The zoopathogenic fungus Wangiella (Exophiala) dermatitidis is one of many form species of the Fungi Imperfecti, which are darkly pigmented (dematiaceous) owing to the deposition of 1,8-dihydroxynaphthalene melanin in their cell walls (22, 45). This fungus has recently become better known as a paradigm for the causative agents of phaeohyphomycosis in humans. To begin a genetic analysis of the initial synthetic steps leading to 1,8-DHN melanin biosynthesis, a 772-bp PCR product was amplified from genomic DNA using primers based on conserved regions of fungal polyketide synthases (Pks) known to produce the first cyclized 1,8-DHN-melanin pathway intermediate, 1,3,6,8-tetrahydroxynaphthalene. The cloned PCR product was then used as a targeting sequence to disrupt the putative polyketide synthase gene, WdPKS1, in W. dermatitidis. The resulting wdkps1Δ disruptants showed no morphological defects other than an albino phenotype and grew at the same rate as their black wild-type parent. Using a marker rescue approach, the intact WdPKS1 gene was then successfully recovered from two plasmids. The WdPKS1 gene was also isolated independently by complementation of the mel3 mutation in an albino mutant of W. dermatitidis using a cosmid library. Sequence analysis substantiated that WdPKS1 encoded a putative polyketide synthase (WdPKS1p) in a single open reading frame consisting of three exons separated by two short introns. This conclusion was supported by the identification of highly conserved Pks domains for a β-ketoacyl synthase, an acetyl-malonyl transferase, two acyl carrier proteins, and a thioesterase in the deduced amino acid sequence. Studies using a neutrophil killing assay and a mouse acute-infection model confirmed that all wdkps1Δ strains were less resistant to killing and less virulent, respectively, than their wild-type parent. Reconstitution of 1,8-DHN melanin biosynthesis in a wdkps1Δ strain reestablished its resistance to killing by neutrophils and its ability to cause fatal mouse infections.
dermatitidis, Verticillium dahliae, Cochliobolus miyabeanus and M. grisea (4, 22, 43, 47, 51, 53). In general, most studies of the pathway leading to 1,8-DHN-melanin biosynthesis have been oriented toward its downstream genes and enzymes and not to the synthesis of the first pentaketide, 1,3,6,8-THN, which appears to be made by a Pks either from malonyl coenzyme A (malonyl-CoA) exclusively or from malonyl-CoA together with acetyl-CoA (19, 21).

In this study, a gene (WdPKS1) that encodes a putative polyketide synthase (WdPKs1p) of W. dermatitidis was cloned, sequenced, and disrupted. The WdPKS1 gene contained a single open reading frame, consisting of three exons separated by two short introns. The predicted WdPKs1p consisted of 2,177 amino acids and showed significant similarities with other polyketide synthases, but particularly those encoded by the pks gene of C. lagenarium, the alb1 gene of A. fumigatus, and the wA gene of Aspergillus nidulans (8, 44, 46). The derived protein also contained sequences for the highly conserved β-ketoacyl synthase, acetyl-malonyl transferase, acyl carrier protein (ACP), and thioesterase domains, which are all characteristic of a type I polyketide synthase (23). Disruption of WdPKS1 produced strains that had albino phenotypes, which strongly indicated that WdPKS1 was involved in 1,8-DHN melanin biosynthesis. Support for this hypothesis was provided by precursor feeding studies and also by complementation experiments that remelanized the albino wdpks1Δ-1 mutant, as well as mutants described previously (13) with mel3 lesions, but not those with mel4 lesions. When tested in human neutrophils or in an acute mouse model, the albino wdpks1Δ mutatants were less resistant to neutrophil killing and less virulent, respectively, than their wild-type parent, as was found previously for other Mel+ mutants of W. dermatitidis tested similarly (18, 41). The importance of WdPKS1 to virulence was further supported by showing that the disruptants had no growth rate defects or other morphological abnormalities. Furthermore, restoration of 1,8-DHN melanin biosynthesis in the wdpks1Δ-1 mutant by complementation with WdPKS1 reestablished its ability to resist killing by human neutrophils and to cause fatal mouse infections.

MATERIALS AND METHODS

Strains and media. The laboratory wild-type strain of W. dermatitidis 8656 (ATCC 34100 [Exophiala dermatitidis CBS 525.76]) and the melanin mutants Mel1 (mel1; ATCC 44502), Mel2 (mel2; ATCC 44503), Mel3 (mel3-1; ATCC 44504), Mel4 (mel3-2; ATCC 58058), and Mc2w-3 (cdc1 mel4 arg1) have been extensively described (11, 12, 13, 15, 22). Routine propagation of these strains was in the rich medium YPD (2% peptone, 1% Bacto Yeast extract, and 2% dextrose), and all transformations were carried out as previously described (49, 58). For the precursor cross-feeding experiments with melanin mutants and for the sctylane (kindly provided by M. H. Wheeler, Texas A&M University, College Station) and acetate feeding experiments, strains were grown in modified Czapek-Dox (MCD) agar (11, 13, 22). For long-term storage, all strains were stored deep frozen (−70°C) in 25% glycerol. Strains cultured for neutrophil analysis of five transformants in which pBF9 was integrated into the WdPKS1 locus, whereas the plasmid was ectopically located in the WdPKS1 chromosome. Genomic DNA of wild type; albino transformants 1, 2, and 3 showed hybridization consistent with site-specific integration of pBF9 into the WdPKS1 locus.

Preparation and analysis of nucleic acid. Genomic DNA of W. dermatitidis was isolated by spheroplasting with Zymolase-20T (ICN Biomedicals, Inc., Aurora, Ohio) followed by detergent lysis, phenol-chloroform extraction, and ethanol precipitation as previously described (55). Total RNA was isolated by the acid-phenol/chloroform method and treated with DNase I. The integrity of the DNA was determined by gel electrophoresis and the integrity of the RNA was determined by polyacrylamide gel electrophoresis.

FIG. 1. (A) Predicted structure for the integration of pBF9 at the WdPKS1 locus. The 772-bp PCR product of WdPKS1 was used as a target sequence to disrupt the gene. The whole gene was then cloned by a marker rescue method. The two rescued plasmids pBF10 and pBF32 are also shown. The arrow labeled WdPKS1 indicates the position of the gene and the direction of its transcription. Prior to transformation, pBF9 was linearized with SacII. (B) Southern hybridization analysis of five transformants in which pBF9 was integrated into the W. dermatitidis genome. Genomic DNA of wild type; albino transformants 1, 2, and 3; and black transformants 4 and 5 (lanes 6 and 1 to 5, respectively) was extracted, digested with XhoI, fractionated on a 0.9% agarose gel, and subjected to Southern blotting analysis using the 772-bp WdPKS1 PCR fragment as a probe. Albino transformants 1, 2, and 3 showed hybridization consistent with site-specific integration of pBF9 into the WdPKS1 locus, whereas the plasmid was ectopically integrated at different genomic positions in black transformants 4 and 5.
FIG. 2. Characterization of the melanin defect in *wdpks1Δ-1* by comparisons with other *Mel^−* strains. (A) Diffusion cross-feeding by *Mel2* (*mel2*): *wdpks1Δ-1* was streaked close to *Mel2* on MCD agar (note blackening of *wdpks1Δ-1* at arrow). (B) Precursor feeding with scytalone: lawn of *wdpks1Δ-1* was first grown on MCD agar for a few days, after which time a few drops of scytalone solution was added to the resulting growth at one point (note blackening of *wdpks1Δ-1* at arrow). (C) Contact cross-feeding by *Mc2w-3* (*mel4*): *wdpks1Δ-1* was cross-streaked from top to bottom through a streak of *Mc2w-3* on MCD agar (note blackening region of the *wdpks1Δ-1* streak where the *Mc2w-3* [*mel4*] cells and *wdpks1Δ-1* cells were mixed and thus were in intimate contact). (D) Effect with acetate: cultures of *wdpks1Δ-1* and strains with either a *mel3* or *mel4* lesion were streaked on MCD agar with 1% (wt/vol) potassium acetate (note that only *Mc2w-3* [*mel4*] is black, indicating lack of identity with *Mel3* [*mel3*] and *wdpks1Δ-1*).
produce the plasmid pCB1004ΔXhoI. A 2.9-kb DNA fragment in plasmid pMO cocoX, which contains the \( \lambda \) bacteriophage cos sequence, was released by CiaI and BamHI double digestion and was inserted into pCB1004ΔXhoI to produce the new cosmid vector pCB1004cos.

**Construction of cosmid library.** A genomic cosmid library of *W. dermatitidis* was constructed using pCB1004cos by the method of Osiewacz (38). After digesting pCB1004cos with XhoI and dephosphorylating the cohesive ends using calf intestine alkaline phosphatase (Promega), the linearized plasmid was cleaved with BamHI to produce two cosmid arms. The cosmid arms were then ligated to DNA fragments generated from high-molecular-weight genomic DNA (~150 kb) of *W. dermatitidis* wild-type 8656 (concentration, 100 \( \mu \)g/ml), which had been partially digested with Sau3AI to generate fragments of 40 to 50 kb, recovered by ethanol precipitation, and resuspended in double-distilled H\(_2\)O.

Incubation of the ligation reaction mixture was for 16 h at 16°C in a total volume of 10 \( \mu \)l, using 5 \( \mu \)g of partially digested genomic DNA and 1 \( \mu \)g of the two cosmid arms. The reaction products were then used immediately for in vitro packaging, which together with the determination of the titer of the cosmid packaging reaction mixture was carried out according to the instruction manual from the Gigapack III XL packaging kit (Stratagene Inc.). The resulting library, consisting of about 5 \( \times \) 10\(^9\) colonies, was pooled and stored in 25% glycerol at –70°C, prior to use in experiments designed to rescue Me\(^{+}\) mutants by complementation.

**Measuring of phagocytosis and oxidative burst by flow cytometry.** To assess phagocytosis, sedimented (3,000 \( \times \) g at 4°C for 5 min) and washed (in 5 ml of sterile 0.9% [w/v] NaCl) yeast cells from SDA broth (5 ml) were incubated with a 1:50000 carbonoyl-chloroacetyl-carbonyloxyreinc-pentacetyloxy-methylester (BCECF-AM) (final concentration, 1 \( \mu \)mol/liter; Roche Biochemicals, Mannheim, Germany) for 30 min at 37°C in phosphate-buffered saline (PBS) (1 ml) as previously described (40, 41). The labeled cells (5 \( \times \) 10\(^9\)) were then incubated at 37°C for a maximum of 120 min with 1 ml of haringenized (10 units of haringen [as defined by the German Pharmacopeia]/ml [equivalent to ca. 5 units of haringen [as defined by the U.S. Pharmacopeia]/ml]) whole blood from healthy donors in a Thermomixer (Eppendorf, Hamburg, Germany) at 1,000 rpm. At 0, 10, 30, and 60 min, samples (100 \( \mu \)l) were removed and immediately mixed with 2 ml of ice-cold lysis buffer (Becton Dickinson, Heidelberg, Germany) to lyse the erythrocytes, and then after 2 h, leukocyte and yeast cell isolations were carried out by centrifugation (10 min, 4°C, 1,300 rpm; Beckman GS-6R centrifuge). After being washed twice in ice-cold PBS, cells were resuspended in PBS (500 \( \mu \)l) and analyzed by flow cytometry, using a FACScan flow cytometer (Becton Dickinson) and Cellquest (Becton Dickinson) software (40, 41). Instruments setting, linear parameters for forward and side scatter, and logarithmic parameters for FL1 and FL2 with the best test performance were revealed to be the same as those set previously for experiments with *W. dermatitidis* and its melanin-deficient mutants (41). Oxidative burst was assessed during the phagocytosis by incubation of unlabeled yeast cells in haringenized blood under conditions identical to those described above. Dihydrorhodamine (DHR) (Molecular Probes, Eugene, Oreg.) was added to samples at a final concentration of 10 \( \mu \)g/ml as described previously (41). The association of neutrophils with the labeled yeast cells and the oxidative burst of the neutrophils induced by unlabeled yeast cells in the presence of DHR were detected by an increase in fluorescence of the neutrophils. The increased fluorescence signal was expressed as a percentage of nonfluorescing neutrophils.

**Neutrophil killing assay.** For quantification of killing by human neutrophils, yeast cells of the strains tested were diluted in PBS to 10\(^5\) to 10\(^6\) CFU per ml. The diluted cells (100 \( \mu \)l) and fresh haringenized human blood (900 \( \mu \)l) were then mixed and rotated at 37°C for 4 h. Initial viable counts and cell counts were determined after 10 min and after 1, 2, 3, and 4 h of rotation by plating samples of yeast cells either undiluted (20 \( \mu \)l) or after dilution with 180 \( \mu \)l of PBS (20 \( \mu \)l) on SDA.

**Microscopy.** To ensure the intracellular location of the yeast cells associated with the neutrophils, representative samples used for determination in flow cytometry were examined by epifluorescence interference contrast microscopy (Leitz DM RB microscope; Leica, Wetzlar, Germany) as described previously (40, 41). From three independent assays of each strain studied, we examined 300 yeast cells with respect to their association with the neutrophils at the beginning, after 30 min, and after 60 min of incubation in haringenized blood.

**Virulence studies with mice.** Test strains (wdPS1Δ1-1, wdPs1Δ1-2, wdps1Δ1-3, wdps1Δ2-3, and wdps1Δ3-3) were cultured in YPD (5 ml) overnight at 30°C with shaking. An aliquot of the overnight culture was used to inoculate 50-ml YPD cultures, which were then grown overnight to mid-log phase. Yeast cells were harvested, washed three times with sterile water, counted on a hemacytometer, and diluted to a final density of 9 \( \times \) 10\(^7\) cells/ml. Virulence of the strains was then tested in an immunocompetent (normal) mouse model system. Male ICR mice (22 to 25 g; Harlan Sprague-Dawley) were housed five per cage; food and water were supplied ad libitum, according to National Institutes of Health guidelines for the use of animals.
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**WdPKS1**

---MEHYVPGFDQTADCRAFFYKTTPRDK---VLLGSLERAGAEVARFDQGMH---SPHKNV 55

**AfPKS**

MEDLRVLYLFQDQCDGDLGNLNLQAKQ---TVFSPFPRPHALQEQITLRLPSQRTF 59

**AnPKS**

MEDPVRVYLFQCQDFVQGLFRLQNL--HLSLSSLQGSRVQESIHLPSRSTF 59

**ClPKS**

MAAATMSYVLDQGSLDTHGFIQAEFCRGNMPSILAKTLLFQAGSMLREIIGDGKLRSKL 60

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**WdPKS1**

PNFTQVLQDYRDPVQAIKADTAESALCVTSQFCHIAGAFRREEPSIQYNPSMDALVRGC 115

**AfPKS**

FRPTSALDLAQR---SGTNPALSGALTCTYLQCDIDYH-QDRHPPSDDD---LGNCESC 116

**AnPKS**

FRPTSQDGLALRHC---SGPPAIAISVCTYLVQCFINITY-DLQGTFSSQIGQVLCGL 117

**ClPKS**

TPFQRLQSLKHQVSAIKHPGIDSALLCTQLAHYFIDR---KEFQACLDNHTFPMGCL 119

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**WdPKS1**

TGLIAATAVASDSLTSILPAVLAEVRAFIARAGVQVAQTECSDKTQMVSTVAADE 175

**AfPKS**

TGMCLCTAVSESNVGLLPLADEVHLTHLGCMRVMRQVDSTESSGSGWILSVEI 176

**AnPKS**

TGLSACAIVASCNIGELLKAPVVVVVVALRLGCRYVRKQFQDQQAPLSNLAVGLG 177

**ClPKS**

TGLIAAATIASTPSVTILAPAVOQLMLAFTQMKQGSMNLALRFLQGQVFSEFNTLH 179

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**WdPKS1**

KS---AQEALDAPHKKEQFSTGFQWISVSVASATSVSFVWPATKALXEXSEFFERTQKSA 233

**AfPKS**

NEADAITLIDQDFDRIQFPPQFQYIIVGSKGLT---SAFLPEILLNIEBPQYDHFK 236

**AnPKS**

SEQSESDIAITQFTPQEFNTPSRFQYISACANTLTQISGFYYQDFFPSIGKNAVM 237

**ClPKS**

KESDAKAELANPRAPNYTWSYAVSVSLSALSPATKLIDQNDGVQ----VAKSTA 238

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**WdPKS1**

VSIFAQYHAHHKHSQDLDKLIRP---QQTIQFQMTRTTFQCVSTGQPHNAENGFLF 290

**AfPKS**

PVQSGQPYYHLHYDREIRNLFS---EDVILBPFRVLFVQSEMTKQVQKSMRDL 293

**AnPKS**

P-IQFQFHASHYLHYKRDVTMILS---VETIRNKHPVFPLSNSTGHELIVVNEGFL 293

**ClPKS**

IPVQGFYYHAAHLSQHATADEKILNRLDPVVEILATKPKSMALPQSGTKWPAETFDY 297

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**WdPKS1**

QALAEIEIIDDPLRWKDLKLVCYAC---KASEAFLAIFQPNLASSVLASAKASSKT 346

**AfPKS**

VKALEELTIKDQCDKTECSSLVQATND---KWRPFLISNSATQLVLATLQRMGNCQZ 348

**AnPKS**

KLAEELLIQDQESKDVTSCISILSKVQDNKPKKLISPTATGQFLHNKLLKGNVIE 353

**ClPKS**

QTVHELCLVDLVQFFQRQKQIECETARDJFQTCMLQVCLQDQFRTLQANVM 357

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**WdPKS1**

EDHSTWSTVFPQ---GTHSKEADIALIVGGRSRPGMADWLEDNLQGQLQGLDLVRHP 402

**AfPKS**

VTDGAGPAQPM---AAPNQANASRSKIALTIMGRSRFPADQIEADFWLLYLQLDLVRHP 411

**AnPKS**

VTDGAGPDAE---QVQONTQRSAKLSAIKIMGRSRFEADSQPDQMLNQLQLDLVRHP 412

**ClPKS**

VTDGAGPDAE---QVQONTQRSAKLSAIKIMGRSRFEADSQPDQMLNQLQLDLVRHP 412

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**WdPKS1**

PDRFFVESHDTPSQKKNKNTSHPQRFNPQKPLDFARFFMNFSREAQTDQMHQLMT 472

**AfPKS**

PERRWADAMHDTQRTKNSTQSKYQPCINHDFARFFMNFSREAQTDQMHQALITA 471

**AnPKS**

EDRRCDADVHDTQRTKNSTQSKYQPCINHDFARFFMNFSREAQTDQMHQALITA 471

**ClPKS**

ADRRCDADVHDTQRTKNSTQSKYQPCINHDFARFFMNFSREAQTDQMHQALITA 471

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**WdPKS1**

XABMAGKPGVQKSTXTKDHQ3QFTQGQTSDKREWVNAAXADITYTFQGQYRPGQ 522

**AfPKS**

YEALMGAFQNPVNSQSDQDRVPRGFPQMNSTSDKREDINQTDIYTFQGQNYRPGQ 531

**AnPKS**

YEALMGAFQNPVNSQSDQDRVPRGFPQMNSTSDKREDINQTDIYTFQGQNYRPGQ 531

**ClPKS**

YEALMGAFQNPVNSQSDQDRVPRGFPQMNSTSDKREDINQTDIYTFQGQNYRPGQ 531

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**WdPKS1**

YFPFQSGPSVTDACSSLSLAMNVAITLSLNAECDVTFTGQANVTLNDSFMGSLRVS 582

**AfPKS**

YFPFQSGPSVTDACSSLSLAMNVAITLSLNAECDVTFTGQANVTLNDSFMGSLRVS 582

**AnPKS**

YFPFQSGPSVTDACSSLSLAMNVAITLSLNAECDVTFTGQANVTLNDSFMGSLRVS 582

**ClPKS**

YFPFQSGPSVTDACSSLSLAMNVAITLSLNAECDVTFTGQANVTLNDSFMGSLRVS 582

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**WdPKS1**

LSRTGSCWTDNADADGRQCGDQGCTVICTJMRLDADLRDVPGLVIGRTGMRHSAEAVS 642

**AfPKS**

LSRTGSCWTDNADADGRQCGDQGCTVICTJMRLDADLRDVPGLVIGRTGMRHSAEAVS 651

**AnPKS**

LSRTGSCWTDNADADGRQCGDQGCTVICTJMRLDADLRDVPGLVIGRTGMRHSAEAVS 651

**ClPKS**

LSRTGSCWTDNADADGRQCGDQGCTVICTJMRLDADLRDVPGLVIGRTGMRHSAEAVS 651

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**WdPKS1**

HPGNAQAPFILKKNALVNPVAYPSYIEMGTOQGAGDIEGEMVESVSVSFAPFRAP 702

**AfPKS**

RPVAGAQAPFILKKNALVNPVAYPSYIEMGTOQGAGDIEGEMVESVSVSFAPFRAP 708

**AnPKS**

RPVAGAQAPFILKKNALVNPVAYPSYIEMGTOQGAGDIEGEMVESVSVSFAPFRAP 708

**ClPKS**

RPVAGAQAPFILKKNALVNPVAYPSYIEMGTOQGAGDIEGEMVESVSVSFAPFRAP 708

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**FIG. 3—Continued.**
FIG. 3—Continued.
DISRUPTION OF WdPKS1 USING A PCR PRODUCT FOR GENE TARGETING PRODUCED ALBINO STRAINS. PCR primers having a design based on fungal wdpks conserved regions allowed amplification of a 772-bp PCR product from genomic DNA of W. dermatitidis, which was then cloned, sequenced, by sequence similarity to encode a putative polyketide synthase (WdPks1p), and used to produce the disruption plasmid pBF9 (Fig. 1A). Among 53 HmB-resistant transformants obtained after electroporation of pBF9 linearized with SacI, five were albino, suggesting, as expected, that WdPKS1 encoded a Pks involved in melanin biosynthesis. Southern analysis of three albino transformants (wdpks1Δ-1, wdpks1Δ-2, and wdpks1Δ-3) and two melanized transformants (211 and 212) using the 772-bp PCR fragment as a probe, showed that the WdPKS1-hybridized DNA band was suitably shifted from a wild-type position of 4.1 kb to a disruptant position of 10 kb among XhoI-digested fragments (Fig. 1B). These results confirmed that the albino phenotype of the transformants was due to site-specific integration and disruption of WdPKS1, whereas the bands larger than 4.1 kb in strains 211 and 212 were indicative of ectopic plasmid integrations.

Diffusion cross-feeding experiments showed that albino wdpks1Δ-1 became blackened and produced melanin when cross-fed by 1,8-DHN melanin precursors produced by the brown strains Mel1 (data not shown) and Mel2 (Fig. 2A) or with the purified melanin biosynthetic pathway intermediate, scytalone (Fig. 2B). These experiments also showed that no visible intermediates were secreted into the medium on which the wdpks1Δ-1 disruption strain was grown. Thus, WdPKS1p is involved in a step before 1,3,6,8-THN reductase functions to produce the compound scytalone or before an oxidase converts 1,3,6,8-THN to the colored shunt product flaviloin (53) and consequently likely participates in the production of 1,3,6,8-THN itself. In W. dermatitidis this process is thought to involve two steps, which are defined by the mutations mel3 and mel4 (13). Therefore, additional experiments using standard protocols (13) were carried out to determine whether strains with either the mel3 or the mel4 lesion could be distinguished from the disruption strain wdpks1Δ-1. From the results of the contact cross-feeding experiment with strains having the mel3 mutation (data not shown) or the mel4 mutation (Fig. 2C) and from the acetate feeding experiment (Fig. 2D), wdpks1Δ-1 was clearly distinguished from the latter (mel4) but not the former (mel3), suggesting for the first time that the mel3 lesion was due to a mutation in WdPKS1.

Cloning of WdPKS1 by a marker rescue approach and by cosmid complementation of the mel3 mutation. Plasmid pBF10 carrying the 5′ end of WdPKS1 was recovered by digesting
genomic DNA of \textit{wdpks1}\(\Delta\)-1 with \textit{KpnI} and then allowing self-ligation in dilute solution. The 12-kb plasmid, pBF10, was then rescued by transformation of \textit{E. coli}, and the 5' end of the gene was cloned (Fig. 1A). Using the same strategy, the 3' end of \textit{WdPKS1} was also recovered in a 13-kb rescue plasmid, pBF32, through \textit{SpeI} digestion (Fig. 1A).

The \textit{WdPKS1} gene was also simultaneously cloned independently by cosmid cloning during a search for other melanin biosynthetic genes. After the cosmid library DNA was used to transform several \textit{W. dermatitidis} Mel\(2\) albino strains, including \textit{Mc2w-3 (cde2 mel4 arg1)}, Mel1 (\textit{mel1}), Mel3 (\textit{mel3-1}), and Mel4 (\textit{mel3-2}), the transformants were selected on hygromycin-containing media. Although over 400 HmB-resistant transformants were obtained with these strains, only one black, putatively complemented strain was obtained out of the nearly 100 transformants of Mel4 (\textit{mel3-2}).

The transforming cosmid responsible for the reversion of the \textit{mel3}-2 mutation in strain Mel4 was recovered by in vitro \(\lambda\) packaging of undigested genomic DNA, followed by transduction of \textit{E. coli} as described by Yelton et al. (56). As expected, retransformation of Mel3 (\textit{mel3-1}) and Mel4 (\textit{mel3-2}) with the cosmid caused most transformants of these two strains to regain melanin production capability (data not shown). PCR analysis using primers for \textit{WdPKS1} yielded a 772-bp product, which was consistent with the possibility that \textit{WdPKS1} was contained in the cosmid (now called cos-Mel3) and was responsible for complementing the \textit{mel3} lesions in strains Mel3 and Mel4 (data not shown).
**WdPKS1 encodes a type 1 Pks.** Restriction enzyme mapping of the cloned inserts in pBF32 and pBF10 (Fig. 3A) and comparisons with the map of the cos-Mel3 (data not shown) confirmed that *WdPKS1* had been cloned independently by two methods. Therefore, only the two rescued *WdPKS1* gene fragments in pBF32 and pBF10 were completely sequenced after a series of subclonings. A single open reading frame was deduced for *WdPKS1* and found to encode 2,177 amino acids. Two putative introns (279 to 337 bp and 6540 to 6596 bp) were also identified in *WdPKS1*, which were confirmed by RT-PCR (Fig. 3B), and by sequence analysis of the RT-PCR products (data not shown). The deduced amino acid sequence showed 46.8, 45.7 and 44.9% identity to *pks1* of *C. lagenarium*, *alb1* of *A. fumigatus*, and *wA* of *A. nidulans*, respectively (Fig. 3C), further indicating that the cloned gene encoded a polyketide synthase. Northern analysis showed constitutive expression of *WdPKS1* (data not shown).

Because eukaryotic Pks are generally thought to be large multifunctional proteins (type I Pks) (23), the predicted amino acid sequence was investigated further. The β-ketoacyl synthase, acetyl-malonyl transferase, and two ACP and thioesterase domains usually found within a type I Pks were all identified (Fig. 4). The putative WdPks1p also showed complete conservation of the putative active site cysteine residue of the β-ketoacyl synthase, the active site serine residue of the acetyl-malonyl transferase, and the pantotheine-binding serine residue of the ACP (3, 8, 20, 31, 36, 44, 57), which provided further evidence that *WdPKS1* was a type I Pks.

**Reconstitution of melanin biosynthesis.** To confirm that the albino phenotype was not due to secondary, hidden mutations, melanin biosynthesis in the albino strain, *wdpks1Δ-1*, was reconstituted by integration of *WdPKS1* into its genome at the *WdPKS1* endogenous site. This was accomplished with the vector pBF50 constructed by subcloning and ligating the 5′ end of the gene from pBF10 to the 3′ end of the gene from pBF32 in vector pCB1532, which contained the sulfonyl-urea (*SUR*) and the ampicillin (*amp*) resistance genes for selection in *W. dermatitidis* and *E. coli*, respectively. Plasmid pBF50 (Fig. 5A), which contained the full-length *WdPKS1* with its endogenous promoter, was then transformed without being linearized into *wdpks1Δ-1* by electroporation. Among the resulting *SUR*-resistant transformants, 2 of 11 were restored to melanin synthesis (data not shown). Southern analysis of the two complemented strains showed the expected DNA band shifts (Fig. 5B), which confirmed that the cloned *WdPKS1* had integrated into the 5′ portion of the disrupted *WdPKS1* gene.

To ensure that any differences detected in susceptibility to killing by neutrophils or loss of virulence in mice was due only to loss of melanin in the *wdpks Δ* mutants, the growth rates of *wdpks Δ-1*, wild-type 8656, and the *WdPKS1*-complemented strain 501 were also compared in rich YPD (Fig. 6). The results confirmed that *wdpks1Δ-1* grew at the same rate as the wild-type strain.
type 8656 and as the WdPKS1-complemented strain 501, both at 25°C (generation time, 3.3 h) and 37°C (generation time, 2.8 h). Microscopic comparisons of these strains with the wild type grown identically also showed no apparent morphological differences (data not shown).

**Fluorescence staining of W. dermatitidis and phagocytosis by human neutrophils.** Staining of the W. dermatitidis strains by incubation for 30 min in PBS containing 1 µmol of BCECF-AM per liter resulted in a stable green fluorescence in the parent strain and all of its isogenic mutants and complemented strains (501 and 502) (data not shown). Although the darkly pigmented, wild-type strain and the respective complemented strains exhibited a slightly lower level of green fluorescence compared to the albino wdpks1Δ-1 mutant (data not shown), accurate quantitative comparisons of the rates of phagocytosis by the human neutrophils of the strains were still possible. The results showed that phagocytosis by the neutrophils was essentially the same for all the strains during three independent assays (Fig. 7). By 10 min of coincubation, >98% of yeast cells of all the strains were localized within the phagocytes as judged by microscopy. No significant difference with respect to the proportion of budding yeast cells in relation to their extracellular or intracellular location was observed by epifluorescence interference contrast microscopy (data not shown), which suggested that a good correlation existed between the flow cytometric assay and microscopic observations.

**Oxidative burst evoked by and killing of W. dermatitidis in human neutrophils.** Phagocytosis of yeast cells was paralleled by an oxidative burst in the phagocytosing neutrophils, as suggested by the increased fluorescence detected by flow cytometry. In no case was a significant difference in oxidative bursts detected among the responses evoked after neutrophil phagocytosis of the wild-type strain 8656, the mutant wdpks1Δ-1, and its respective complemented strain 501 (Fig. 8). In contrast, the wdpks1Δ mutants were considerably more susceptible to killing by the neutrophils than the melanized strains (Fig. 9). In every case, the killing of the melanin-deficient mutants was significantly greater (P < 0.005) after 120, 180, and 240 min of coincubation with whole blood compared to the wild-type strain 8656 and the respective complemented strains. However, no differences were detected among the rates of killing of the three wdpks1Δ mutants tested in five independent experiments (Fig. 9).

**Disruption of WdPKS1 causes loss of virulence in mice.** Animal studies were carried out to compare the degrees of virulence of the wild type, the wdpks1Δ disruptants, and the WdPKS1-complemented strains. In at least three independent experiments, mice injected with the wdpks1Δ disruptants showed a dramatic reduction in mortality compared to that of the wild type and the WdPKS1-complemented strains. Lethality in mice infected with strain 8656 began on days 4 and 5, with mortality rates of 90 to 100% by days 6 and 7 (Fig. 10). In contrast, mice injected with the wdpks1Δ disruptants showed only 0 to 10% mortality at the end of the experiments (day 13). However, the WdPKS1-complemented strains all recovered full virulence; mortality started on day 5 and reached 70 to 80% by the termination date (Fig. 10). In addition, the Mel4 (mel3-2) cos-WdPKS1-complemented strain was also found to have recovered full virulence (data not shown).

**DISCUSSION**

Prior studies have strongly indicated that virulence of dematiaceous fungi is influenced by the presence of 1,8-DHN melanin (13, 15–18, 24, 41). The results of this study provide additional support for this concept. After the melanin biosyn-
the synthetic pathway gene, WdPKSI, of W. dermatitidis was cloned and disrupted, the resulting wdpks1Δ mutants were shown to be more susceptible to killing by neutrophils and to be less virulent in an acute mouse model. One wdpks1Δ mutant was then shown to regain resistance to neutrophil killing and to exhibit normal virulence in mice when melanin biosynthesis was reconstituted by complementation with WdPKSI. To our knowledge, WdPKSI is the first melanin biosynthetic pathway gene cloned in W. dermatitidis, or any other dematiaceous pathogen of humans, and shown to contribute directly to virulence.

The WdPKSI gene was cloned by a PCR method coupled with a marker rescue approach and then sequenced. The predicted WdPKSIp showed highly significant sequence similarity with type I Pks (23) and shared domains with other Pks for a diketoacyl synthase, an acetyl-malonyl transferase, two ACPs, and a thioesterase (3, 8, 20, 31, 36, 44, 57). This result strongly suggested that the WdPKSI gene encodes a polyketide synthase involved in melanin biosynthesis in W. dermatitidis. In addition, the WdPKSI gene was also found to be constantly expressed throughout the W. dermatitidis growth cycle at both 25 and 37°C and confirmed to be nonessential, as would be expected of a gene involved in melanin biosynthesis by secondary metabolism. Phenotypic analyses of wdpks disruption mutants further showed that these albino strains had both wild-type growth rates and cellular morphologies in vitro.

Recently, flow cytometry of BCECF-AM-stained W. dermatitidis was found to be suitable for determination of phagocytosis rates of yeast cells by incubation with neutrophils in whole, heparinized, human blood: BCECF leads to a stable intracellular stain of the yeast cells (41). To ensure an intracellular location of the yeast cells found associated with neutrophils, epifluorescence microscopy was carried out in combination with interference contrast microscopy of representative experimental samples. In accordance with the prior results (41), no differences were found in the degrees and rates of phagocytosis between the W. dermatitidis wild-type strain and the WdPKSI-complemented and noncomplemented wdpks1Δ mutants. Evoked oxidative burst, estimated by a DHR method that was also recently validated for W. dermatitidis (41), similarly showed that the amounts and rates of the oxidative bursts induced by the same strains were comparable. This again was taken as confirmation of equal internalization of unstained yeast cells by the human granulocytes, because all the neutrophils involved exhibited a bright green fluorescence in the presence of DHR, which is indicative of a comparable degree of oxidative burst.

The rates of phagocytosis and subsequent amounts of killing of W. dermatitidis determined in a bioassay were also in excellent agreement with the prior results (41) but were obtained with the genetically uncharacterized W. dermatitidis albino mutant Mel3 (mel3-1), which was generated previously by UV mutagenesis (22). Statistically significant differences between the darkly pigmented wild-type strain and the wdpks1Δ-1-complemented strain and the albino wdpks1Δ mutants were clearly detected after exposure of yeast cells of these strains in whole heparinized human blood for more than 10 min. This observation clearly demonstrated that the presence of intact melanin is the main factor contributing to the difference in killing of the different strains by human neutrophils. Thus, the albino mutant strains of W. dermatitidis are killed by neutrophils in a comparable fashion to Candida albicans and Saccharomyces cerevisiae (unpublished data), which can be taken as an indication that melanin synthesis contributes directly to the virulence of this black yeast species.

The most intriguing phenotype of the wdpks1Δ disruption strains beyond their loss of pigmentation was their significant loss of virulence in a mouse model of acute infection compared to those of their wild-type parent and the two complemented strains in the wdpks1Δ-1 background. Such reduced virulence in the mouse model was also previously observed with the albino strain Mel3 (mel3-1) (15). However, because the UV used to generate this mutant could have given rise to multiple gene defects not easily detected in an asexual fungus, it might be argued that firm conclusions about the relationship of melanin with virulence in this strain are not be warranted (16-18). This concern is now moot because of our ability to complement the mel3 lesion with WdPKSI, which clearly established the equivalency of strains with mel3 lesions with our newly derived and less virulent wdpks1Δ disruption strains.

The importance of a polyketide synthase involved in melanin biosynthesis in the human pathogen W. dermatitidis is now documented for the first time to be equivalent to that of similar fungi found among the plant pathogens. In the plant pathogenic fungi C. lagenarium and M. grisea, deficient 1,8-DHN melanin biosynthesis resulted in nonmelanized appressoria (27, 32, 39). In both cases, those mutants lost their ability to penetrate plant leaf tissue and thus became avirulent due to the fact that melanin is required for the rigidity of appressoria (2). Similarly, it was reported recently that invasive hyphal growth in W. dermatitidis is dependent on melanin biosynthesis.
Brush and Money hypothesized that melanized hyphae exert larger turgor-derived forces at their apices than nonmelanized cells in *W. dermatitidis*, explaining their propensity for fast substrate invasion (5).

Previous studies demonstrated by parasexual genetic methods that strains with the *mel3* and *mel4* genotypes are representative of mutants with defects in two different enzymes that function during the conversion of acetyl-CoA or malonyl-CoA to 1,3,6,8-THN (12, 13). Strains with mutations in these genes can be differentiated also by physiological methods (13). Thus, comparisons of *wdpks1Δ-1* with strains with one or the other of these two mutations, plus complementation studies with the cloned *WdPKS1* gene itself, can be used to distinguish such mutants. For example, although none of the strains with the *mel3*, *mel4*, or *wdpks1Δ* mutations secrete visible intermediates into the culture medium, the *wdpks1Δ-1* strain contact crossfeeds with strains with the *mel4* mutation (Fig. 2C), whereas *wdpks1Δ-1* does not do the same with strains with the *mel3* mutation (13). Also, whereas the latter two mutant types are both very white, strains with the *mel4* mutation are more beige than white with time on YPD or MCD agar, which previously suggested that *mel3* represents a lesion in an enzyme that functions prior to *mel4* (13). Finally, when grown on 1% (wt/vol) acetate-supplemented MCD agar, *wdpks1Δ-1* and strains with the *mel3* lesion remained white, whereas strains with the *mel4* lesion turned black (Fig. 2D). Although the basis of these contact cross-feeding and acetate effects is not clear, these physiological tests support the hypothesis that two enzymes are involved in the conversion of acetyl-CoA or malonyl-CoA to 1,3,6,8-THN in *W. dermatitidis* and that the *wdpks1Δ* disruption mutants are equivalent to the previously described mutants with *mel3* lesions (13). Furthermore, although *WdPKS1* complemented strains with the *mel3* lesion, it did not complement a strain with a *mel4* lesion. Thus, although the nature of the *mel3* mutation is now clear, that of *mel4* remains unknown. In this respect, in most fungal 1,8-DHN melanin systems, there is only one structural polyketide synthase gene known to contribute at this step in the pathway (19, 21, 29, 50). The cloning of this gene in the future will enable us to understand better 1,8-DHN melanin biosynthesis in the human pathogen *W. dermatitidis* and the differences between the pathways in *W. dermatitidis* and in other black fungi. Unfortunately all attempts to clone this gene by complementation or by insertional mutagenesis and marker rescue have failed (unpublished data).

Genes involved in 1,8-DHN melanin synthesis are clustered in some fungi and not in others. In *A. alternata*, a melanin pathway gene cluster has been identified that contains at least three pathway biosynthetic genes within a 30-kb region (25), which encode the polyketide synthase, the scytalone dehydratase, and the 1,3,8-THN reducetase. Cloning of these three genes in *C. lagenarium* was also reported (28, 39, 44). However, in contrast to *A. alternata* it appears that these same genes are not closely linked in *C. lagenarium* (27). In *M. grisea*, classical genetic analysis with melanin mutants indicates none of the pathway genes are closely linked (10). Although pathway genes have not been isolated for *C. miyabeanae* and *Cochliobolus heterostrophus*, classical genetics analysis indicates that in both organisms the polyketide synthase and the 1, 3, 8-THN reductase genes but not the scytalone dehydratase gene, are linked (28). In *W. dermatitidis*, two independently isolated cosmids clones containing *WdPKS1* were used to transform mutants with *mel1*, *mel2*, and *mel4* lesions. However, in no case was complementation observed. Thus, it appears that the *mel4* mutation (representative of the unknown enzyme in the pathway) and the genes for the scytalone reductase and for the 1,8-DHN oxidase (polymerase) are not closely linked with *WdPKS1*. More extensive searches to identify these genes and others related to 1,8-DHN melanin biosynthesis in *W. dermatitidis* are in progress.

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