The sexual spore pigment asperthecin is required for normal ascospore production and protection from UV light in Aspergillus nidulans

Jonathan M. Palmer1,4, Philipp Wiemann1, Claudio Greco3, Yi Ming Chiang2, Clay C. W. Wang2, Daniel L. Lindner4, Nancy P. Keller1,3

1Department of Medical Microbiology & Immunology, University of Wisconsin–Madison, Madison, WI 53706, USA
2Departments of Chemistry and Pharmacology & Pharmaceutical Sciences, University of Southern California, Los Angeles, CA 90089, USA
3Department of Bacteriology, University of Wisconsin–Madison, Madison, WI 53706, USA
4Northern Research Station, USDA Forest Service, Madison, WI 53726, USA

Correspondence should be addressed to: Nancy P. Keller, 3467 Microbial Sciences, 1550 Linden Drive, Madison, WI 53706. Phone: +(608) 262–9795. Email: npkeller@wisc.edu

Abstract: Many fungi develop both asexual and sexual spores that serve as propagules for dissemination and/or recombination of genetic traits. Asexual spores are often heavily pigmented and this pigmentation provides protection from UV light. However, little is known about any purpose pigmentation that may serve for sexual spores. The model Ascomycete Aspergillus nidulans produces both green pigmented asexual spores (conidia) and red pigmented sexual spores (ascospores). Here we find that the previously characterized red pigment, asperthecin, is the A. nidulans ascospore pigment. The asperthecin biosynthetic gene cluster is composed of three genes: aptA, aptB, and aptC, where deletion of either aptA (encoding a polyketide synthase) or aptB (encoding a thioesterase) yields small, misshapen hyaline ascospores; while deletion of aptC (encoding a monooxygenase) yields morphologically normal but purple ascospores. ΔaptA and ΔaptB but not ΔaptC or wild type ascospores are extremely sensitive to UV light. We find that two historical ascospore color mutants, dAG and cBI, possess mutations in aptA and aptB sequences, respectively.

Keywords: Polyketide, UV protection, Ascospore, Cleistothecia, Fungi

Introduction

Sporulation is an essential developmental program for most fungi. A common characteristic of fungi, particularly members of the taxon Ascomycota, is the differentiation in time and space of asexual (mitotically derived) and sexual (meiotically derived) spores (Han et al., 2020; Zhang et al., 2020). Termed conidia and ascospores respectively, these two spore types are usually associated with long distance spread (conidia) or recombination in the sexual fruiting body, the ascocarp. Ascospores may or may not be airborne depending on the mechanism of ascocarp release. All Aspergillus spp. with a known sexual stage bear ascospores in enclosed ascocarps with dissolution of ascocarp cell walls/membranes leading to ascospore release.

Both spore types can be pigmented or hyaline (lacking pigmentation). Typically, airborne conidia are heavily pigmented, a finding that led to early speculation that pigments protected the conidia from UV radiation. This hypothesis has been largely borne out in studies of numerous fungi where pigment deficient conidia are more sensitive to UV radiation. Examples include Aspergillus fumigatus (Blachowicz et al., 2020), Aspergillus niger (Estelin et al., 2013), Cochliobolus (Bipolaris) heterostrophus (Leonard, 1977), Ashbya gossypii (Stahmann et al., 2001), Alternaria brassicicola (Cho et al., 2012), Ustilago violacea (Will et al., 1984), and Neurospora crassa (Blanc et al., 1976). In contrast, while some ascospores are airborne, particularly those produced by a subset of plant pathogenic Ascomycetes (Churchill, 2011; Gimeno et al., 2020), most ascospores are not commonly found in the air but formed in the organic debris of soils where ascocarps serve as resistant fungal tissues favorable for overwintering or surviving other harsh environmental conditions (Jeger, 1984; Mmbaga, 2000).

Although ascospores can be pigmented, few studies have examined either the genetic pathway of ascospore pigmentation or any role of pigments in sexual spore survival. The ascospores of the genetic model Aspergillus nidulans are deep red in color. One of the first studies to address the chemistry of ascospore pigmentation in this fungus found that the pigment was a polyketide-derived dimeric hydroxylated anthraquinone (Brown & Salvo, 1994). Genetic studies of A. nidulans identified both colorless (c) and blue (bl) variants of the red ascospore color (Apirion, 1963; Peintner & Rainer, 1999). The genes in the pigmentation pathway of conidia in A. nidulans are not physically linked (Mayorga & Timberlake, 1990; O’Hara & Timberlake, 1989), unlike the melanin gene cluster of the grey/blue polyketide A. fumigatus conidial pigment (Tsai et al., 1999) and most other fungal specialized metabolic pathways (Kjaerboiling et al., 2019). Therefore we investigated if the polyketide pathway of the anthraquinone red ascospore pigment would be arranged in a gene cluster and contain genes representing the c and bl mutants of previous genetic studies. We present our findings that the ascospore pigment of A. nidulans is formed by the previously characterized three gene asperthecin biosynthetic gene cluster (BGC) and that two historical c1 mutants represent mutants in the polyketide synthase Apta and the metallo-β-lactamase-type thioesterase AptB. Loss of Apta or AptB yields misshapen, small ascospores highly sensitive to UV radiation.

Received: May 13, 2021. Accepted: August 3, 2021.
© The Author(s) 2021. Published by Oxford University Press on behalf of Society of Industrial Microbiology and Biotechnology. This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted reuse, distribution, and reproduction in any medium, provided the original work is properly cited.
Table 1 Aspergillus nidulans Strains Used in This Study

| Strain        | Genotype                              | Source                                      |
|---------------|---------------------------------------|---------------------------------------------|
| RJMP103.5     | Wild type                             | (Palmer et al., 2013)                       |
| LO2131        | pyroA4, pyrG89, riboB2, ΔaptA:pyroA4, ΔsumO:pyrG, Δnkua, veA+ | (Szewczyk et al., 2008)                     |
| LO2435        | pyroA4, pyrG89, riboB2, ΔaptB:pyroA4, ΔsumO:pyrG, Δnkua, veA+ | (Szewczyk et al., 2008)                     |
| LO2440        | pyroA4, pyrG89, riboB2, ΔaptC:pyroA4, ΔsumO:pyrG, Δnkua, veA+ | (Szewczyk et al., 2008)                     |
| RDIT55.3      | pyroA4, veA+                          | (Tsitsigiannis et al., 2005)                |
| RJMP240.8     | ΔaptA, veA+                           | This study                                 |
| RJMP238.5     | ΔaptB, veA+                           | This study                                 |
| RJMP239.7     | ΔaptC, veA+                           | This study                                 |
| TJMP190.4     | pyrG89, pyroA4, ΔmatB:pyrG, Δnkua, veA+ | This study                                 |
| RJMP290.2     | ΔmatB, veA+                           | This study                                 |
| RJMP291.12    | ΔmatB, ΔaptC, Δnkua, veA+             | This study                                 |
| RJMP239.13    | pyroA4, pyrG89, metG1, veA+           | This study                                 |
| FGSCA674      | clB1, yA2, wA2, scC12                 | FGSC                                        |
| FGSCA280      | clA6, proA1, pabaA1, yA2, palB7       | FGSC                                        |
| FGSCA268      | blA1, yA2, wA3, thiA4, cmeE16, adeD3  | FGSC                                        |
| RJMP250.1     | pyroA4, veA+                          | This study                                 |
| RJMP251.3     | clA6, pyroA4, veA+                    | This study                                 |
| RJMP252.14    | clB1, pyroA4, veA+                    | This study                                 |
| RJMP250.11    | blA1, pyroA4, veA+                    | This study                                 |
| RDIT88.13     | ΔnsdD, veA+                           | This study                                 |
| RJMP4.9       | pyroA4, pyrG89, Δnkua, veA+           | (Tsitsigiannis et al., 2004)                |
| RCCG1.4       | pyroA4, pyrG89, riboB2, ΔaptC:pyroA4, ΔsumO:pyrG, Δnkua, veA+ | (Shaaban et al., 2010)                     |
| TCGG1.1       | pyroA4, pyrG89, riboB2, ΔaptC:pyroA4, ΔsumO:pyrG, Δnkua, veA+ | This study                                 |
| TCGG9.1       | pyroA4, Δnkua::agB, ΔaggA::pyroA4, veA+ | This study                                 |

Materials and Methods

Fungal Strains

Fungal strains are listed in Table 1 and primers are listed in Supplementary Table S1. All fungal strains were maintained on glucose minimal media (GMM) and, when appropriate, medium was supplemented for growth of auxotrophic strains according to standard protocols. Creation of isogenic asperthecin mutant strains was done by crossing RDIT55.3 (pyroA4, veA+) to each of the single gene deletion strains of the asperthecin cluster (LO2131–LO2440 and LO2440–ΔaptC) (Szewczyk et al., 2008). The single ΔaggA mutant was obtained by deletion of aggA using pyroA as a marker. The double deletion strain ΔagitC/ΔaggA was created by deletion of aggA with riboB. The single ΔaggA deletion mutant was confirmed by Southern analysis (Supplementary Fig. S1) while the double mutant was confirmed via diagnostic PCR (data not shown).

Quantification of Ascospores

Sexual development was induced in A. nidulans by overlay inoculation of 1 × 10^6 spores on Champe’s medium (Champe & el-Zayat, 1989) agar petri plates and subsequently incubated in the dark at 37°C for 7 days. Ascospores were quantified from sexual developmentally induced plates by homogenization of three 10-mm cores from each plate in 3 ml of sterile water and enumerated using a hemacytometer. If asci were present with visible immature ascospores inside, each ascus was counted as eight ascospores. Four biological replicates were conducted for each fungal strain and statistical significance was calculated with an ANOVA using Tukey multiple comparison posttest with Prism 6 (GraphPad). Sensitivity to UV-induced DNA damage was compared to sensitivity to methyl methanesulfonate (MMS) by spot plating serial dilutions of ascospores onto GMM plates that were treated with UV light or onto GMM plates amended with different concentrations of MMS.

Developmental mRNA Expression of Asperthecin Cluster

Wild-type A. nidulans (RJMP103.5) was overlay inoculated on Champe’s medium at a density of 1 × 10^6 spores per petri dish. Asexually induced cultures were incubated at 37°C in constant light and sexually induced cultures were incubated at 37°C in constant darkness. Tissue was harvested at several time points by scraping mycelia/conidia/cleistothecia from the surface of petri dishes using a glass slide. Tissue was lyophilized overnight and total RNA was extracted using the TRIZol Plus RNA Purification Kit (ThermoFisher #12183555) according to the manufacturer’s recommendations. Northern analysis was conducted and radiolabeled probes were generated from ~1 kb PCR products specific to each gene (Supplementary Table S2).

Secondary Metabolites Extraction

Different strains were point inoculated (10^6 spores) on Champe’s media and grown at 37°C in the dark for 18 days. Water, filtered through sterile miracloth, and ascospores were enumerated using a hemacytometer. Approximately 75 ascospores were spread plate onto solid agar medium and exposed to UV-C (254 nm) irradiation using a UV crosslinker, incubated at 37°C for 24–48 h, and colony forming units (CFUs) were counted. All CFU data was normalized to percent survival by comparing CFUs on the treated plates to nontreated control plates for each strain. Four biological replicates were conducted for each fungal strain and statistical significance was calculated at the 50 ml/cm² treatment with an ANOVA using Tukey multiple comparison posttest with Prism 6 (GraphPad).
Cleistothecia from sexually induced cultures were scrapped off the surface using a glass slide, transferred to a glass vial, homogenized, and extracted using acetonitrile/formic acid/dimethyl sulfoxide (98:5:0.5:1). The rest of the plate was blended and extracted with ethyl acetate (100 ml). After 2 h, the solid was removed using vacuum filtration and the organic layer was washed with water (3 × 50 ml). The combined organic phases were dried over anhydrous magnesium sulfate and concentrated under reduced pressure. The crude extracts were resuspended in LCMS-grade acetonitrile to obtain a concentration of 10 mg/ml, filtered through a PTFE 0.2 μm Teflon syringe filter.

HPLC-DAD

HPLC-DAD for analysis was performed on Gilson GX-271 Liquid Handler with system 322 H2 Pump connected to a 171 Gilson Diode Array Detector and fraction collector. A XBridge BEH C18 XP Column (130 Å, 2.5 μm, 4.6 mm × 100 mm) with XBridge BEH C18 XP VanGuard Cartridge (130 Å, 2.5 μm, 3.9 mm × 5 mm) was used for analytical run with a flow rate of 0.8 ml/min. HPLC-grade water with 0.5% formic acid (solvent A) and HPLC-grade acetonitrile with 0.5% formic acid (solvent B) were used with the following gradients: 0 min, 20% Solvent B; 2 min, 20% Solvent B; 15 min, 95% Solvent B; 18 min, 95% Solvent B; 20 min, 20% Solvent B. Data acquisition and procession for the HPLC-DAD were controlled by TRILUTION LC V3.0.

UHPLC-HRMS

UHPLC-HRMS was performed on a Thermo Scientific-Vanquish UHPLC system connected to a Thermo Scientific Q Exactive Orbitrap mass spectrometer in ES+ and ES− mode between 200 m/z and 1000 m/2 to identify metabolites. A Zorbax Eclipse XDB-C18 column (2.1 × 150 mm, 1.8 μm particle size) was used with a flow rate of 0.2 ml/min for all samples. LCMS-grade water with 0.5% formic acid (solvent A) and LCMS-grade acetonitrile with 0.5% formic acid (solvent B) were used with the following gradients: 0 min, 20% Solvent B; 2 min, 20% Solvent B; 15 min, 95% Solvent B; 18 min, 95% Solvent B; 20 min, 20% Solvent B. Nitrogen was used as the sheath gas. Data acquisition and procession for the UHPLC-HRMS were controlled by Thermo Scientific Xcalibur software.

Whole Genome Resequencing of Historical Ascospore Color Mutants

Three historical ascospore color mutants (clA6, clB1, and blA1) were obtained from the Fungal Genetics Stock Center (McCluskey et al., 2010) and each color mutant was subsequently crossed with RJMP139.13 to create single mutant strains in a similar genetic background (pyrA4, veA+). One isolate from each cross as well as a wild-type isolate obtained from one of the crosses (RJMP250.1, RJMP250.11, RJMP251.3, and RJMP252.14) were sequenced on the Ion Torrent Personal Genome Machine. High molecular weight DNA was isolated as described in Palmer et al. (2014) and DNA-sequencing libraries for each isolate were constructed using the Ion Xpress Plus Fragment Library Kit (#4471269), templated onto Ion Sphere Particles using the Ion PGM Template OT2 400 Kit (#4479878), and sequenced using the Ion PGM 400bp Sequencing Kit (#4482002) using an Ion 318v2 (#4484355) chip according to manufacturer’s recommendations. Single nucleotide polymorphisms (SNPs) were identified against the A. nidulans FGSCA4 reference genome (obtained from www.aspgd.org) using CLC genomics Workbench 8.5.1 (Qiagen) as previously described (Pfannenstiel et al., 2017).

Table 2 Genes Deleted in blA1 Mutant

| Gene name | Putative function |
|-----------|------------------|
| AN3492    | hypothetical, putative end of chromosome |
| AN3493    | hypothetical |
| AN3494    | hypothetical |
| AN3495    | hypothetical |
| AN3496    | hypothetical |
| AN3497    | putative glutathione S transferase |
| AN3498    | putative cyclohexane monoxygenase |
| AN3499    | putative ER transporter homology |
| AN3500    | putative retropepsin |

Note. Fellutamide biosynthetic gene cluster shown in bold.

Results

Ascopercin is the Ascospore Pigment

The sexual spore (ascospore) pigment of A. nidulans has previously been chemically characterized as the polyketide ascoquinone A (Brown & Salvo, 1994); however, no secondary metabolite gene cluster has been linked to production of this compound. A forward genetics screen by Apirion (1963) identified three ascospore color mutants in A. nidulans: clA6 produced clear ascospores, clB1 also produced clear ascospores, and blA1 produced blue ascospores. To determine which of the secondary metabolite gene clusters of A. nidulans was involved in ascospore pigmentation, we obtained strains harboring these mutations from the Fungal Genetics Stock Center (Table 1) and subsequently outcrossed each strain once to generate near isogenic backgrounds (pyrA4, veA+). Whole genome sequencing was employed for each mutant strain (RJMP251.3, RJMP252 15, RJMP250 11) as well as a near isogenic wild-type strain (RJMP250 1). Using SNP analysis from these data, we were able to identify mutations in the clear ascospore mutants, clA6 and clB1. Both strains identified SNPs in the previously characterized three-gene cluster responsible for asperpercin production (Szweczyk et al., 2008). The clA6 mutant (RJMP251.3) has a two base pair insertion in the coding region of FKS-encoding aptA that results in a frame-shift mutation at Leu-1151. In the clB1 mutant, we identified a G → A mutation at position 902 in the coding region of the stand-alone thioesterase-encoding aptB, which resulted in mutating the Trp-301 to an early termination signal. We were unable to identify mutations in the aptC gene or surrounding genes in the blue ascospore mutant blA1. Through sexual crosses, the blA1 mutation was mapped to the right arm of chromosome II (Apirion, 1963). We were unable to identify a specific causative mutation in this region; however, we did identify a large deletion near the right telomere of chromosome 2 from AN3500 to the end of the chromosome (∼100 kb) containing ca 30 putative genes including the characterized fellutamide gene cluster (Yeh et al., 2016 and Table 2). The apt cluster is located on chromosome 3. To determine if the asperpercin gene cluster was turned on during sexual development, we looked at expression levels during darkness (inducing sexual development) and in light (inducing asexual development) in a wild-type A. nidulans genetic
Fig. 1 Asperthecin cluster genes are expressed during sexual development but not in ascospores. (Top panel) Aspergillus nidulans development in light and dark regimes. Green conidia (mitotic spores) are produced on asexual conidiophores and red ascospores (meiotic spores) are produced with in the sexual fruiting body, the cleistothecium. (Bottom panel) mRNA extracted from light and dark regimes show that the three asperthecin genes (aptA, aptB, and aptC) are highly expressed during sexual development but not in ascospores. BrlA is a transcription factor required for conidiophore initiation and VosA is a regulatory protein required for maturation of both conidia and ascospores.

Szewczyk et al. (2008) characterized the asperthecin gene cluster in a ΔsumO deletion background, which renders the fungus very “sick” and unable to induce sexual development. We constructed new asperthecin gene cluster ΔaptA, ΔaptB, ΔaptC deletion mutants (Table 1) in a veA+ genetic background through sexual crosses. Morphological characterization of cleistothecia in these genetic backgrounds revealed that deletion of the polyketide synthase, PKS (aptA), or the metallo-β-lactamase-type thioesterase (aptB) resulted in clear (hyaline) ascospores, matching their forward genetic counterparts, clA6 and clB1. Deletion of the flavin-dependent monooxygenase (aptC) resulted in ascospores that were visually blue-purple in color (Fig. 2A). Quantification of ascospores from these mutant strains also revealed that deletion of either aptA or aptB resulted in a reduction in the number of ascospores produced (Fig. 2B). Moreover, ascospores from these strains appeared to be immature as they were often found still inside asci even after prolonged incubation, and those ascospores that were released were misshapen (Fig. 2A). However, the blue-purple ascospores produced by ΔaptC strains appeared to be developmentally mature and were produced at wild-type levels (Fig. 2).

Asperthecin and Pigmented Precursor Confer Resistance to UV Radiation

Many spore pigments are known to protect spores from UV radiation. Therefore we tested the ability of asperthecin to confer resistance to UV light. Ascospores were exposed to different exposures of UV-C (254 nm) light and survival was measured by taking a ratio of CFUs from UV treated plates versus untreated control plates. The clear ascospores from ΔaptA and ΔaptB displayed an increased sensitivity to UV light compared to wild-type red ascospores at 50 mJ/cm² treatment (Fig. 3A, B). Interestingly, the blue-purple ascospores from the ΔaptC mutant was slightly more resistant to UV light than the wild type. UV light induces pyrimidine-dimer damage in DNA and must be repaired in order for the fungus to survive, thus the UV light survival assay is
Fig. 2 Asperthecin gene cluster deletion mutants have ascospore pigment and morphology phenotypes. (A) Deletion of the polyketide synthetase AptA or the beta-lactamase AptB results in hyaline and immature ascospore production. Cleistothecial cell walls are lighter in pigmentation compared to wild type and ascospores are commonly found contained in asci. Deletion of AptC, a putative monooxygenase, results in purple-blue ascospore pigmentation but wild-type ascospore and asci morphology. (B) Mutants with hyaline ascospore production are produced at an order of magnitude less than wild-type or ΔaptC mutants.

Fig. 3 Asperthecin protects sexual spores from UV light. (A) ΔaptC ascospores are slightly more resistant than wild type to UV light, while both ΔaptA and ΔaptB ascospores are very sensitive to UV damage. Error bars represent standard error of four biological replicate experiments. Statistical significance was calculated using the 50 mJ/cm² treatment with an ANOVA and Tukey multiple comparison posttest (P < 0.001). (B) Comparison of dilution plating of ascospores from wild type and apt mutants from UV and methyl methanesulfonate (MMS) (DNA damaging agent) treatment demonstrates that UV sensitivity is a result of loss of pigment protection and not of DNA repair machinery.

measuring two distinct “resistance” pathways, the first being the ability of the spore metabolites (pigments) to absorb UV light thereby protecting the DNA from damage, while the survival assay is also measuring the ability of the fungus to repair damaged DNA. These two pathways can be distinguished by measuring sensitivity to MMS, a chemical mimic of pyrimidine-dimer damage. Fungal mutants that are differentially sensitive to both UV light and MMS damage would be indicative of a defect in DNA repair pathway, whereas sensitivity to only UV light and not MMS is indicative of a fungal mutant that is defective in “physical” protection, that is, pigments in the spore. None of the asperthecin gene cluster mutants were susceptible to MMS in an ascospores spot dilution plate assay (Fig. 3B), thus supporting a role of physical protection for asperthecin.

Apt Enzymes are Expressed in Developing Cleistothecia

Based on gene expression analysis, the asperthecin genes aptA, aptB, and aptC were specifically turned on during cleistothecial maturation of sexual development (Fig. 1). Moreover, these data showed that transcripts from all three enzymes are absent from ascospores as well as from asexual developmental tissue (Fig. 1), suggesting that the biosynthetic cluster is active during ascosporogenesis.

Characterization of the ΔaptC Pigment and Noninvolvement of Putative Aperthecin Tailoring Genes

In order to learn more about the chemical profiles of the deletion mutants, we extracted metabolites under sexual development inducing conditions. HPLC-DAD analysis of metabolites extracted from crushed cleistothecia of the wild-type strain, various deletion mutants, and an asperthecin standard revealed the loss of asperthecin (Fig. 4: peak 3) in ΔaptA, ΔaptB, ΔaptC strains as well as in the corresponding mutants clA6, clB1, and blA1 (Fig. 4). We were surprised to see an accumulation of a new metabolite (Fig. 4: peak 7) that appeared in all deletion mutants except for blA1 (Fig. 4). Since the PKS (AptA) that synthesizes the carbon
Fig. 4 HPLC-DAD analysis of cleistothecial metabolites. Cleistothecia were collected, crushed, and extracted for metabolites from indicated strains of *Aspergillus nidulans*. (A) HPLC-PDA chromatograms of the crude extracts from each strain compared to the chromatogram of the standard asperthecin (3). Key peaks are highlighted by a dashed line and details about acospore production of each mutant are provided on the left. (B) UV-Vis spectrum of the eight and compared to the UV-Vis spectrum of asperthecin (3) shown by comparison to a standard.

Backbone of asperthecin is absent in the ΔaptA strain, peak 7 is therefore not derived from the Apt pathway. We also analyzed knockout strains of two genes involved in sexual development in *A. nidulans*, the positive acting GATA-type transcription factor encoding gene nsdD (Han et al., 2001) and the gene, MAT-1 (matB), encoding the “alpha-box” containing protein (Dyer et al., 2003), as well as a matB/aptC double deletion strain. Our data show an overproduction of asperthecin in ΔmatB and an absence of this compound in the ΔnsdD strain. Noteworthy is absence of the new metabolite (Fig. 4A: peak 7) in the ΔnsdD mutant. Analysis of ΔΔmatB/aptC mutant displayed almost the exact profile as the ΔaptC single deletion strain (Fig. 4).

As mentioned earlier, the original chemical characterization of the ascospore pigment resulted in structure elucidation of ascoquinone A (Brown & Salvo, 1994). However, this structure varies from asperthecin and a putative downstream metabolite of asperthecin, although no mass for ascoquinone A has been characterized experimentally (Brown & Salvo, 1994). We were most interested in trying to identify the structure of the purple pigment of the ΔaptC ascospores since the proposed functions of AptA, B, and C were biochemically elucidated in a heterologous expression host (Li et al., 2011). LCMS analysis of the extracts detected four metabolites, two prominent ones with respective m/z values of 355.0460 (5, C18H11O8), and 299.0196 (6, C15H7O7) in negative ionization mode (Supplementary Fig. S2); however, none of these metabolites correspond to the known intermediates described in the Apt pathway (Li et al., 2011). These data suggest that the metabolites detected are either shunt products of the Apt pathway, from different pathways, or represent an uncharacterized intermediate. Since asperthecin only contains the chemical makeup of a decarboxylated octaketide (C15), we speculated that during biosynthesis additional chain shortening had to occur.

Precedent for chain shortening reactions during pigment biosynthesis comes from *A. fumigatus* and *Botrytis cinerea*, where the hydrolase Ayg1 catalyzes such reactions (Fujii et al., 2004; Schumacher, 2016; Tsai et al., 2001). BlastP analysis of *A. fumigatus* Ayg1 querying the *A. nidulans* FGSC A4 genome resulted in one hit, AN9171 (63.7% identity, e-value: 4e-115), located on chromosome VI unlinked to the asperthecin gene cluster. As comparison, *A. fumigatus* Ayg1 and *B. cinerea* Ayg1 share 52.4% identity (e-value: 5e-161). To determine if AygA was indeed involved in the hypothesized chain shortening reaction, we created ΔaygA and analyzed the secondary metabolite profile under sexually inducing conditions. However, the wild-type strain and the ΔaygA strains did not
have any apparent difference and both still produced asperthecin (Supplementary Fig. S2). Therefore, the mechanism of chain shortening remains elusive.

**Discussion**

In the past, secondary metabolites have been defined as metabolites that are not required for normal growth and development. However, many studies across fungal taxa show clear biological roles of secondary metabolites in fungal biology that are exceptions to this simplistic definition (Demain & Fang, 2000). For example, iron-binding siderophores are often defined as secondary metabolites; however, they play a key role in scavenging iron from environments, which is necessary for growth of many microbes (Haas et al., 2008). Xanthocillins, encoded by an isocyaniyne gene cluster, participate in copper homeostasis (Raffa et al., 2021). Some fungal secondary metabolites are required for asexual spore development (Zhang et al., 2017) and some even suppress fungal growth such as fusaristatin A produced by certain isolates of Fusarium pseudogroarum (Khudhair et al., 2020). Additionally, the literature is rich with examples of how fungal natural products provide protection/secures environmental niches when utilized as “weapons” against other microbes (Keller, 2019). Here we describe the “secondary” metabolite gene cluster responsible for production of the sexual spore pigment in A. nidulans, show it is required for normal ascosporogenesis, and protects ascospores from UV light.

The switch from vegetative growth to asexual or sexual development is a genetic regulatory pathway that is controlled by many factors in filamentous fungi. In a general sense, environmental stimuli such as changes in carbon, nitrogen, pH, and light are recognized by fungi which in term elicit a morphological change in growth. For the model fungus, A. nidulans, several stimuli are known to induce asexual or sexual development. Asexual development is induced by light through a genetic pathway involving a series of transcription factors (Yu, 2010) in which the end result is formation of conidiophores bearing specialized progenitor cells called phialides that produce conidia. Phialides are in essence the “stem cells” of asexual reproduction in Aspergillus as they repeatedly produce mitotically derived propagules. While the exact mechanisms are not understood, phialides also act as the “packaging center” for conidia where components of the cell wall are integrated, including conidial cell wall pigments. Whereas structural elucidation of the A. nidulans conidial pigment has remained elusive (postulated to be a complex polyketide polymer), the genes involved in production of the pigment are well known, a polyketide synthase yA (Mayorga & Timberlake, 1990) that synthesizes YWA1 intermediate which is then hypothesized to be polymerized by a laccase yA to form the green-colored pigment (O’Hara & Timberlake, 1989). Both of the enzymes, WA and YA, are specifically expressed in phialides but absent from conidia, indicating that the polymerized pigment is integrated into the conidial cell wall inside the phialide (Aramayo & Timberlake, 1993; O’Hara & Timberlake, 1989). Numerous studies have reported the role of the conidial pigments for structural integrity of the conidial cell wall and protection from UV light in various fungi, particularly in the genus Aspergillus (Blachowicz et al., 2020; Geib et al., 2016; Wang et al., 2020; Zhang et al., 2017).

Sexual development is in turn induced by growth in the dark in which the coordinated activity of several transcription factors (Han, 2009) that govern formation of cleistothecia composed of ascogenous hyphae give rise to asci each containing eight ascospores. Consistent with the conidial pigment, we show that the sexual spore pigment is produced by the asperthecin PKS containing gene cluster (Fig. 2). Additionally, the asperthecin cluster is specifically expressed in cleistothecia (Fig. 1). Moreover, asperthecin protects ascospores from UV light (Fig. 3). In order to test if asperthecin specifically protects the ascospores from UV light-based DNA damage, we also tested the ascospores for sensitivity to a chemical mimics of UV light that induces the same DNA damage (pyrimidine-dimer formation). As expected, there was no differential sensitivity to MMS between the deletion mutants of the asperthecin gene cluster. These data indicate that the general DNA damage repair pathway is therefore not affected in asperthecin cluster deletion mutants; but rather, that asperthecin specifically protects ascospores from UV light. This is consistent with asperthecin absorbing light in the UV range (Fig. 4B). The ΔaptA mutant metabolites also provide protection in the UV range, whereas, notably, there is an absence/diminution of any UV absorbing metabolites in the ΔaptA and ΔaptB mutants. We speculate that the enhanced resistance of ΔaptC ascospores to UV light, compared to wild type ascospores (Fig. 5), may be due to the numerous UV light absorbing metabolites made by this strain (Fig. 4B).

A consistent theme with spore pigments is that they form polymers which are thought to add structural rigidity. This is clearly documented in the fungus Pestalotiopsis fici where deletion of the conidal PKS results not only in loss of conidal pigmentation but also multicell conidial integrity (Zhang et al., 2017). Similarly, the polyketide-derived fusarubins contribute the dark colorization of the perithecial cell wall and their structural integrity (Studt et al., 2012). DHN melanin is an example of a spore pigment that is found in several filamentous fungi and is known to form large polymers (Ao et al., 2019; Pal et al., 2014; Schumacher, 2016). Due to the propensity for these pigments to polymerize, chemical characterization of spore metabolites is difficult. Brown and Salvo (1994) identified the sexual spore pigment of A. nidulans as ascoquinone A (MW = 619.0724); however, this identification was inferred by UV spectra and no mass corresponding to ascoquinone A was identified. We have repeatedly looked for a mass consistent with ascoquinone A and have also been unable to identify it via mass spectrometry of extracts. Instead, we were able to consistently identify asperthecin (MW = 318.0376) by HPLC and mass spectrometry. Interestingly, ascoquinone A is composed of two asperthecin molecules joined with an ester linkage. While we were unable to generate evidence to definitely support the claim that asperthecin polymerizes as a structural component of the ascospore cell wall—given the polymerization theme in other spore pigments, the requirement of asperthecin for normal ascospore cell wall—given the polymerization theme in other spore pigments, the requirement of asperthecin for normal ascospore cell wall maturation and protection from UV light.

Surprisingly, the ΔaptC mutant accumulated several unique masses although none were the known intermediates of the Apta pathway reconstituted in Saccharomyces cerevisiae (Li et al., 2011). Finally, we were able to assign two historical clear ascospore mutants (clA6 and clB1) to specific mutations in the aptA and aptB genes, respectively. What remains a mystery is the explanation of the b1A1 mutant, which despite producing ascospores that superficially resemble the ΔaptC ascospores, we were unable to find any mutations in aptC.

**Supplementary Material**

Supplementary material is available online at JIMB (www.academic.oup.com/jimb).
Funding
This work was supported in part by National Institutes of Health under grant 2R01GM112739-05A1 to N.P.K and by USDA Forest Service, Northern Research Station.

Conflict of Interest
The authors declare no conflict of interest.

Data Availability
Raw sequencing data is available via the NCBI Small Read Archive under the SRP326812 accession and the corresponding BioProject PRJNA743544 accession.

References
Ao, J., Bandyopadhyay, S., & Free, S. J. (2019). Characterization of the Neurospora crassa DHN melanin biosynthetic pathway in developing ascospores and peridium cells. Fungal Biology, 123(1), 1–9.
Apiron, D. (1963). Formal and physiological genetics of ascospore colour in Aspergillus nidulans. Genetical Research, 4(2), 276–283.
Aramayo, R. & Timberlake, W. E. (1993). The Aspergillus nidulans yA gene is regulated by abaA. EMBO Journal, 12(5), 2039–2048.
Blachowicz, A., Raffa, N., Bok, J. W., Choera, T., Knox, B., Lim, F. Y., Hutenlocher, A., Wang, C. C. C., Venkateswaran, K., & Keller, N. P. (2020). Contributions of spore secondary metabolites to UV-C protection and virulence vary in different Aspergillus fumigatus strains. mBio, 11(1), e03415–19.
Blanc, P. L., Tuveson, R. W., & Sargent, M. L. (1976). Inactivation of carotenoid-producing and albino strains of Neurospora crassa by visible light, blacklight, and ultraviolet radiation. Journal of Bacteriology, 125(2), 616–625.
Brown, D. W. & Salvo, J. J. (1994). Isolation and characterization of sexual spore pigments from Aspergillus nidulans. Applied and Environmental Microbiology, 60(3), 979–983.
Champe, S. P. & el-Zayat, A. A. (1989). Isolation of a sexual sporation hormone from Aspergillus nidulans. Journal of Bacteriology, 171(7), 3982–3988.
Cho, Y., Srivastava, A., Ohm, R. A., Lawrence, C. B., Wang, K. H., Grigoriev, I. V., & Marahatta, S. P. (2012). Transcription factor Amr1 induces melanin biosynthesis and suppresses virulence in Alternaria brassicicola. PLoS Pathogens, 8(10), e1002974.
Churchill, A. C. (2011). Mucosphaerella fijensis, the black leaf streak pathogen of banana: progress towards understanding pathogen biology and detection, disease development, and the challenges of control. Molecular Plant Pathology, 12(4), 307–328.
Dernain, A. L. & Fang, A. (2000). The natural functions of secondary metabolites. Advances in Biochemical Engineering/Biotechnology, 69, 1–39.
Dyer, P. S., Paoletti, M., & Archer, D. B. (2003). Genomics reveals sexual secrets of Aspergillus. Microbiology (Reading, England), 149(9), 2301–2303.
Estelin, J., Mallea, S., Ram, A. F., & Carlin, F. (2013). Role of pigmentation in protecting Aspergillus niger conidiospores against pulsed light radiation. Photochemistry and Photobiology, 89(3), 758–761.
Fujii, I., Yasuoka, Y., Tsai, H. F., Chang, Y. C., Kwon-Chung, K. J., & Ebizuka, Y. (2004). Hydrolytic polyketide shortening by ayg1p, a novel enzyme involved in fungal melanin biosynthesis. Journal of Biological Chemistry, 279(43), 44613–44620.
Geib, E., Gressler, M., Viedieriukova, I., Hillmann, F., Jacobsen, I. D., Nietzsche, S., Hertweck, C., & Brock, M. (2016). A non-canonical melanin biosynthesis pathway protects Aspergillus terreus conidia from environmental stress. Cell Chemical Biology, 23(5), 587–597.
Gimeno, A., Kagi, A., Drakopoulos, D., Banziger, I., Lehmann, E., Forrer, H. R., Keller, B., & Vogelsang, S. (2020). From laboratory to the field: biological control of Fusarium graminearum on infected maize crop residues. Journal of Applied Microbiology, 129(3), 680–694.
Haas, H., Eisendle, M., & Turgeon, B. G. (2008). Siderophores in fungal physiology and virulence. Annual Review of Phytopathology, 46(1), 149–187.
Han, K. H. (2009). Molecular genetics of Emricicella nidulans sexual development. Mycobiology, 37(3), 171–182.
Han, K. H., Han, K. Y., Yu, J. H., Chae, K. S., Jahng, K. Y., & Han, D. M. (2001). The nsd1 gene encodes a putative GATA-type transcription factor necessary for sexual development of Aspergillus nidulans. Molecular Microbiology, 41(2), 299–309.
Han, X., Chen, L., Li, W., Zhang, L., Zhang, S., Zou, S., Liang, Y., Yu, J., & Dong, H. (2020). Endocytic FgEde1 regulates virulence and autophagy in Fusarium graminearum. Fungal Genetics and Biology, 141, 103400.
Keller, N. P. (2019). Fungal secondary metabolism: regulation, function, and drug discovery. Nature Reviews: Microbiology, 17, 167–180.
Khudhair, M., Kazan, K., Thatcher, L. F., Obanor, F., Rusu, A., Sorensen, J. L., Wollenberg, R. D., McKay, A., Giblot-Ducray, D., Simpfendorfer, S., Aitken, E., & Gardner, D. M. (2020). Fusaristatin A production negatively affects the growth and aggressiveness of the wheat pathogen Fusarium pseudogrumeaum. Fungal Genetics and Biology, 136, 103314.
Kjaerbolting, I., Mortensen, U. H., Vesth, T., & Andersen, M. R. (2019). Strategies to establish the link between biosynthetic gene clusters and secondary metabolites. Fungal Genetics and Biology, 130, 107–121.
Leonard, K. J. (1977). Virulence, temperature optima, and competitive abilities of isolines of races T and O of Bipolaris maydis. Phytopathology, 67(10), 1273–1279.
Li, Y., Chooi, Y. H., Sheng, Y., Valentine, J. S., & Tang, Y. (2011). Comparative characterization of fungal anthracenone and napthacenedione biosynthetic pathways reveals an alpha-hydroxylation-dependent C1aisen-like cyclization catalyzed by a dimanganese thioesterase. Journal of the American Chemical Society, 133(39), 15773–15785.
Mayorga, M. E. & Timberlake, W. E. (1990). Isolation and molecular characterization of the Aspergillus nidulans uA gene. Genetics, 126(1), 73–79.
McCluskey, K., Wiest, A., & Flannam, M. (2010). The Fungal Genetics Stock Center: a repository for 50 years of fungal genetics research. Journal of Biosciences, 35(1), 119–126.
Mmbaga, M. T. (2000). Winter survival and source of primary inoculum of powdery mildew of dogwood in Tennessee. Plant Disease, 84(5), 574–579.
O’Hara, E. B. & Timberlake, W. E. (1989). Molecular characterization of the Aspergillus nidulans yA locus. Genetics, 121(2), 249–254.
Pal, A. K., Gajjar, D. U., & Vasavada, A. R. (2014). DOPA and DHN pathway orchestrate melanin synthesis in Aspergillus species. Medical Mycology, 52, 10–18.
Palmer, J. M., Kubatova, A., Novakova, A., Minnis, A. M., Kolarik, M., & Lindner, D. L. (2014). Molecular characterization of a heterothallic mating system in Pseudogymnoascus destructans, the fungus causing white-nose syndrome of bats. G3, 4(9), 1755–1763.
Palmer, J. M., Theisen, J. M., Duran, R. M., Grayburn, W. S., Calvo, A. M., & Keller, N. P. (2013). Secondary metabolism and development is mediated by LlmF subcellular localization in Aspergillus nidulans. PLoS Genetics, 9(1), e1003193.

Peintner, U. & Rainer, J. (1999). A blue ascospore colour variant of Emericella nidulans. Mycotoxin, 70, 445–451.

Pfannenstiel, B. T., Zhao, X., Wortman, J., Wiemann, P., Throckmorton, K., Spraker, J. E., Soukup, A. A., Luo, X., Lindner, D. L., Lim, F. Y., Knox, B. P., Haas, B., Fischer, G. J., Choera, T., Butchko, R. A. E., Bok, J. W., Affeldt, K. J., Keller, N. P., & Palmer, J. M. (2017). Revitalization of a forward genetic screen identifies three new regulators of fungal secondary metabolism in the genus Aspergillus. mBio, 8(5), e01246–17.

Raffa, N., Won, T. H., Sukowaty, A., Candor, K., Cui, C., Halder, S., Dai, M., Landero-Figueroa, J. A., Schroeder, F. C., & Keller, N. P. (2021). Dual-purpose isocyanides produced by Aspergillus fumigatus contribute to cellular copper sufficiency and exhibit antimicrobial activity. Proceedings of the National Academy of Sciences of the United States of America, 118(8), e2015224118.

Schumacher, J. (2016). DHN melanin biosynthesis in the plant pathogenic fungus Botrytis cinerea is based on two developmentally regulated key enzyme (PKS)-encoding genes. Molecular Microbiology, 99(4), 729–748.

Shaaban, M. I., Bok, J. W., Lauer, C., & Keller, N. P. (2010). Suppressor mutagenesis identifies a velvet complex remediator of Aspergillus nidulans secondary metabolism. Eukaryotic Cell, 9(12), 1816–1824.

Stahmann, K. P., Arst, H. N., Jr., Althofer, H., Revuelta, J. L., Monschau, N., Schlueter, C., Gatgens, C., Wiesenburg, A., & Schlosser, T. (2001). Riboflavin, overproduced during sporulation of Ashbya gossypii, protects its hyaline spores against ultraviolet light. Environmental Microbiology, 3(9), 545–550.

Studt, L., Wiemann, P., Kleigrewke, K., Humpef, H. U., & Tudosynski, B. (2012). Biosynthesis of fusarubins accounts for pigmentation of Fusarium fujikuroi perithecia. Applied and Environmental Microbiology, 78(12), 4468–4480.

Szewczyk, E., Chiang, Y. M., Oakley, C. E., Davidson, A. D., Wang, C. C., & Oakley, B. R. (2008). Identification and characterization of the asperthecin gene cluster of Aspergillus nidulans. Applied and Environmental Microbiology, 74(24), 7607–7612.

Tsai, H. F., Fujii, I., Watanabe, A., Wheeler, M. H., Chang, Y. C., Yauko, Y., Ebizuca, Y., & Kwon-Chung, K. J. (2001). Pentaketide melanin biosynthesis in Aspergillus fumigatus requires chain-length shortening of a heptaketide precursor. Journal of Biological Chemistry, 276(31), 29292–29298.

Tsai, H. F., Wheeler, M. H., Chang, Y. C., & Kwon-Chung, K. J. (1999). A developmentally regulated gene cluster involved in conidial pigment biosynthesis in Aspergillus fumigatus. Journal of Bacteriology, 181(20), 6469–6477.

Tsitsigiannis, D. I., Kowieski, T. M., Zarnowski, R., & Keller, N. P. (2004). Endogenous lipogenic regulators of spore balance in Aspergillus nidulans. Eukaryotic Cell, 3(6), 1398–1411.

Tsitsigiannis, D. I., Kowieski, T. M., Zarnowski, R., & Keller, N. P. (2005). Three putative oxylipin biosynthetic genes integrate sexual and asexual development in Aspergillus nidulans. Mycobiology (Reading, England), 151(6), 1809–1821.

Wang, J., Ma, Y., Liu, Y., Tong, S., Zhu, S., Jin, D., Pei, Y., & Fan, Y. (2020). A polyketide synthase, BbpksP, contributes to conidial cell wall structure and UV tolerance in Beauveria bassiana. Journal of Invertebrate Pathology, 169, 107280.

Will, O. H., Newland, N. A., & Reppe, C. R. (1984). The photosensitivity of pigmented and non-pigmented strains of Ustilago violace. Current Microbiology, 10(5), 295–301.

Yeh, H. H., Ahuja, M., Chiang, Y. M., Oakley, C. E., Moore, S., Yoon, O., Hajovsky, H., Bok, J. W., Keller, N. P., Wang, C. C., & Oakley, B. R. (2016). Resistance gene-guided genome mining: serial promoter exchanges in Aspergillus nidulans reveal the biosynthetic pathway for Fellutamide B, a proteasome inhibitor. ACS Chemical Biology, 11(8), 2275–2284.

Yu, J. H. (2010). Regulation of development in Aspergillus nidulans and Aspergillus fumigatus. Mycobiology, 38(4), 229–237.

Zhang, P., Wang, X., Fan, A., Zheng, Y., Liu, X., Wang, S., Zou, H., Oakley, B. R., Keller, N. P., & Yin, W. B. (2017). A cryptic pigment biosynthetic pathway uncovered by heterologous expression is essential for conidial development in Pestalotiopsis fici. Molecular Microbiology, 105(3), 469–483.

Zhang, X., Gonzalez, J. B., & Turgeon, B. G. (2020). Septins are required for reproductive pigment development and virulence of the maize pathogen Cochliobolus heterostrophus. Fungal Genetics and Biology, 135, 103291.