* fumonisin cluster was revealed by comparison to the *Gibberella moniliformis* fumonisin gene cluster (Baker, 2006; Frisvad et al., 2007; Pel et al., 2007).

Several approaches for activating cryptic clusters have recently been described in *Aspergillus* (Chiang et al., 2009a; Scherlach and Hertweck, 2009). One strategy utilizes pathway-specific transcription factors which are found in many secondary metabolite gene clusters. Forced expression of these genes through promoter replacement can lead to the activation of the entire associated gene cluster and production of new metabolites (Bergmann et al., 2007; Chiang et al., 2009b). A second strategy has utilized a global regulator of secondary metabolism, LaeA. Microarray analysis of *laeA* deletion and overexpression in *A. nidulans* led to the discovery of a co-expressed cluster of genes responsible for the production of terrequinone A, a metabolite not previously associated with this fungus (Bok et al., 2006a). Inspired by the function of LaeA, a third strategy involves modifying chromatin landscapes, or “epigenome manipulation” (Cichewicz, 2010). This could be achieved by culturing the *Aspergillus* in the presence of histone deacetylase or DNA methyltransferase inhibitors (Henrikson et al., 2009; Shwab et al., 2007), or by deleting genes involved in modifying chromatin structure and allowing the expression of previously silent gene clusters (Bok et al., 2009). These strategies have been quite successful in uncovering new biosynthetic gene clusters in *A. nidulans*.

However, accessing the large, secondary metabolite potential of *A. niger* using these strategies is complicated by the large quantity of naphtho- γ -pyrones found after growth under a variety of conditions. In addition to limiting the availability of common polyketide building blocks (e.g. malonyl and acetyl-CoA), the presence of these metabolites may mask the presence of other materials during chromatography detection methods. To enable the successful genomic mining of *A. niger* cryptic secondary metabolites, it is necessary to identify genes responsible for the biosynthesis of naphtho- γ -pyrones and create mutant strains that lack these major aromatic polyketides.

MATERIALS AND METHODS

Fungal strains, growth conditions, and molecular genetic manipulations

A. niger strains used in this study are listed in Table 1. Construction of fusion PCR products, protoplast production and transformation were carried out as described. All DNA insertions into the *A. niger* genome were performed using protoplasts and standard PEG transformation. A strain of ATCC 11414 was altered in order to facilitate the process of gene deletion. First, an auxotrophic strain was generated by mutating the *pyrG* locus. This *pyrG* auxotrophic strain (KB1002, $\Delta pyrG$) was created by directly transforming *A. niger* ATCC strain 11414 with a PCR fragment generated by amplifying two regions of the *pyrG* gene and fusing them while leaving out approximately thirty codons between the two fragments. Stop codons were also inserted within the region of the missing codons to ensure the creation of a null mutation that could no longer produce a functioning enzyme. The mutation was selected by growth on media supplemented with uracil and 5-fluoro anthranilic acid (FOA). Only cells lacking the *pyrG* gene can survive in the presence of FOA. The recipient mutant strain with enhanced homologous integration (KB1001, $\Delta kusA$) was created by inserting the *A. fumigatus pyrG* gene into the *kusA* locus of strain KB1002, as described by Nielsen et al. (2008). The following gene replacements were performed by insertion of the hygromycin resistance marker in place of the targeted gene. Hygromycin deletion cassettes were generated using the double joint PCR technique (Yu et al., 2004). For construction of the fusion PCR amplicon, two ~1000 base pair fragments, upstream and downstream of the targeted gene, were amplified from genomic *A. niger* DNA by PCR. Primers used in this study are listed in Table 2. A fusion PCR reaction was set up with the two amplified flanking sequences and the hygromycin phospho-transferase gene (*hph*) marker cassette amplified from plasmid pCB10003 (Fungal Genetics Stock Center) as template DNA. The three fragments were fused to create a single molecule and amplified with two nested primers creating the gene deletion cassette (Table 2). The *albA* and *aygA* deletions were generated by replacing each gene with the *hph* selectable marker gene in KB1001 ($\Delta kusA$). Correct deletion of the target gene was determined by diagnostic PCR and Southern hybridization analysis (Fig. S2).

Fermentation and LC-MS analysis

All *A. niger* strains were cultivated at 30°C on solid glucose minimal medium [GMM, 6 g/l NaNO₃, 0.52 g/l KCl, 0.52 g/l MgSO₄•7H₂O, 1.52 g/l KH₂PO₄, 10 g/l D-glucose supplemented with 1 ml/l of a trace element solution] at 10 × 10⁶ spores per 10 cm plate. After 5 days, agar was chopped into small pieces and the material was extracted with 50 ml of MeOH followed by 50 ml of 1:1 CH₂Cl₂/MeOH each with 1 h sonication. The extract was evaporated *in vacuo* to yield a residue, which was suspended in H₂O (25 ml) and partitioned with ethyl acetate (EtOAc, 25 ml × 2). The combined EtOAc layer was evaporated *in vacuo*, re-dissolved in 1 ml of 20% DMSO/MeOH and a portion (10 µl) was examined by high performance liquid chromatography-photodiode array detection-mass spectrometry (HPLC-DAD-MS) analysis. HPLC-MS was carried out using a ThermoFinnigan LCQ Advantage ion trap mass spectrometer with a RP C₁₈ column (Alltech Prevail C18 3 mm 2.1 × 100 mm) at a flow rate of 125 µl/min. The solvent gradient system for HPLC and the condition for MS analysis were carried out as described previously (Bok et al., 2009).

Isolation and identification of secondary metabolites

For production and structure elucidation of WT *A. niger* metabolites, thirty-one 15-cm GMM plates (~40 ml medium per plate) inoculated with WT *A. niger* (2.25 × 10⁷ spores per plate) were grown for 5 days at 30°C and extracted with EtOAc as described above. In order to extract most of the acidic secondary metabolites due to the acidic nature of polyphenol,

we acidified the water layer to pH 2 and extracted with EtOAc a third time. The three EtOAc extracts were combined and evaporated *in vacuo*. The crude extract in EtOAc layer (339 mg) was coated on 5.1 g SiO₂ gel (Merck 230-400 mesh, ASTM) which was then suspended in CH₂Cl₂ and applied to a SiO₂ gel column (20 × 75 mm). This column was then eluted with CH₂Cl₂-MeOH mixtures of increasing polarity (fraction A, 1:0, 350 ml; fraction B, 49:1, 350 ml; fraction C, 19:1, 350 ml; fraction D, 9:1, 350 ml; and fraction E, 7:3, 350 ml). All fractions were analyzed by HPLC-DAD-MS. Fraction B containing the major metabolites found in HPLC profile was subjected to semi-preparative reverse phase HPLC [Phenomenex Luna 5 μm C18 (2), 250 × 10 mm] and separated into nine subfractions (B1 ~ B9) with a flow rate of 5.0 ml/min and monitored by a UV detector at 254 nm (Fig. 3a). The gradient system was MeCN (solvent B) in 5 % MeCN/H₂O (solvent A) both containing 0.05 % TFA: 40 to 68 % B from 0 to 28 min, 68 to 100 % B from 28 to 30 min, maintained at 100 % B from 30 to 33 min, 100 to 40 % B from 33 to 34 min, and re-equilibration with 40 % B from 34 to 37 min. Subfractions B1 (**5**; 5.0 mg), B2 (**6**, **7**, **10**, and **11**; 3.2 mg), B3 (**6**, **7**, **10**, and **11**; 4.0 mg), B4 (**8**, **9**, **12**, and **13**; 2.9 mg), B5 (**8**, **9**, **12**, and **13**; 3.5 mg), B6 (**10**, 4.7 mg), B7 (**11**, 4.2 mg), B8 (**12**, 3.2 mg), and B9 (**13**, 2.7 mg) were eluted at 12.9, 15.0, 16.5, 17.9, 19.7, 21.2, 23.7, 24.7, and 27.2 min, respectively. Fraction E containing compound **16** was subjected to semi-preparative reverse phase HPLC as described above. The gradient system was 20 % B from 0 to 5 min, 20 to 33 % B from 5 to 18 min, 33 to 100 % B from 18 to 20 min, maintained at 100 % B from 20 to 22 min, 100 to 20 % B from 22 to 24 min, and re-equilibration with 20 % B from 24 to 28 min. Compound **16** (pestalamide B, 3.1 mg) was eluted at 14.4 min.

Compound identification

Optical rotation and Infrared (IR) spectra were recorded on a JASCO P-200 digital polarimeter and a GlobalWorks Cary 14 Spectrophotometer. NMR spectra were collected on a Varian Mercury Plus 400 spectrometer. High resolution electrospray ionization mass spectrum was obtained on Agilent 6210 time of flight LC-MS.

Pestalamide B (16): colorless oil; $[\alpha]_D^{22} +15.9^\circ$ (MeOH, c 0.2); For UV-Vis and ESIMS spectra see Fig. S1; For ¹H and ¹³C NMR data (acetone-*d*₆), see Table S1; HRESIMS positive mode, $[M+H]^+$ *m/z* found 343.1290; calcd for C₁₈H₁₉N₂O₅: 343.1288.

RESULTS

Analysis of the secondary metabolite profile of wild type *A. niger* ATCC11414

The secondary metabolite profiles of the organic extracts from wild type *A. niger* ATCC 11414, a non-mutagenized derivative of the genome sequenced *A. niger* strain ATCC 1015, cultured in GMM were analyzed by HPLC-DAD-MS (Fig. 1). Since secondary metabolites from *A. niger* have been comprehensively reviewed by Frisvad's group (Nielsen et al., 2009), we first compared the UV-visible (Vis) and mass spectra (MS) of major metabolites with those reported (Fig. S1). This resulted in the identification of compounds eluting at 12.5 min (**1**, nigragillin), 15.4 min (**2**, nigerazine B), 23.1 min (**3**, funalenone), 25.8 min (**4**, aurasperone B), 27.3 and/or 27.7 min (**6**, fonsecinone B), 28.6 and/or 28.8 min (**8**, forsecinone C), 29.8 min (**10**, fonsecinone A), 30.6 min (**11**, asperpyrone B), 31.1 min (**12**, aurosporene A), and 31.8 min (**13**, asperpyrone C) (Fig. 2). Interestingly, compounds eluting at 27.3 and 27.7 min had similar UV-Vis and MS to the known compound, fonsecinone B (**6**); and compounds eluting at 28.6 and 28.8 min had similar UV-Vis and MS to the known compound, fonsecinone C (**8**) (Fig. S1). These data suggested that compounds eluting at 27.3 and 27.7 min are isomers that have the same chromophore and molecular formula, as well as compounds eluting at 28.6 and 28.8 min. Moreover, these data also suggested that the majority of UV active metabolites produced by *A. niger* ATCC 11414 in solid GMM are

dimeric naphtho- γ -pyrones. In order to confirm the identified structures of dimeric naphtho- γ -pyrones, these compounds were purified from large scale culture followed by column chromatography. The dimeric naphtho- γ -pyrone enriched B fraction was further purified by semi-preparative reverse phase HPLC and a total of nine subfractions (B1 ~ B9) were obtained (Fig. 3a). After obtaining the spectral data of subfractions B1 ~ B9, we identified that subfraction B1 is kotanin (**5**) (Cutler et al., 1979), and confirmed that subfractions B6, B7, B8, and B9 are fonsecinone A (**10**) (Priestap, 1984), asperpyrone B (**11**) (Akiyama et al., 2003), aurosperone A (**12**) (Priestap, 1984), and asperpyrone C (**13**) (Akiyama et al., 2003), respectively, by comparing their UV-Vis, MS (Fig. S1), and ^1H NMR spectra (data not shown). Subfractions B2 and B3 had similar ^1H NMR spectra which exhibited a mixture of dimeric naphtho- γ -pyrone derivatives, and so did subfraction B4 and B5 (data not shown). Subfractions B1 ~ B9 were then analyzed by HPLC-DAD-MS with the same condition as in Fig. 1. As shown in Fig. 3b, subfraction B1 eluted compound **5**; subfractions B2 and B3 both eluted a mixture of compounds **6**, **7**, **10**, and **11**; subfractions B4 and B5 both eluted a mixture of compounds **8**, **9**, **12**, and **13**; and subfractions B6, B7, B8, and B9 eluted compounds **10**, **11**, **12**, and **13**, respectively. Surprisingly, no aurasperone B (**4**) was isolated from this naphtho- γ -pyrone enriched fraction although **4** was a major compound in B fraction after column chromatography (data not shown). Aurasperone B (**4**) which has two hemiketal groups was found to dehydrate easily to give aurasperone A (**12**) by heating in DMSO or in the presence of Lewis acid Al_2O_3 (Priestap, 1984). Linear naphtho- γ -pyrone YWA1 (**14**) was known in equilibrium with the side-chain open form (**15**). After recyclization, angular naphtho- γ -pyrone (**16**) can be formed in the presence of C-14 phenol group (Fig. 4). Taken together, these results suggested that **4** is not stable and dehydrate to generate compounds **6** – **13**, especially under aqua acidic condition during the HPLC purification process. The irreversible dehydration of hemiketal from **4** produces four stable dimeric naphthopyrones (**10** – **13**). In addition, considering that subfractions B2 and B3 both can convert to compounds **10** and **11**, B2 and B3 are an equilibrium mixture of **6** and **7**, as compounds **6**, **7**, **10**, and **11** all have angular naphtho- γ -pyrone in the “southern” part of the molecules. Similar to this, subfractions B4 and B5 both can convert to compounds **12** and **13**, B4 and B5 are an equilibrium mixture of **8** and **9**. These different naphtho- γ -pyrones elute over a large time frame from 25 minutes to 33 minutes which hinders the detection of other secondary metabolites produced in smaller quantities or are less UV active. For example from large scale isolation we were able to isolate the polyketide kotanin (**5**) which was obscured by compound **4**.

Besides the major naphtho- γ -pyrones, three minor compounds (**17** – **19**) eluting around 19 min were also detected by HPLC-DAD-MS (Fig. 1). Compounds **17** – **19**, however, have not been reported from *A. niger* so far after comparing the UV-Vis and MS spectra of compounds **17** – **19** with those of known metabolites from *A. niger* (Nielsen et al., 2009). We thus attempted to solve the chemical structures of these minor compounds. While compound **19** could be purified from large scale culture, compounds **17** and **18** were unstable and decomposed after SiO_2 column chromatography. The molecular formula of compound **19** was established to be $\text{C}_{18}\text{H}_{18}\text{N}_2\text{O}_5$ by its ^{13}C NMR, gHSQC, and HRESIMS spectral data. ^1H and ^{13}C NMR data of compound **19** including the mono-substituted phenyl group, the *para*-disubstituted pyridin-4-one moiety, and the α -methylsuccinic group (Fig. S5 and S6) are similar to those of the known compound, nygerone A, isolated from *A. niger* (Nielsen et al., 2009; Henrikson et al., 2007). This suggested that compound **19** is a derivative of nygerone A. Long range ^1H - ^{13}C correlation NMR data (gHMBC) of compound **19** allowed us to fully construct the chemical structure (Fig. S7) and assign the chemical shift of individual protons and carbons of compound **19** (Table S1). The assigned chemical structure of compound **19** has been identified from a plant pathogenic fungus *Pestalotiopsis theae* and named as pestalamide B (Ding et al., 2008). Although our ^1H NMR data of compound **19** are almost identical with the published data of pestalamide B, our ^{13}C

NMR data of compound **19** is different from what is reported in the literature (Ding et al., 2008), specifically on C-2 and C-6 of pestalamide B (Table S1). In addition to pestalamide B, Ding et al. also isolated pestalamide A which is a 4H-pyran-4-one derivative of pestalamide B in the same article. Oddly, Ding et al. reported that the ¹³C NMR data of both pestalamide A and B are identical (Table S1). Our data suggest that the ¹³C NMR data for pestalamide B reported by Ding et al. is incorrect. The electronegativity of the nitrogen atom is smaller than the oxygen atom and this causes about 20 ppm upfield shifted of C-2 and C-6 in compound **19** (pestalamide B) when compared with pestalamide A. Compounds **17** and **18** have similar UV-Vis and MS spectra suggesting that these two compounds are stereoisomers (Fig S1). The molecular masses of compounds **17** and **18** are 18 Da greater than that of pestalamide B (**19**). This together with the fact that compounds **17** – **19** have the same daughter ion at *m/z* 229 (Fig S1) suggested that compounds **17** and **18** might dehydrate to become pestalamide B (**19**) after SiO₂ column chromatography.

Identification of the naphtho-γ-pyrone polyketide synthase in *A. niger*

In order to facilitate gene function studies for use in a genome mining approach, the *kusA* gene was mutated by insertional mutagenesis (Nielsen et al., 2008) (Nayak et al., 2006). To determine if the deletion of *kusA* alters the secondary metabolite profile of a strain, we compared the profile of a control *kusA*+*A. niger* ATCC 11414 strain with that of the *kusA* deletion strain (KB1001). After finding no significant differences in the UV 254 nm active secondary metabolites of the two strains (Fig. 1), we used strain KB1001 as the parental strain for further studies.

Fungal polyketides are produced by multidomain type I polyketide synthases (PKS) which are iterative in nature. In general, it is possible to examine the domains encoded in a fungal PKS gene and separate them into three categories, non-reducing (NR), partially reducing (PR) and highly reducing (HR) PKS (Chiang et al., 2010; Cox, 2007). The aromatic structure of naphtho-γ-pyrones suggests that a NR-PKS gene with the following domain organization is likely responsible for their biosynthesis: starter unit ACP transacylase (SAT), β-ketoacyl synthase (KS), acyl transferase (AT), product template (PT), acyl carrier protein (ACP) and thiolesterase/Claisen-cyclase (TE/CLC). Examination of the 32 PKSs in *A. niger* ATCC 1015 revealed that three PKS genes, Aspni1:56896, Aspni1:191422 and Aspni1:51499 (using the JGI *A. niger* designation) contain all the necessary domains and do not contain unnecessary tailoring domains. The *alb1* gene in *A. fumigatus* has been shown to be a naphtho-γ-pyrone synthase when heterologously expressed in *A. oryzae* (Watanabe et al., 2000). The predicted protein corresponding to gene aspni1:56896 shares the greatest amino acid similarity with the Alb1 protein. In order to determine if aspni1:56896 is involved in naphtho-γ-pyrone synthesis in *A. niger*, its coding sequence was replaced with a gene conferring resistance to hygromycin B. The *AlbA* deletion strain (*albA*Δ; KB1008) was first verified first by diagnostic PCR and then confirmed by Southern hybridizations (Fig. S2).

The secondary metabolite profile of Δ*albA* was analyzed by HPLC-DAD-MS (Fig. 1). The observation that the Δ*albA* did not accumulate dimeric naphtho-γ-pyrones as compared to its parent strain but did accumulate compounds **1** and **2** indicate that *A. niger* albA (*AlbA*) is responsible for the biosynthesis of all the different naphtho-γ-pyrones (**4**, and **6** – **13**). Kinetic studies comparing Δ*kusA* and Δ*albA* metabolite profiles further confirmed that Δ*albA* did not accumulate dimeric naphtho-γ-pyrones after culture for a duration from day 4 to day 12 (Fig. S3). The peak intensity of stable dimeric naphtho-γ-pyrones (**10** – **13**) of Δ*kusA* gradually increased from day 4 to day 12 suggesting that compounds **10** – **13** can be produced *in vivo*. Interestingly, production of kotanin (**5**) and funalenone (**3**) decrease in Δ*albA* (Fig. 1 and S3). Kotanin (**5**) in Δ*albA* can be detected either by DAD (Fig. 1) or MS in selected ion monitoring (SIM) mode (Fig. S4a). However, funalenone (**3**) was hardly

detectable by DAD (Fig. 1) but can be detected by MS in single ion monitoring (SIM) mode (Fig. S4a). Comparison of the same MS/MS fragments for funalenone (**3**) from the parental strain with those from $\Delta albA$ clearly shows that compound **3** is present in the $\Delta albA$ (Fig. S4b). Moreover, the peak intensity of three compounds (**17** – **19**) eluting around 19 min also dramatically decreased in the $\Delta albA$ (Fig. 1 and S3). These three compounds, however, can also be detected by MS in extracted ion chromatogram (EIC) mode in $\Delta albA$ (Fig. S4c). The production of funalenone (**3**), kotanin (**5**), and pestalamide B (**19**) in $\Delta albA$ confirmed that *albA* is not the PKS responsible for the biosynthesis of these compounds.

Phenotype of $\Delta albA$, $\Delta aygA$ and control strain

On solid GMM media, the *A. niger* $\Delta kusA$ control strain produced black conidia that is the hallmark of *A. niger*. Under the same growth conditions $\Delta albA$ displayed a white or colorless conidial phenotype (Fig. 5) consistent with the *albA* PKS being responsible for both melanin and naphtho- γ -pyrones production. Moreover, the *ayg1* gene has been shown in *A. fumigatus* to be responsible for polyketide chain shortening and converts a 14 carbon naphtho- γ -pyrone PKS product to the 10 carbon 1,3,6,8-THN intermediate in the biosynthesis pathway leading to the spore pigment (Fujii et al., 2004; Tsai et al., 2001). In *A. niger*, a putative ortholog of *A. fumigatus* *ayg1*, *aygA*, has been identified (Baker, 2008). As predicted, *A. niger* $\Delta aygA$ mutant was minimally affected in the ability to synthesis naphtho- γ -pyrones (Fig 1) and generated orange pigmented conidia (Fig. 5).

DISCUSSION

Bioinformatic analysis of the *A. niger* genome sequence revealed the presence of over 50 core genes that could play a role in the synthesis of different secondary metabolites (Fisch et al., 2009; Pel et al., 2007). Chemical surveys of *A. niger* culture extracts have identified only a limited number of biosynthetic pathways (Nielsen et al., 2009). In this study, we found by the HPLC-DAD-MS analysis, that the most abundant group of metabolites in culture extracts were a family of dimeric naphtho- γ -pyrones (**4**, and **6** – **9**) that may non-enzymatically dehydrate to a more stable form (**10** – **13**, respectively). The significant quantities of the different naphtho- γ -pyrones make the detection of other metabolites by HPLC challenging as they could be obscured. For example, the bis-coumarin kotanin (**5**) appeared as a minor peak on the shoulder of a major peak corresponding to aurasperone B (**4**) (Figure 1). In our effort to explore the biosynthetic function of the unknown secondary metabolite genes in *A. niger*, we sought to first create a strain that was deficient in naphtho- γ -pyrone biosynthesis. Towards this goal, we deleted *albA*, a homolog of a PKS shown in *A. fumigatus* to be responsible for the synthesis of spore pigment intermediate 1,8-dihydroxynaphthalene (DHN). The identification of the gene responsible for naphtho- γ -pyrone biosynthesis and the creation of a strain devoid of naphtho- γ -pyrone biosynthesis could be extremely useful for further genome mining of secondary metabolites in *A. niger*.

Perhaps one of the most interesting aspects of our study is the finding that a single polyketide synthase produces a precursor for two major classes of compounds, DHN-melanin and the naphtho- γ -pyrones. Our data is consistent with the prediction that *A. niger* melanin production is similar to the pathway elucidated for *A. fumigatus* (Tsai et al., 1997; Tsai et al., 1999; Tsai et al., 2001; Langerfelder et al., 1998; Fujii et al., 2004; Sugareva et al., 2006.; Baker, 2008). When we delete the orthologue of *A. fumigatus* *ayg1*, melanin production is blocked but naphtho- γ -pyrones are still synthesized. In contrast to *A. fumigatus*, the genes for melanin biosynthesis are not clustered in *A. niger* - although *albA* and *aygA* are both on chromosome 1, they are on different chromosomal arms (Baker, 2008). These results show that a single PKS can produce a precursor molecule for two different biosynthetic pathways: the DHN derived spore pigments and the naphtho- γ -pyrone family of chemicals (Fig. 4). The bifurcation of pathways from a single molecule illustrates

the amazing variety of chemical structures produced by ascomycete fungi. Perhaps the accumulation of so much of the dimeric naphtho- γ -pyrones in *A. niger*, as compared to *A. fumigatus*, is because the *A. niger* *aygA* does not work well. Another possibility of the dimeric naphtho- γ -pyrones produced in *A. niger* might be that the enzyme responsible for the dimerization is very active and is able to compete with *aygA* in *A. niger*.

The naphtho- γ -pyrone biosynthetic pathway in *A. niger* has not been determined previously and our identification of the PKS gene involved allows us to propose a likely pathway (Fig. 4). The TE/CLC domain in AlbAp catalyzes the Claisen cyclization and releases the heptaketide **15**. The two phenol groups at C-6 and C-14 can cycle to form either a linear (**14**) or an angular naphtho- γ -pyrone (**16**) and, before dehydration, these two structures are in equilibrium. The linear and/or angular naphtho- γ -pyrones are then methylated and dimerized to generate **4**. We were not able to identify using homology analysis the genes coding for the enzymes responsible for the methylation and dimerization. It is likely that these genes are not clustered with the PKS gene. Finally the dehydration of the naphtho- γ -pyrone prevents the equilibrium and allowed us to isolate four different dimeric naphtho- γ -pyrones representing the four different combinations of angular and linear rings.

In conclusion, we have demonstrated that a single polyketide synthase is able to produce a precursor for two different classes of polyketides, DHN-melanin and the naphtho- γ -pyrones. Moreover, we have generated a “clean background” *A. niger* strain. Since the products of cryptic secondary metabolite pathways could be new compounds with potential biological activities, eliminating the major metabolites would benefit the discovery of cryptic pathways via genome mining approach.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This project was supported by grants PO1GM084077 to CW from the National Institute of General Medical Sciences. Research conducted at the Pacific Northwest National Lab was supported by the Department of Energy, Office of the Biomass Program. We are grateful to the DOE Joint Genome Institute for generation of the genome sequence of *Aspergillus niger* strain ATCC 1015 which was performed under the auspices of the US Department of Energy's Office of Science, Biological and Environmental Research Program and the University of California, Lawrence Berkeley National Laboratory under contract no. DE-AC02-05CH11231, Lawrence Livermore National Laboratory under contract no. DE-AC52-07NA27344, and Los Alamos National Laboratory under contract no. DE-AC02-06NA25396. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of General Medical Sciences or the National Institutes of Health.

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Fig. 2.
Chemical structures of secondary metabolites identified from *A. niger* ATCC 11414.

Fig. 3.

(a) Purification of naphtho- γ -pyrones from enriched B fraction by using a semi-preparative HPLC. (b) HPLC profiles of subfractions B1 ~ B9 with the same condition as in Fig 1.

Fig. 4. Proposed biosynthesis pathway of DHN melanin and dimeric naphtho- γ -pyrones. The DHN melanin pathway was proposed based on Fujii et al. (2004).

Fig. 5.
A. niger phenotype in solid media.

TABLE 1*A. niger* strains used in this study (PNNL)

Fungal strain or transformants	Parent Strain	Genotype
11414		Parental
KB1001	<i>A. niger</i> 11414	<i>pyrG</i> ⁻ , <i>kusA::pyrG</i>
KB1002	<i>A. niger</i> 11414	<i>pyrG</i> ⁻
KB1008	<i>A. niger</i> 11414	<i>pyrG</i> ⁻ , <i>kusA::pyrG</i> , <i>albA</i> ⁻ , <i>hyg</i> ^R
KB1009	<i>A. niger</i> 11414	<i>pyrG</i> ⁻ , <i>kusA::pyrG</i> , <i>aygA</i> ⁻ , <i>hyg</i> ^R

TABLE 2

PCR PRIMERS

Primer name	Primer Sequence
<i>AlbA</i> deletion	
ALBF1	GCAAGGCAAATGAACCGGCC
ALBF2	ATCCGTCGGTCAGGCTGCTT
ALBR3	TGACCTCCACTAGCTCCAGCACACGAGATGGACCCTCCAT
ALBHPHF	ATGGAGGGTCCTATCTCGTGTGCTGGAGCTAGTGGAGGTCA
ALBHPHR	TGTTGTCAGTCCTAGCGGGACGGTCGGCATCTACTCTATT
ALBF4	AATAGAGTAGATGCCGACCGTCCCGCTAGGACTGACAACA
ALB5R	CTCCCTCGTATTGCGCTCCT
ALB6R	AACCGACCCAGACGCCACTC
<i>AygA</i> deletion	
AYGF1	GGCACACTTGTCTGGTTCTG
AYGF2	AAGGGGGCCAAATCGCAAGG
AYGR3	TGACCTCCACTAGCTCCAGCCGAGGATCCAAGGAGCCAT
AYGHPHF	ATGGCTCCTTGGATCCTCGGGCTGGAGCTAGTGGAGGTCA
AYGHPHR	GCTCTTGGGGCGAGCGCAGACGGTCGGCATCTACTCTATT
AYGF4	AATAGAGTAGATGCCGACCGTCTGCGCTCGCCCCAAGAGC
AYG5R	GTACTACACCACCAGACCCA
AYG6R	TGGGACTGTTGGGGATTGTG
<i>pyrG</i> mutation	
PG1F	GCAGGGAAAAATACGAGCTCCAATG
PG2F	AACCTGGGTGTGGCAACTTCAATGG
PG4R	CACCCGTCGCCATTTGCTCTACGCA
PG5R	AAGCTTATCACCGTCCCTTATCAGC
PG3RB	TATGGGCTCACTTATCTAGAATTGCTTCTGGACAGTGTGCCAAT
PG3FB	AGAAGCAATTCTAGATAAGTGAGCCCATATCATCAACTGCAGCA
<i>kusA</i> insertion	
T1F	ATGGCGGACGGTAACCCACA
T2R	GTGAAGAGCATTTGTTTGGGCAATCCACATGCCAGGAGGGT
T3F	GCCTCCTCTCAGACAGAATGCTGTTTCCGATCTCACGCC
T4R	GTCAGTCTCCAAGAACTCC
M1F	GCCTCAAACAATGCTCTTCAC
M2R	GGAAGAGAGGTTACACC
M3F	TGATACAGGTCTCGGTCCC
M4R	ATTCTGTCTGAGAGGAGGC