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

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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 non-reducing 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.

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1. 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, naphtha-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 PKS-NRPS hybrid 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 transcrip-

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tion 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 *Aspergillus 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., 2006). 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.

2. Materials and methods

2.1. 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, Δ*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, Δ*kusA*) was created by inserting the *Aspergillus 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 (Δ*kusA*). Correct deletion of the target gene was determined by diagnostic PCR and Southern hybridization analysis (Fig. S2).

2.2. Fermentation and LC–MS analysis

All *A. niger* strains were cultivated at 30 °C on solid glucose minimal medium [GMM, 6 g/l $NaNO_3$, 0.52 g/l KCl, 0.52 g/l $MgSO_4 \cdot 7H_2O$, 1.52 g/l KH_2PO_4, 10 g/l D-glucose supplemented with 1 ml/l of a trace element solution] at 10×10^6 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 CH_2Cl_2/MeOH each with 1 h sonication. The extract was evaporated *in vacuo* to yield a residue, which was suspended in H_2O (25 ml) and partitioned with ethyl acetate (EtOAc, 25 ml × 2). The combined EtOAc layer was

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

Fungal strain or transformants	Parent strain	Genotype
11414		Parental
KB1001	*A. niger* 11414	*pyrG*⁻, *kusA::pyrG*
KB1002	*A. niger* 11414	*pyrG*⁻
KB1008	*A. niger* 11414	*pyrG*⁻, *kusA::pyrG, albA*⁻, *hyg*^{R}
KB1009	*A. niger* 11414	*pyrG*⁻, *kusA::pyrG, aygA*⁻, *hyg*^{R}

Table 2
PCR primers.

Primer name	Primer sequence
AlbA deletion	
ALBF1	GCAAGGCAAATGAACCGGCC
ALBF2	ATCCGTCGGTCAGGCTGCTT
ALBR3	TGACCTCCACTAGCTCCAGCACACGAGATGGACCCTCCAT
ALBHPHF	ATGGAGGGTCCTATCTCGTGTGCTGGAGCTAGTGGAGGTCA
ALBHPHR	TGTTGTCAGTCCTAGCGGGACGGTCGGCATCTACTCTATT
ALBF4	AATAGAGTAGATGCCGACCGTCCCGCTAGGACTGACAACA
ALB5R	CTCCCTCGTATTCGCCTCCT
ALB6R	AACCGACCCAGACGCCACTC
AygA deletion	
AYGF1	GGCACACTTGTCTGGTTCTG
AYGF2	AAGGGGGCCAAATCGCAAGG
AYGR3	TGACCTCCACTAGCTCCAGCCCGAGGATCCAAGGAGCCAT
AYGHPHF	ATGGCTCCTTGGATCCTCGGGCTGGAGCTAGTGGAGGTCA
AYGHPHR	GCTCTTGGGGCGAGCGCAGACGGTCGGCATCTACTCTATT
AYGF4	AATAGAGTAGATGCCGACCGTCTGCGCTCGCCCCAAGAGC
AYG5R	GTACTACACCACCAGACCCA
AYG6R	TGGGACTGTTGGGGATTGTG
pyrG mutation	
PG1F	GCAGGGAAAAATACGAGCTCCAATG
PG2F	AACCTGGGTGTGGCAACTTCAATGG
PG4R	CACCCGTCGCCATTTGCTCTACGCA
PG5R	AAGCTTATCACCGTCCCTTATCAGC
PG3RB	TATGGGCTCACTTATCTAGAATTGCTTCTGGACAGTGTTGCCAAT
PG3FB	AGAAGCAATTCTAGATAAGTGAGCCCATATCATCAACTGCAGCA
kusA insertion	
T1F	ATGGCGGACGGTAACCCACA
T2R	GTGAAGAGCATTGTTTGAGGCAATCCACATGCCAGGAGGGT
T3F	GCCTCCTCTCAGACAGAATGCTGTTTCCGATCTCACGCC
T4R	GTCACTGCTCCAAGAACTCC
M1F	GCCTCAAACAATGCTCTTCAC
M2R	GGAAGAGAGGTTCACACC
M3F	TGATACAGGTCTCGGTCCC
M4R	ATTCTGTCTGAGAGGAGGC

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