New Biosynthetic Step in the Melanin Pathway of \textit{Wangiella (Exophiala) dermatitidis}: Evidence for 2-Acetyl-1,3,6,8-Tetrahydroxynaphthalene as a Novel Precursor$\dagger$

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The predominant cell wall melanin of \textit{Wangiella dermatitidis}, a black fungal pathogen of humans, is synthesized from 1,8-dihydroxynaphthalene (D2HN). An early precursor, 1,3,6,8-tetrahydroxynaphthalene (T4HN), in the pathway leading to D2HN is reportedly produced directly as a pentaketide by an iterative type I polyketide synthase (PKS). In contrast, the bluish-green pigment of \textit{Aspergillus fumigatus} is produced after the enzyme Ayg1p converts the PKS product, the heptaketide YWA1, to T4HN. Previously, we created a new melanin-deficient mutant of \textit{W. dermatitidis}, WdBrm1, by random molecular insertion. From this strain, the altered gene WdYG1 was cloned by a marker rescue strategy and found to encode WdYG1p, an ortholog of Ayg1p. In the present study, two gene replacement mutants devoid of the complete WdYG1 gene were derived to eliminate the possibility that the phenotype of WdBrm1 was due to other mutations. Characterization of the new mutants showed that they were phenotypically identical to WdBrm1. Chemical analyses of mutant cultures demonstrated that melanin biosynthesis was blocked, resulting in the accumulation of 2-acetyl-1,3,6,8-tetrahydroxynaphthalene (AT4HN) and its oxidative product 3-acetylflavin in the culture media. When given to an albino \textit{W. dermatitidis} strain with an inactivated WdPKS1 gene, AT4HN was mostly oxidized to 3-acetylflavin and deacetylated to flavin. Under reduced oxygen conditions, cell-free homogenates of the albino converted AT4HN to D2HN. This is the first report of evidence that the hexaketide AT4HN is a melanin precursor for T4HN in \textit{W. dermatitidis}.

Melanins are dark pigments widely produced by fungi and other organisms. In fungi, they are frequently found in the cell wall. While not essential for growth and development, these complex polymers seem to enhance the survival and competitive abilities of fungi in certain environments. They are composed of various types of phenolic monomers and are often complexed with protein and, less often, carbohydrates (12, 22, 29). The melanins in fungi are named according to their composition and the way they are synthesized and include dihydroxyphenylalanine melanin, catechol melanin, $\gamma$-glutamyl-4-hydroxybenzene melanin, and 1,8-dihydroxynaphthalene (D2HN) melanin (23, 26). The best characterized of these melanins is probably D2HN melanin, which is synthesized from various types of phenolic monomers and are often complexed with protein and, less often, carbohydrates (12, 22, 29). The melanins in fungi are named according to their composition and the way they are synthesized and include dihydroxyphenylalanine melanin, catechol melanin, $\gamma$-glutamyl-4-hydroxybenzene melanin, and 1,8-dihydroxynaphthalene (D2HN) melanin (23, 26). The best characterized of these melanins is probably D2HN melanin, which is synthesized from related polyketide pathways (Fig. 1 and 2A and B). The D2HN melanin pathways start with one acetyl-coenzyme A (acetyl-CoA) molecule and four malonyl-CoA molecules, or solely with malonyl-CoA molecules, which undergo a head-to-tail joining and cyclization catalyzed by an iterative type I polyketide synthase (PKS) to initially form 1,3,6,8-tetrahydroxynaphthalene (T4HN) (16). From T4HN, multiple sequential enzyme-catalyzed steps produce D2HN, which is then polymerized to form melanin by a poorly characterized oxidase/laccase reaction (5, 7, 22).

In \textit{Colletotrichum lagenarium}, T4HN is made directly from malonyl-CoA by PKS1p, as shown in Fig. 2B (16). In contrast, in the bluish-green fungus \textit{Aspergillus fumigatus}, a pigment-producing polyketide synthase, Alb1p, uses acetyl-CoA and/or malonyl-CoA to produce a heptaketide naphthopyrone designated YWA1 (Fig. 1), which is then enzymatically converted to T4HN, as shown in Fig. 2C (17, 40). YWA1 is metabolized to T4HN by a post-PKS polyketide shortening mechanism (17) involving a protein called Ayg1p. This reaction occurs upstream of the T4HN reductase step that is required for the synthesis of scytalone. Other compounds made in \textit{A. fumigatus} include 1,3,8-trihydroxynaphthalene (T3HN), vermelone, and D2HN (6, 17). The reductase that converts T4HN to scytalone can be blocked by a specific pathway inhibitor known as tricyclazole (20). Using this blocking tool, the reductase step was shown to be present only in the bluish-green fungus \textit{A. fumigatus} but also in all the brown to black fungi that make D2HN melanins (5, 10, 36, 40). In \textit{A. fumigatus}, a second type of polymeric subunit made upstream from YWA1 (Fig. 2C) is also believed

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FIG. 1. Structures and abbreviated names for metabolites in the D2HN melanin biosynthetic pathway and related shunt pathways in *W. dermatitidis*. Previously, T4HN was believed to be made directly from acetyl-CoA and malonyl-CoA; however, the present study shows that T4HN is a deacetylation product of AT4HN, which is made via a WdPks1p enzyme from malonyl-CoA and acetyl-CoA or possibly malonyl-CoA only (16). The metabolites shown in the two rectangles with dashed lines have not previously been reported for *W. dermatitidis* or other brown/black fungi; however, YWA1 is a known melanin precursor in *A. nidulans* and *A. fumigatus*. The reactions that metabolize T4HN to D2HN and then melanin have been reported for *Verticillium dahliae*, *Magnaporthe grisea*, and other fungi (5, 7), as well as for *W. dermatitidis* (5, 20, 44). Reaction types are indicated as follows: (Ac), deacetylation; (O), oxidation; (H), reduction; and −H₂O, dehydration. Tricyclazole (Tr) specifically inhibits the pathway at the sites indicated. The proposed intermediates in brackets are extremely unstable and have not been isolated from fungi. Other abbreviations: 3AF, 3-acetylflaviolin; T3HN, 1,3,8-trihydroxynaphthalene; 4-HS, 4-hydroxyscytalone; 5-HS, 5-hydroxyscytalone; 2-HJ, 2-hydroxyjuglone; 3-HJ, 3-hydroxyjuglone; 4,8-DHT, 4,8-dihydroxytetralone; 3,4,8-THT, 3,4,8-trihydroxytetralone; YWA1, 2,3-dihydro-2,5,6,8-tetrahydroxy-2-methyl-4H-naphtho[2,3-b]pyran-4-one.

to be incorporated into the final melanin polymer (17, 39). Details of this second pathway are unknown at this time. In contrast to the results with *A. fumigatus*, the fungus *Aspergillus nidulans* does not make T4HN or D2HN (Fig. 2D), but instead, it uses YWA1 directly in the synthesis of its green conidial pigment (42). Again, the polymerization reaction that forms the melanin is poorly characterized.

The D2HN melanin biosynthetic pathway was initially discovered in *Verticillium dahliae* (4) and then identified in many other phytopathogens and saprophytes (45). The existence of the pathway in human-pathogenic fungi was demonstrated first in *Wangiella dermatitidis*, a dematiaceous (melanized) agent of phaeohyphomycosis (20), and shortly thereafter in a number of other related species (36). The pathway in *W. dermatitidis* from T4HN to D2HN and then to melanin (Fig. 1) was characterized (20, 44) via the chemical identification of key intermediates or shunt metabolites that accumulated in cultures of different melanin-deficient (Mel⁻) mutant strains, the inhibitor tricyclazole, metabolic cross-feeding by the different Mel⁻ strains, and enzymatic studies. Subsequently, the D2HN melanin was found to be polymerized exclusively in the cell walls of *W. dermatitidis*, and the earliest enzymatic step identified in its synthesis was determined to involve a PKS encoded by the gene WdPKS1 (14, 44). Importantly, the pathway has been documented to be a virulence factor in *W. dermatitidis* by virtue of the finding that all Mel⁻ mutants tested thus far are less virulent than the wild type in mouse models of acute infection (12, 13, 14), less resistant to the phagolysosomal activities of human neutrophils (14, 33), and more susceptible to antifungal compounds (30). The results of these studies of *W. dermatitidis* suggest that the D2HN melanins, and possibly also melanins synthesized from L-3,4-dihydroxyphenyalanine, of the more than 100 other dematiaceous pathogens of humans known or suspected to be melanized by D2HN melanin are important to virulence (30, 34, 35).

The objective of the present study was to delineate further the biosynthesis of D2HN melanin in *W. dermatitidis*. Of interest were early reactions in the pathway that occur prior to
FIG. 2. Related polyketide pigment pathways that synthesize T4HN, AT4HN, and/or YWA1 in *W. dermatitidis* (A), *C. lagenarium* (B), *A. fumigatus* (C), and *A. nidulans* (D). Early PKSp enzymes for these fungi are designated WdPks1p, PKS1p, Alb1p, and WAp, respectively. The enzymes that convert AT4HN and YWA1 to T4HN are named WdYg1p in *W. dermatitidis* and Ayg1p in *A. fumigatus*. Chemical structures for the metabolites depicted are shown in Fig. 1. Note that *A. fumigatus* reportedly makes melanin from YWA1 via two pathways, as shown in panel C, and that AT4HN may similarly contribute to melanin production in *W. dermatitidis* via two pathways, as shown in panel A.

synthesis of T4HN and that might provide evidence to confirm our previous preliminary result (47) that suggested that the early part of the pathway to D2HN melanin in *W. dermatitidis* involves WdYg1lp, an ortholog of Ayg1p in *A. fumigatus*. As such, metabolites of the disruption strain WdBrm1, generated by a random genetic insertion in the preliminary study, and two related melanin-deficient gene deletion strains, generated for this study, were identified. In addition, the relationships among those compounds and the other known D2HN melanin metabolites were determined.

**MATERIALS AND METHODS**

**Strains.** The eight strains of *W. dermatitidis* used in this study are listed in Table 1. They include the well-described laboratory wild-type strain 8656 (ATCC 34100; *Eosophila dermatitidis* CBS 525.76), which is usually black and sometimes dark brown, and an albino mutant, WdPk1 (WdPKS1 Δ-1 strain), derived previously from the wild type (14, 24). Also listed are WdBrm1 (WdYg1 Δ-1), which is an integrative disruption mutant strain described previously (47); two additional yellow-to-brown, pigment-producing deletion strains, designated WdYe12 (WdYg1 Δ-2) and WdYd13 (WdYG1 Δ-3) and the same three WdYg1 Δ mutants complemented with the wild-type WdYG1 gene, designated the WdYg1 Δ-1WdYG1, WdYg1 Δ-2WdYG1, and WdYg1 Δ-3WdYG1-reconstituted strains. An *Escherichia coli* strain DH5, was used for all *W. dermatitidis* DNA cloning and manipulation procedures. The *Aspergillus oryzae* strain M-2-3 (21), which is unable to make T4HN or other D2HN metabolites, was used as the heterologous host for the expression of the melanin WdPKS1 gene from *W. dermatitidis*.

**Media and growth conditions.** Routine culture of *W. dermatitidis* for most of the molecular studies was done at 25°C with yeast-peptone-dextrose agar and broth media (2) or with synthetically defined broth or agar (SDA) medium containing 0.17% (wt/vol) yeast nitrogen base without amino acids and ammonium sulfate, 0.2% ammonium nitrate, 0.1% asparagine, and 1% glucose (49). When selection of transformants was required, media were supplemented with 50 μg/ml hygromycin B (Sigma, St. Louis, MO) or 50 μg/ml chlorimuron ethyl (VWR, West Chester, PA), thus allowing the determination of resistance in strains having acquired the hygromycin phosphotransferase (*hph*) gene or the sulfonylurea receptor (*sur*) gene, respectively. Cultures of *E. coli* were grown at 37°C in Luria-Bertani (LB) medium (2), which was supplemented as necessary with 100 μg/ml ampicillin or 25 μg/ml chloramphenicol for selection of transformants. Experimental cultures of *W. dermatitidis* and *A. oryzae* for physiological and biochemical studies were initiated from stock cultures maintained in the dark for 7 to 14 days at 25°C on 25 ml Difco Czapek Dox agar (Becton Dickinson, Sparks, MD) with 0.1% (wt/vol) Difco yeast extract (CDYA) and on Difco potato dextrose agar (PDA), respectively, for 14 to 30 days at 25°C. Cultures prepared to investigate the accumulation of melanin metabolites produced by *W. dermatitidis* were grown in Difco Czapek Dox broth with 0.1% (wt/vol) Difco yeast extract (CDYB) or on CDYA without or with tricyclazole, which was added to the autoclaved medium in ethanol (EtOH) at a final concentration of 30 μg/ml tricyclazole (Dow Elanco, Indianapolis, IN), unless otherwise stated. Control cultures without tricyclazole contained the same amount of EtOH (1% [vol/vol]). AOIC medium was used to grow the *A. oryzae* strain expressing the WdPKS1 gene and the WdYg1 Δ strains in order to produce 2-acetyl-1,3,6,8-tetrahydroxynaphthalene (AT4HN) and 3-acetylflaviolin (3-AF), which was identified in AOIC cultures used in this study. The AOIC medium consisted of 3 g NaNO₃, 2 g KCl, 1 g KH₂PO₄, 0.5 g MgSO₄*·*7H₂O, 0.01 g FeSO₄*·*7H₂O, 10 g peptone P (United States Biological, Swampscott, MA), and 20 g soluble starch (Sigma-Aldrich, St. Louis, MO). Antibiotics were added at 50 μg/ml chloramphenicol for the *W. dermatitidis* strains to make AT4HN; the soluble starch was omitted and EtOH was added (1% [vol/vol]). For the accumulation of 3-AF, AOIC cultures that lacked both stachy and exogenous EtOH was used. The resulting CDYB and AOIC cultures were generally grown in 250-ml Erlenmeyer flasks after inoculation of 50 ml of media with 0.3 ml H₂O containing 1 × 10⁷ *W. dermatitidis* propagules obtained from 24-h-old CDYB cultures via a previously described
serial transfer technique (20). Unless stated otherwise, CDYB and AOIC cultures without added EtOH were grown in the dark in a reciprocating shaker at 200 rpm and 28°C for 5 days, whereas AOIC cultures amended with EtOH were grown for 84 h. CDYA was inoculated with 0.2 ml of evenly spread propagules (2 × 10^5) of W. dermatitidis from CDYB cultures, after which the resulting plates were incubated in the dark for 10 days at 25°C. To grow A. oryzae for metabolite analysis, conidia (1 × 10^9) obtained from PDA cultures were added to 25 ml of growth medium in a 125-ml Erlenmeyer flask and incubated for 3 days in the dark at 200 rpm and 28°C. The growth medium contained dextrose (10 g corn (Sigma, St. Louis, MO)), 10 g peptone, 5 g Difco yeast extract, 5 g KH₂PO₄, and 0.5 g MgSO₄-7H₂O brought up to 1,000 ml in deionized H₂O. Fungal hyphae and other cells from cultures were then collected on Whatman no. 3 filter paper (Whatman International Ltd., Maidstone, England) and washed with H₂O. Approximately half of the fungal material was transferred to 50 ml AOIC in a 250-ml flask and then incubated at 28°C and 200 rpm for 2 days.

Nucleic acid manipulations and computational analyses. The E. coli transformation cultures with W. dermatitidis DNA were done by the TSS method (9). The methods for transformation of W. dermatitidis by electroporation of intact, competent yeast cells were described previously (41, 49). The isolation of genomic DNA from W. dermatitidis was done by a glass bead method adapted from the work of Ausubel et al. (2) as described previously (27). Total RNA from W. dermatitidis was isolated by Tri reagent (Sigma, St. Louis, MO) extraction of cells according to the manufacturer's instructions and prior to spheroplasting with Zymolyase 20T (ICB Biomedicals, Inc., Aurora, OH). DNA contamination was removed from the RNA by incubation with RQ1 DNase (Fisher Scientific, Suwanee, GA) at 37°C for 1 h, followed by acid phenol-chloroform extraction, ethanol precipitation, and washing. Southern blotting experiments were carried out using standard methods (2). Prior to transfer to nylon Nitran N membranes (Schleicher & Schuell, Keene, NH), genomic DNA was digested with appropriate restriction enzymes and separated in agarose gels. DNA dots were obtained by using a Bio-Dot SF microfiltration apparatus (Bio-Rad, Hercules, CA) and its manufacturer's protocol. After genomic DNA (0.5 to 1 μg) was transferred to the Nitran N membranes, hybridization was performed according to standard procedures (2). Blots were then hybridized and washed under low-stringency conditions at 65°C. Assembly and editing of DNA sequences were performed using Lasergene software (DNASTAR, Madison, WI). Sequence similarity searches were carried out using the BLAST algorithm (1) and the GenBank nucleotide and protein databases (http://www.ncbi.nlm.nih.gov). Protein sequences were aligned using the CLUSTAL W program (37). The phylogram was constructed using MegaAlign (DNASTAR, Madison, WI).

PCR and RT-PCRs. PCR and RT-PCR products were carried out with a model 2720 thermal cycler (Applied Biosystems, Foster City, CA) and a Perkin-Elmer PCR system (Norwalk, CT). PCR mixtures usually contained 50 pmol of each primer, 100 ng of sample DNA, deoxynucleoside triphosphates at a final concentration of 200 μM each, 1.5 mM MgCl₂, 0.2 mM each of TaKaRa gene amplification kit (TaKaRa Bio Inc., Tokyo, Japan). The thermocycling conditions used for the PCR were as follows: initial denaturation at 94°C for 2 min; 36 cycles of denaturation (94°C for 40 s), annealing (55°C for 1 min), and extension (72°C for 2 min); and a final extension at 72°C for 8 min. Specific gene probes for the Southern and dot blot analyses were prepared by PCR amplifications using the following primers: for the 267-bp YGI probe, forward primer FYGI (5′-GCAAATGCTCCTGGAATGG-3′) and reverse primer RYGI (5′-CCCTTAGTAATGTCTCCTCCTCCTCGCTGCCC-3′); and for the 293-bp hph probe (HG), FHYG (5′-ATGGACGCGGCGCAGCAGCTGCAGTGGTCTTCTGCCCC-3′) and RHYG (5′-GCTCAGAATGTCACTGATGCGAATGGCTCCGCCC-3′), which carried the specific restriction sites (underlined) for NotI and XbaI, respectively. The latter also facilitated the cloning into the complementation plasmid pDACYG (Fig. 3E). All probes were labeled with [32P]dATP, using a Deca Prime II DNA labeling kit (Ambion, Austin, TX). Reverse transcription-PCRs (RT-PCRs) were carried out using the RT-PCR kit (Invitrogen, Carlsbad, CA) and specific primers enclosing the WdYGI gene. To generate the WdYGI cDNA and to aid in cloning, primer RTF (5′-GTCTCAAGATGCTGGAATCAGAACAAAATATGGAAGAACCCG-3′) was designed to add an XbaI restriction site (underlined) at the 5′ end, and primer RTR (5′-GACTAGTAAATGTCACTGCTGATCTTCTCTTCGA-3′) was designed to add an Sphl restriction site (underlined) at the 3′ end. Each RT-PCR involved RT at 50°C for 30 min, after which the PCR was begun as described above. To ensure that the samples were not contaminated with genomic DNA, PCR was also carried out with total RNA and Taq polymerase. The PCR and RT-PCR products were eluted from gels, ligated with the pGEM-T Easy (Promega, Madison, WI) or pBluescript SK(−) (New England Biolabs, Ipswich, MA) vector, cloned, and then analyzed by DNA sequencing. Sequencing of the PCR and RT-PCR gene products was carried out by the core facility of the Institute for Cellular and Molecular Biology, University of Texas at Austin (Austin, TX).

Identification of the gene disrupted in WdBrm1 and the production of related strains. The WdYeI2 and WdYeI3 strains were produced by gene manipulations based in part on preliminary results about the disrupted gene in WdBrm1, which was isolated by a marker rescue approach and then sequenced (47). The production of the WdYel2 strain itself was produced by the unintended insertion into the WdYGI gene of W. dermatitidis of an XbaI-linearized disruption vector, pZL70 (48, 49), which was constructed by incorporating a 500-bp PCR fragment of the WdURA5 gene into the multiple cloning site of pCB1004, which contains genes for resistance to chloramphenicol and hygromycin B. Among the hygromycin B-resistant mutants produced, WdBrm1 was retained for further study because it secreted a yellow-brown pigment. After the genomic DNA was extracted from WdBrm1, the WdYGI gene was cloned by a marker rescue strategy. Briefly, this strategy involved the digestion of the WdBrm1 genomic DNA with SacI and KpnI, followed by self-ligation of the resulting two fragments with DNA ligase and, finally, the rescue of the two cyclized plasmids, pCQBrmK1 and pCQBrmS16, in E. coli by selection with chloramphenicol. Sequencing then showed that pCQBrmK1 contained considerable lengths of both the 5′-end and 3′-end portions of the entire WdYGI gene, whereas pCQBrmS16 contained only a single large portion of WdYGI starting from its 5′-end (data not shown). For construction of the WdYGI replacement knockout vector (Fig. 3A), a homologous upstream 700-bp region (H1) and a homologous downstream 290-bp region (H2) for targeting the WdYGI gene were generated by PCR amplifications, using primers H1F (5′-GCCCTAAGCAATGTACATGGTAAAACTGG-3′) and H1R (5′-GGAATTCGAGCTTCCTGATGGTTGCCAACAGG-3′), which introduced sites (underlined) for XbaI and EcoRI, respectively, for the H1 fragment amplification. Also used were primers H2F (5′-CCGCTCAGAATGAGCTGCGGCGGC-3′) and H2R (5′-GGCCCCAGTTCGCTCACTGCGGCGC-3′), which introduced sites (underlined) for XhoI and KpnI, respectively, for H2 fragment amplification. After amplification, the H1 and H2 targeting fragments were cloned into the corresponding sites of vector pCB1068 (kindly provided by J. Sweigard, Dupont Co., Wilmington, DE), which has the hph gene marker for selection, to produce the knockout replacement vector pDAD11H2. After linearization with NotI and KpnI, pDAD11H2 was used to transform competent W. dermatitidis cells by electroporation. Identification of potential WdYGI disruption strains was achieved by observation of resistance to hygromycin on SDA selection medium and by their production of yellow-brown secretion products. The specific gene deletions were verified by PCR, using specific primers for WdYGI gene amplifications, and by Southern blotting analyses using probes for both WdYGI and hph. The complementation vector pDACYG (Fig. 3E) was constructed by ligating a 0.2-kb hph gene fragment, generated by digestion with NotI and XbaI, and a 2.1-kb DNA fragment, generated by PCR with primers HI F and H2 R (5′-GACATGTCGCTAATGGTGGATGGATTGTATACGCTGCGG-3′), which introduced sites (underlined) for XhoI and KpnI, respectively, for H2 fragment amplification. After amplification, the H1 and H2 targeting fragments were cloned into the corresponding sites of vector pCB5132 (Fungal Genetics Stock Center, Kansas City, KS). Thus, a 0.7-kb homologous targeting region (H1) and the 1.4-kb full-length WdYGI gene were inserted. After the resulting pDACYG complementation vector was linearized with XbaI, it was transformed into the WdBrm1 and the WdYGI mutant strains by electroporation. Putative reconstituted WdPKS1 strains were selected by observation of resistance on SDA to both hygromycin B and chloramphenicol ethyl and by their wild-type phenotype. Transformants were subsequently confirmed to contain the intact WdYGI gene by PCR using the FC and RC primers and also by Southern blotting using a WdYGI probe.

Analysis and isolation of melanin metabolites from W. dermatitidis and A. oryzae. To determine the accumulated known D2HN melanin precursors or related metabolites, the W. dermatitidis or A. oryzae cultures were extracted with ethyl acetate (EtOAc), and the extracts were dried in vacuo. The resulting residues were dissolved in methanol (MeOH) and then analyzed by high-performance liquid chromatography (HPLC) and, occasionally, thin-layer chromatography (TLC) (20, 46). Authentic D2HN melanin metabolite standards obtained previously, including YWA1 and AT4HN, were used to confirm HPLC peak and TLC spot identities. Standards of known 3-AF and the shunt metabolites 1,2,4,5,7-pentahydroxynaphthalene (1,2,4,5,7-PHNN), 1,2,4,5-tetrahydroxynaphthalene (1,2,4,5-THNN), and 1,4,5-trihydroxynaphthalene (1,4,5-THN), however, were not available for the study. HPLC was also performed directly on super-
FIG. 3. Strategies used for the deletion of WdYG1 and for mutant complementation in W. dermatitidis and Southern blot analyses. (A) Deletion of WdYG1 in the wild-type strain was done by gene replacement with a hygromycin phosphotransphorase (hph) selection marker contained in pDAH1H2. Construction of pDAH1H2 was carried out by inserting targeting sequences H1 and H2 into pCB1636 at positions flanking the hph selection marker and into engineered restriction sites for XbaI/EcoRI and XhoI/KpnI, respectively. (B) Southern dot blot analysis of genomic DNA with the 32P-labeled YG1 probe. (C) Southern dot blot analysis of genomic DNA hybridized with the 32P-labeled HG probe to detect the hph cassette. (D) Genomic DNAs isolated from mutants WdYel2 and WdYel3 were digested with BamHI (B) and XhoI (Xh) and hybridized with the 32P-labeled HG probe. (E) Complementation with WdYG1 was done by transformation of the null WdYG1 mutant strains and WdBrm1 with linearized pDACYG, which was derived from vector pCB1532 containing the full-length WdYG1 gene, the 2.8-kb sur selection marker, and portions of the targeting WdYG1 (H1) and hph (HG) sequences adequate to bring about complementation by site-directed integration into the WdYG1 genomic locus. (F) Southern blot analysis of genomic DNA with a specific YG1 32P-labeled probe. Genomic DNAs from the wild-type (WT) and WdYG1-complemented strains (WdYG1Δ-2WdYG1) were digested separately with XbaI (Xb), EcoRI (E), SalI (S), and BamHI.
NADPH was added, and the tube was sealed and backfilled with N₂ gas. Enzymes T4HN, and flaviolin, and substrate. They were then extracted and analyzed by HPLC for metabolites. shaker at 25°C and 250 rpm in the dark for 36 h after the second addition of 1 mg of AT4HN or 3-AF dissolved in 0.05 ml MeOH were added to 10 ml of the fed to CDYB cultures of the albino Wd
pks1
with 4.9 ml of 20 mM potassium phosphate buffer at pH 6.8. Next, 5 ml of cell-free albino homogenate in a 20-ml culture tube was diluted were provided AT4HN, 3-AF, T4HN, and flaviolin under reduced oxygen con-

Studies were also carried out with a combined cellular suspension of the albino WdYG1 and WdYel3) had the same phenotypic characteristics as

The sequence of Wd
PKS1
/-H9004
mutant showed that except for their pigment differ-

Because the WdYG1 gene in WdBrm1 was disrupted by the unintended integration of a vector in the W. dermatisidis
genome, a complete deletion of the gene was carried out by an allelic one-step insertion-replacement strategy to confirm that the phenotype of WdBrm1 was not due to other mutations (Fig. 5A). Among the transformants produced, two (WdYe12 and WdYel3) had the same phenotypic characteristics as WdBrm1 (Fig. 5A). Comparisons of the microscopic characteristics of the transformants and those of the wild type and the Wdpks1Δ mutant showed that except for their pigment differences, all were identical (data not shown). The deletion of

Cross-feeding between the albino Wdpks1Δ strain and the WdYG1Δ and re-

RESULTS

Genetic characterization of Wdypg1Δ mutant strains. The first yellow-to-brown pigment-secretting strain, WdBrm1, was generated by an unintended insertion into the W. dermatisidis
genome of pLZ70, which was designed with the intention of disrupting its WdUR45 gene (47, 48, 49). The WdBrm1 mutant was maintained for further study because it was obviously different from the wild-type strain and any previously derived Mel− and Wdpks1Δ mutant strains with defects in the D2HN melanin biosynthetic pathway (10, 14, 20). After the gene putatively disrupted in WdBrm1 was isolated by a marker rescue strategy, sequencing showed that it was a homolog of the Ayg1 gene of A. fumigatus. This gene in A. fumigatus encodes an enzyme responsible for the chain shortening of the heptaketide generated in a pathway leading to the bluish-green pigment of its conidial cell walls (38, 39). Therefore, the gene identified in W. dermatisidis was named WdYG1, and its encoded protein was named WdYG1p. Sequencing of a cDNA clone corre-

The derived protein sequence of WdYG1p had its closest relationship (56% identity) with the deduced sequence of a hypothetical protein from Neurospora crassa (GenBank accession no. XP_960081) and significant sequence similarity (38.7% identity) with Ayg1p of A. fumigatus (Fig. 4A). Phylogenetic and sequence analyses revealed additional homologies with other functionally characterized and uncharacterized or-

Nucleotide sequence accession numbers. The sequence of WdYG1 was sub-

muted to the GenBank database (accession number AY6667610). The revised sequence of Wdpks1 was also submitted to the GenBank database (accession number AF130093). (B) Lane L, 2-hog DNA ladder (New England Biolabs). In panels A and E, the shaded YG1 boxes and HG nonshaded arrow show the locations of corresponding probes used for Southern blotting. The arrows FS and RS and the arrows FC and RC indicate the primers used for the PCR amplifications and for screening and verification of the knockout transformants and the complemented strains, respectively. The bold arrows H1F, H1R, H2F, H2R, and H2RC show the locations of the primers used for PCR amplification of DNA fragments used for construction of disruption and recombination vectors. Arrows RTF and RTR show the primers used for DNA amplification by RT-PCR. The thin horizontal lines show the sizes of DNA expected by Southern analysis after restriction enzyme digestion of DNAs from the wild-type, WdYe12, WdYel3, and Wdypg1Δ-2WdYG1 complemented strains; here it should be noted that the complementation of WdBrm1, unlike the complementation of the null WdYG1 mutants, was apparently ectopic, as indicated by Southern analyses (data not shown). Restriction enzymes are abbreviated as follows: Xb, Xhai; E, EcoRI; K, KpnI; Xh, Xhol; B, BamHI; S, SalI; N, NotI; Sp, SpeI.
FIG. 4. Amino acid alignment and phylogenetic analysis of WdYgLp with other yellowish-green fungus-like orthologs. (A) Alignment of WdYgLp of *W. dermatitidis* with Ayg1p of *Aspergillus fumigatus* (GenBank accession no. AAF03353) and NcYgLp of *Neurospora crassa* (GenBank accession no. XP_960081). In the consensus line, identical amino acids are marked by asterisks, conserved substitutions are marked by colons, and semiconserved substitutions are marked by dots. The numbers at the right represent the number of amino acids depicted from the first methionine for each derived protein. The boxed areas identify the Aes enzymatic domain, in which the conserved amino acids for Aes family members are indicated by the dark shading. (B) The phylogenetic tree was inferred by applying a Clustal W algorithm by the neighbor-joining method (32). The units at the bottom of the tree indicate the approximate numbers of substitution events. The fungal source and accession number of each sequence are as follows: BfYgL, *Botryotinia fuckeliana*, XP_001552894.1; SsYgL, *Sclerotinia sclerotiorum*, XP_001597188.1; CgYgL, *Chaetomium globosum*, XP_001222736.1; MgYgL, *Magnaporthe grisea*, XP_001522572.1; AcYgL, *Aspergillus clavatus*, XP_001276032.1; AoYgL, *A. fumigatus*, AF116902_1; AoYgL, *A. oryzae*, BA55407.1; AnYgL, *A. niger*, XP_001401158.1; PnYgL, *Phaeosphaeria nodorum*, EAT80224.2; WdYgL, *Wangiella (Exophiala) dermatitidis*, AAT81166.2; NcYgL, *Neurospora crassa*, XP_965534.2; UmYgL, *Ustilago maydis*, XP_758182.1.
WdYGI was then confirmed by PCR amplifications using primers FS and RS, which flanked the upstream (H1) and downstream (H2) homology regions, respectively, and by DNA sequencing (data not shown). Southern blotting analyses showed that the WdYGI signals were absent from the WdYe12 and WdYe13 strains, but not the WdBrm1 strain, when the blots were probed with the YG1 probe (Fig. 3B). Additionally, the hph replacement fragments were present in all three strains when the blots were probed with the hph probe (Fig. 3C and D). Southern blotting also confirmed that WdYGI existed as a single gene in the W. dermatitidis wild-type genome (Fig. 3F). Evidence for the importance of WdYg1p to melanin biosynthesis in W. dermatitidis was provided by showing that both WdBrm1 and the WdYe12 null mutants (WdYe12 and WdYe13) were restored to the wild-type colony phenotype (Fig. 5B) after transformation with a linearized complementation plasmid, pDACYG, containing the full WdYGI open reading frame and the sur selection marker (Fig. 3E) and selection on yeast-peptone-dextrose agar that contained both hygromycin B and chlorimuron ethyl. PCR using primers FC and RC, DNA sequencing (data not shown), and Southern blotting with the YG1 probe (Fig. 3F) confirmed that all three complemented WdYg1Δ strains contained the WdYGI gene.

**AT4HN and 3-AF are identified in pigment secretions of WdYg1Δ mutants.** All three WdYg1Δ strains (WdBrm1, WdYe12, and WdYe13) had cells that were light brown and secreted soluble yellow pigments during the first 2 to 4 days of growth at 25°C in CDYB. By 5 to 6 days, the cells were a darker brown and the soluble pigments were brown instead of yellow. In contrast, the wild type and the reconstituted WdYg1Δ strains at the end of 5 to 6 days had cells that were dark gray to black and did not produce appreciable amounts of soluble pigment. Cells and pigments obtained from 10-day-old CDYA cultures were similar in appearance to those obtained from 6-day-old CDYB cultures. Analysis of the extracts from CDYB cultures showed that the WdYg1Δ strains had not produced any known melanin metabolites, except for occasional trace amounts of flavinol. They did, however, secrete compounds not previously reported in studies with *W. dermatitidis*. Specifically, extracts of the WdYg1Δ cultures usually contained (Fig. 6A) a large amount of an unknown compound (HPLC *t*ₚ = 19.4 min) with a UV-visible spectrum (Fig. 6B) distinctly different from those of AT4HN, YWA1, and any of the other relevant melanin metabolites. From WdYe12 cultures grown in AOIC, this compound was extracted into EtOAc, but upon concentration of the extract, a dark, almost black, solid precipitated. While the concentrated extract dried to an orange solid that was soluble in organic solvents (e.g., EtOAc, McOH, EtOH, acetone, etc.), the black precipitate was found to be soluble only in water, where it produced an orange color. Upon acidification of the water solution to pH 3 to 4, the orange material could be partitioned into EtOAc and the resulting extract dried to an orange solid. The unknown compound, analyzed via probe insertion mass spectrometry, had a parent ion peak at *m/z* 248 and the fragmentation pattern shown in Fig. 7. Experiments revealed that the unknown compound appeared to be formed from AT4HN (molecular weight, 234) by nonenzymatic oxidation. Interestingly, the conversion of AT4HN to the unknown compound was strongly influenced by pH. That is, only a small amount of AT4HN was converted to the unknown in 0.2 M potassium phosphate at pH 5.0 over 60 min, whereas AT4HN in pH 7.0 buffer was converted completely to the unknown in 10 min. These results led to identification of the unknown compound as 3-AF.

Importantly, although AT4HN was usually not detected in extracts of CDYB and CDYA cultures, it was found in supernatants of 4-day-old CDYB cultures of all three WdYg1Δ strains when the supernatants were immediately and directly analyzed by HPLC (Fig. 6C). In contrast, when there was a delay between sampling and analysis, the quantity of AT4HN decreased or disappeared, while 3-AF appeared or increased (Fig. 6D). The presence of tricyclazole did not appreciably affect production of AT4HN or 3-AF in CDYA cultures of the WdYg1Δ strains (without the albino) and did not cause flavinol or other known DHN melanin metabolites to appear in the cultures.

Cultures of the wild-type and reconstituted WdYg1Δ strains did not secrete appreciable amounts of soluble melanin metabolites, except during growth in the presence of tricyclazole, when they accumulated flavinol, 3,3'-biflavinol, and 2-hydroxyjuglone (2-HJ) (Fig. 6E to H). The accumulation of the melanin metabolites with the addition of tricyclazole to the CDYB or CDYA medium coincided with the appearance of a reddish brown coloration, not the gray-black appearance of the wild-type and reconstituted WdYg1Δ cells (Fig. 6E and F; Table 2). **AT4HN is produced by WdPKS1 expressed in A. oryzae** and in *W. dermatitidis* WdYg1Δ strains cultured in AOIC. An *A. oryzae* strain expressing WdPKS1 was used to identify any WdPKS1p reaction product(s) that could be produced upon the expression of the cDNA of WdPKS1. This strain was used
FIG. 6. Representative HPLC traces (A and C to J) showing 3-AF, AT4HN, and other compounds from cultures of *W. dermatitidis*. (B) UV-visible spectrum of 3-AF. Only the 5- to 25-min regions of the full 39-min chromatograms acquired are shown, and all have the same response scale. The samples include the following: extract from a CDYB culture of WdYel2 grown for 6 days, showing the 3-AF peak (1) (A); supernatant from a 4-day-old culture of WdYel2 revealing the presence of AT4HN (2) in addition to 3-AF (1) (C); supernatant from the analysis in panel C after 16 h at room temperature, showing the loss of AT4HN during storage (D); extracts from tricyclazole-treated 6-day-old CDYB cultures of the wild-type and Wd yg1/H9004-2Wd YG1 strains, respectively, showing the presence of scytalone (3), flavilin (4), tricyclazole (5), 2-HJ (6), and 3,3'-biflaviolin (7) (E and F); extracts from 6-day-old CDYB (no tricyclazole) cultures of the wild-type and Wd yg1/H9004-2Wd YG1 strains, respectively, with no melanin metabolites detectable (G and H); and extracts from 3-day-old AOIC cultures of the WdYel2 strain that were pretreated with EtOH (1% [vol/vol]) or not, respectively, illustrating how culture conditions can be used to selectively produce either AT4HN or 3-AF (I and J). Panel I also shows the presence of trace amounts of YWA1 at approximately 18 min (8).
because it does not contain enzymes of the D2HN melanin pathway and thus is unable to make T4HN or other downstream precursors. When grown in AOIC at 28°C for 2 to 3 days, the Wdpks1-expressing strain of A. oryzae produced moderate to large amounts of AT4HN, small amounts of 3-AF, and occasionally, trace levels of YWA1. Surprisingly, when the WdgΔ strains were grown similarly in AOIC, a medium not previously used for the culture of W. dermatitidis, they produced enhanced amounts of AT4HN and 3-AF and trace amounts of YWA1. The relative quantities of AT4HN, 3-AF, and YWA1 obtained from the WdgΔ cultures depended on the presence or absence of EtOH in the AOIC medium. Extracts of EtOH-treated cultures generally contained large amounts of AT4HN, small or undetectable amounts of 3-AF, and trace amounts of YWA1. The relative quantities of AT4HN, 3-AF, and YWA1 were detected and flaviolin was present in smaller amounts in W. dermatitidis cultures grown without EtOH in AOIC and existed mostly as light brown, budding yeast-type cells.

Metabolites secreted by the WdgΔ strains are metabolized by the albino Wdpks1Δ strain. Cross-feeding experiments on CDYA between alternating parallel streaks of the albino and WdgΔ strains showed that the WdgΔ strains in the presence of 15 μg/ml tricyclazole secreted yellow-brown metabolites that diffused through the medium at the same time that the albino darkened (Fig. 5C). Under these conditions, the albino apparently metabolized one or more compounds from among the diffusible pigments, became brown in color, and made flaviolin, 3,3′-biflaviolin, 2-HJ, and trace amounts of 5-hydroxyscytalone (5-HS), as shown by HPLC analysis of culture extracts (Table 3). These metabolites were not found in streaked control cultures of the WdgΔ strains where the albino strain was absent or in cultures of the albino by itself. We suggest that these results demonstrate that the WdgΔ strains produced one or more upstream substrates that were converted by WdYg1p in the albino to T4HN or flaviolin and other related melanin metabolites. However, when pairings were made in the absence of tricyclazole, flaviolin, 4,8-dihydroxytetralone (4,8-DHT), and/or 4-hydroxyscytalone (4-HS) were detected and flaviolin was present in smaller amounts (Table 3). In contrast, the reconstituted WdgΔ strains and the wild type did not secrete visible yellow-brown compounds in CDYA when they were streaked next to the albino (Fig. 5D). As a consequence, the albino failed to darken in these studies and did not produce downstream metabolites (Table 3). The results obtained on CDYA were similar to those obtained when propagules of the WdgΔ strains and the albino were cocultured in CDYB medium and the albino made known DHN melanin metabolites from secretions of the WdgΔ strains (compare Tables 2 and 3).

AT4HN and 3-AF are metabolized by albino Wdpks1Δ cultures and homogenates. When CDYB cultures of the albino were provided with AT4HN in the absence of tricyclazole, the AT4HN disappeared within 4 h, and in its place appeared 3-AF, flaviolin, 4-HS, and scytalone. In cultures amended with

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**TABLE 2. Melanin-related products from extracts of CDYB cultures containing single and combined strains of Wangiella dermatitidis**

| Strain(s) | Products |
|-----------|----------|
| Wild type | ND       |
| Wild type | Flaviolin, biflaviolin, 4-HS, 5-HS, 2-HJ, scytalone |
| WdPks1   | ND       |
| WdYel2   | ND       |
| WdYel2*  | 3-AF, flaviolin |
| WdYel2*  | 3-AF, flaviolin |
| WdgΔWdYGI | ND       |
| WdgΔWdYGI* | Flaviolin, biflaviolin, 4-HS, 5-HS, 2-HJ, scytalone |
| WdYel2 + WdPks1 | ND |
| WdYel2 + WdPks1* | Flaviolin, biflaviolin, 4-HS, 5-HS, 2-HJ, scytalone, 4,8-DHT |
| Wild type + WdPks1 | ND |
| WdgΔWdYGI + WdPks1 | ND |

* The cultures were grown for 10 days in the dark at 25°C. The cultures of the paired strains had 15 alternating parallel streaks. Cultures contained 15 μg/ml tricyclazole. 5-HS was present only in trace quantities. ND, not detected.

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**FIG. 7. Mass spectrum of 3-AF. The parent ion at m/z 248 fits that for 3-AF. As described for similar compounds by Becher et al. (3), the fragments at m/z 220 and 205 may arise from the sequential loss of a carbonyl and methyl group or the loss of a carbonyl group and an acetyl group (evidenced by the m/z 43 peak), respectively.**
Because AT4HN had not previously been reported as a melanin precursor in any fungus and because *W. dermatitidis* was assumed to make T4HN directly from either a combination of acetyl-CoA and malonyl-CoA or malonyl-CoA by itself (14, 20), Previously, AT4HN had only been identified in studies with a molecularly derived strain of *A. oryzae*, where it was described as a novel hexaketide produced from a chimeric PKS composed of WA from *A. nidulans* and PKS1 of *C. lagenarium* (43). While the *A. oryzae* strain used in that study produced three other known polyketides, it did not make YWA1 and T4HN, which are the usual products of WA and PKS1, respectively (Fig. 2B and C). Also of interest is the fact that 3-AF has not previously been reported as a metabolite in melanin biosynthesis. In our study, its identification was based on its mass spectrometry fragmentation pattern, together with its metabolic relationships with AT4HN and flavolin (Fig. 1). Its mass spectrometric fragments at *m/z* 220 and 205 may have occurred because of the sequential loss of a carbonyl and methyl group and the loss of a carbonyl group and an acetyl group (evidenced by the *m/z* 43 peak), respectively, as described by Becher et al. (3) for other closely related compounds. In addition, the ions at *m/z* 69 and 137 are probably identical to those reported previously in the mass spectrum of flavolin (4). The possibility that the unknown may be 2,5,7-trihydroxy-6-acetyl-1,4-naphthoquinone (6-acetylflavin), another oxidation product of AT4HN, was eliminated based on the distinctly different UV-visible and mass spectra of 6-acetylflavin (3, 28).

The eight strains of *W. dermatitidis* and the single strain of *A. oryzae* grown on a variety of media in this study showed significant differences in the accumulation of AT4HN and 3-AF. For example, in AOIC containing EtOH, the Wdypks1Δ strains usually accumulated AT4HN instead of 3-AF, whereas in AOIC without EtOH or in CDYB or CDBA, they accumulated 3-AF instead of AT4HN. We suspect that this difference was because the AOIC cultures containing EtOH grew under more acidic conditions than the other cultures, thus slowing the oxidation of AT4HN to 3-AF. The apparent oxidation process of AT4HN to 3-AF is supported by the HPLC results, wherein AT4HN was found in culture supernatants but not in extracts of the same cultures, which instead had 3-AF. It is noteworthy that this is probably the first reported instance of HPLC analyses being performed directly on melanin metabolite-containing culture supernatants. The analyses were easily carried out, and the only disadvantage noted was that concentrations of metabolites were reduced compared to those in extracts, to the extent that trace amounts of the metabolites might not be detectable.

The oxidation of AT4HN to 3-AF explains why the albino Wdypks1Δ strain made smaller amounts of melanin than expected in the cross-feeding experiments with the Wdypks1Δ strains on CDYA or in CDYB. That is, whereas some AT4HN was made in the cultures and metabolized to melanin, most was oxidized to 3-AF and then metabolized to flavolin and its known products, e.g., 3,3′-biflavolin, 4-HS, 5-HS, and 2-HJ. Strong supporting evidence for AT4HN as a precursor to D2HN melanin was obtained from anaerobic studies with cell-free homogenates of the albino. These experiments showed that D2HN was made in the same manner from either AT4HN or T4HN and confirmed that T4HN was a deacetylation product of AT4HN. In addition, the albino homogenate studies with 3-AF established that 3-AF is deacetylated to flavolin, not

| Substrate | Product(s) |
|-----------|------------|
| AT4HN     | D2HN*      |
| T4HN      | D2HN*      |
| 3-AF      | 4-HS, 5-HS, 2-HJ, 3,4,8-THT, 4,8-DHT, juglone* |
| Flavolin  | 5-HS, 2-HJ, 3,4,8-THT, 4,8-DHT, juglone* |

* Initial concentration of AT4HN and 3-AF was 1.0 mM; initial concentration of T4HN and flavolin was 0.5 mM. Reactions were carried out in 10-mL assay systems.

b *: product production was blocked in assays containing 30 μg/ml tricyclazole.
T4HN, which resulted in the homogenates accumulating the same products as those made from flavinol.

A somewhat unexpected result in the present study was our finding that AT4HN was metabolized to compounds in addition to T4HN (Fig. 1). For example, in the WdYg1Δ cultures, AT4HN was converted to 3-AF and other compounds, some with UV-visible spectra similar to that of AT4HN. In addition, a portion of the available AT4HN in W. dermatitidis may have been incorporated as part of the melanin polymer before it was deacetylated to T4HN, as indicated by the appearance of a brown pigment in the WdYg1Δ strains when they were grown in CDYB or on CDYA. The latter suggestion means that AT4HN in W. dermatitidis and YWA1 in A. fumigatus may be used similarly as melanin precursors because in A. fumigatus some YWA1 is believed to be incorporated directly into melanin before being converted into T4HN (39).

It is important that although the fungi studied produce T4HN differently, each PKS, along with WAp of A. nidulans (42), is known to share a high degree of sequence similarity based on BLAST analysis (8, 14). CLUSTAL W analysis, which was found to be more selective than BLAST analysis, showed that the PKSs of W. dermatitidis and C. lagenarium and those of A. fumigatus and A. nidulans fall into two different groups based on their deduced amino acid relationships (8). Based on sequence similarity, W. dermatitidis and A. fumigatus are now known to have similar enzymes for converting AT4HN and YWA1, respectively, to T4HN (amino acid identity, 39%; similarity, 55%). These results suggested the (highly) different substrate specificities of WdYG1p and AYG1p. In the present study, WdYG1p poorly converted YWA1 to T4HN, as indicated by the fact that YWA1 was not appreciably metabolized to T4HN when it was fed to albino cultures or used to treat cell-free homogenates. In contrast, in an earlier in vitro study, AYG1p from A. fumigatus converted YWA1 to T4HN very efficiently (17).

Our demonstration in this study that melanin biosynthesis in W. dermatitidis requires AT4HN as a precursor of T4HN is of special interest because fungi are now known to make T4HN in a third manner. The two previous ways are as follows: (i) the bluish-green fungus A. fumigatus makes T4HN from the hepatetkide precursor YWA1 by loss of acetoacetic acid (17, 40), and (ii) the brown fungus C. lagenarium makes T4HN directly as a pentaketide from malonyl-CoA without using either AT4HN or YWA1 as a precursor (16). Although we presently know that a large number of other plant- and human-pathogenic fungi make melanin from D2HN via T4HN, we do not know which of the three pathways they use. Additional studies with important plant pathogens, such as V. dahliae and Magnaporthe grisea, and melanized human pathogens, such as Spo- (25, 36), require to be carried out to determine how they make T4HN. Specifically, it would be helpful to know if they make AT4HN by PKS in a manner similar to that for WdPKS1p in W. dermatitidis (14), YWA1 via a PKS in a manner similar to that for Alb1p in A. fumigatus (38), or T4HN directly by an enzyme, as reported for PKS in C. lagenarium (16). Additional studies with important plant pathogens such as V. dahliae and Magnaporthe grisea need to be carried out to determine if they make T4HN directly or from AT4HN or YWA1. Similar studies should also be carried out with the many human-pathogenic fungi known to synthesize D2HN to see if they make melanin in the same manner as W. dermatitidis. Specifically, do all these different types of fungi make AT4HN via the PKS referred to as WdPKS1p (14), do some also make it in the manner in which A. fumigatus makes YWA1, via the PKS known as Alb1p (38), and do others produce T4HN directly by its PKS in the manner of C. lagenarium (16)? Information about the different types of upstream precursors involved in melanin biosynthesis will help to confirm the relationships among the melanized fungi and should provide additional information that is useful in future studies of host-parasite interactions.

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