

The Developmentally Regulated *alb1* Gene of *Aspergillus fumigatus*: Its Role in Modulation of Conidial Morphology and Virulence

HUEI-FUNG TSAI,¹ YUN C. CHANG,¹ RONALD G. WASHBURN,² MICHAEL H. WHEELER,³
AND K. J. KWON-CHUNG^{1*}

Laboratory of Clinical Investigation, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20892¹;
Section of Infectious Diseases, Department of Internal Medicine, Wake Forest University School of Medicine,
Winston-Salem, North Carolina 27006²; and Cotton Pathology Research Unit, Southern Crops
Research Laboratory, USDA Agricultural Research Service,
College Station, Texas 77845³

Received 12 February 1998/Accepted 14 April 1998

Aspergillus fumigatus, an important opportunistic pathogen which commonly affects neutropenic patients, produces conidia with a bluish-green color. We identified a gene, *alb1*, which is required for conidial pigmentation. The *alb1* gene encodes a putative polyketide synthase, and disruption of *alb1* resulted in an albino conidial phenotype. Expression of *alb1* is developmentally regulated, and the 7-kb transcript is detected only during the conidiation stage. The *alb1* mutation was found to block 1,3,6,8-tetrahydroxynaphthalene production, indicating that *alb1* is involved in dihydroxynaphthalene-melanin biosynthesis. Scanning electron microscopy studies showed that the *alb1* disruptant exhibited a smooth conidial surface, whereas complementation of the *alb1* deletion restored the echinulate wild-type surface. Disruption of *alb1* resulted in a significant increase in C3 binding on conidial surfaces, and the conidia of the *alb1* disruptant were ingested by human neutrophils at a higher rate than were those of the wild type. The *alb1*-complemented strain producing bluish-green conidia exhibited inefficient C3 binding and neutrophil-mediated phagocytosis quantitatively similar to those of the wild type. Importantly, the *alb1* disruptant had a statistically significant loss of virulence compared to the wild-type and *alb1*-complemented strains in a murine model. These results suggest that disruption of *alb1* causes pleiotropic effects on conidial morphology and fungal virulence.

Aspergillus fumigatus, a ubiquitous fungus, causes allergy, noninvasive colonization, or life-threatening invasive pulmonary aspergillosis (25). Recently, the incidence of invasive pulmonary aspergillosis caused by *A. fumigatus* has significantly increased secondary to increased use of immunosuppressive therapy and prolongation of life for neutropenic patients (7, 8, 13, 14). In nature, *A. fumigatus* survives as a saprophyte and propagates by highly dispersible conidia. The ability to adapt to broad ranges of environmental conditions may account for its worldwide distribution. Conidia, which are the first fungal structures encountered by the human host, are small (2 to 3.5 μm in diameter), extremely hydrophobic, and bluish green in color (32, 41). The inhaled conidia can cause invasive aspergillosis, a life-threatening disease, if the immune system is impaired, especially in patients with prolonged neutropenia (25, 56).

To establish an infection in the human host, inhaled conidia must protect themselves from the host defense system. At the initial stage of the *A. fumigatus*-host interaction, conidial surface components may play an important role in evasion of the host immune system. One of the visible conidial surface components is the bluish-green pigment. Fungal pigments have been shown to contribute to the survival and longevity of fungal propagules in the environment (61). Recently, we showed that complement component C3 binds more efficiently to conidia of a reddish-pink conidial color mutant of *A. fumigatus* than to bluish-green wild-type conidia (54). Analysis of the mutation led to the isolation of the *alb1* gene, encoding a

homolog of scytalone dehydratase, which is involved in the dihydroxynaphthalene (DHN)-melanin pathway. DHN-melanin has been shown to be a pathogenic factor in plant pathogenic fungi such as *Magnaporthe grisea* and *Colletotrichum lagenarium* (21, 23, 28, 37). Furthermore, melanin has been shown to be important for virulence in human pathogenic fungi, including *Cryptococcus neoformans* and *Wangiella dermatitidis* (10, 26, 27, 42, 55, 61).

Most studies of pigment biosynthesis in fungi have focused on the brown and black fungi, since these fungi destroy many important agricultural crops (1). In the well-studied DHN-melanin pathway in the brown and black fungi, polyketides are synthesized by polyketide synthases. Using acetate as a precursor, 1,3,6,8-tetrahydroxynaphthalene (1,3,6,8-THN) is synthesized and subsequently reduced to form scytalone (Fig. 1) (5, 61). Scytalone is then dehydrated to produce 1,3,8-trihydroxynaphthalene (1,3,8-THN), which is converted to 1,8-dihydroxynaphthalene (1,8-DHN) after additional reduction and dehydration steps. Finally, 1,8-DHN is polymerized to form DHN-melanin. Both reduction steps are sensitive to tricyclazole, a fungicide which specifically inhibits THN reductases involved in the DHN-melanin pathway. In contrast to pigment biosynthesis in brown and black fungi, little is known about pigment biosynthesis in green and bluish-green fungi. In *Aspergillus nidulans*, a green-spored species, two conidial pigmentation genes, *wA* and *yA*, have been isolated (2, 29, 30, 34). The *wA* gene encodes a putative polyketide synthase, and *yA* encodes a laccase. Disruption of *wA* and *yA* individually resulted in white and yellow conidial-color mutants, respectively. Interestingly, although tricyclazole does not inhibit the conidial pigment biosynthesis of *A. nidulans*, it does inhibit conidial pigmentation in *A. fumigatus* (53, 54, 61, 62). That finding

* Corresponding author. Mailing address: NIH/NIAID, Building 10, Room 11C304, 10 Center Dr., MSC 1882, Bethesda, MD 20892-1882. Phone: (301) 496-1602. Fax: (301) 402-1003. E-mail: June_Kwon-Chung@NIH.GOV.

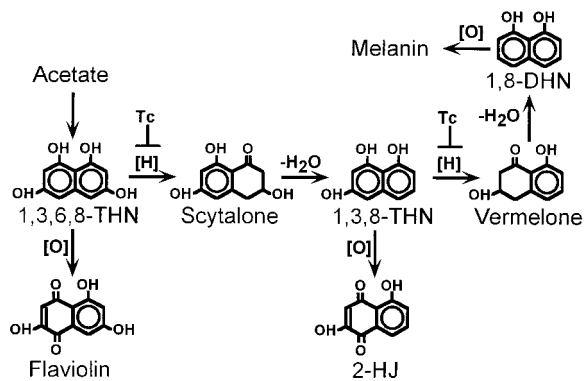


FIG. 1. Biosynthetic pathway of DHN-melanin in brown and black fungi. [H], a reduction step; [O], an oxidation step; $-H_2O$, a dehydration step; Tc, a reduction step which can be inhibited by the fungicide tricyclazole.

suggests that unlike *A. nidulans*, *A. fumigatus* uses a DHN-melanin pathway for conidial pigmentation.

During analysis of the *alb1* gene, we found that *alb1* is linked to a gene that likely encodes a polyketide synthase. The molecular genetics of polyketide biosynthesis has been most thoroughly analyzed in bacteria (15, 16). These studies showed that polyketide synthases can be divided into two types (18, 33). Type I enzymes are large, multifunctional polypeptides, while type II enzymes consist of several single-function polypeptides associated in a complex. To date, all the fungal polyketide synthases that have been characterized are type I enzymes (65) and are involved in the synthesis of pigments and/or mycotoxins.

Here, we report an additional dimension of fungal polyketide synthase which includes modulation of conidial morphology and virulence in *A. fumigatus*.

MATERIALS AND METHODS

Strains and media. *A. fumigatus* B-5233 is a clinical isolate that produces conidia with a bluish-green color. B-5233/RGD12-8 is an *alb1* disruptant which produces white conidia. RGD12-8/PKS33-3, obtained by transforming B-5233/RGD12-8 with pPKS33, produces bluish-green conidia. *Aspergillus* minimal medium contains 1% glucose, 10 mM $NaNO_3$, and trace elements (20). Malt extract medium contains 2% glucose, 2% malt extract, and 0.1% peptone. Cultures were grown at 37°C. Asparagine-sucrose agar (ASA) medium is identical to a previously described alkaline medium (TM medium [64]). This medium was required for the growth of *A. fumigatus* under slightly alkaline conditions, near pH 7.5, to optimize flaviolin and 2-hydroxyjuglone (2-HJ) production. Scytalone and tricyclazole (Eli Lilly Research Laboratories, Greenfield, Ind.) were dissolved in absolute ethanol (EtOH). ASA medium was modified to contain 1% EtOH without tricyclazole or scytalone, with 30 μ g of tricyclazole per ml, with 1 mM scytalone, or with a combination of 30 μ g of tricyclazole per ml and 1 mM scytalone. Controls with 1% EtOH alone or no EtOH were compared to exclude the possibility that 1% EtOH affects morphology or conidial pigment production.

Preparation and analysis of nucleic acids. Isolation of total DNA and RNA from *Aspergillus* cultures was performed as previously described (52). Cultures were harvested at 0 h (hyphal stage) and 14 h (conidiophores and conidial chains were observed) after induction of conidiation for RNA preparation (36). DNA sequencing was done with a Sequenase version 2.0 kit (U.S. Biochemical, Cleveland, Ohio) and an ABI automatic DNA sequencing system (Perkin-Elmer, Foster City, Calif.). A GeneClean II kit (Bio101, Vista, Calif.) was used to purify recovered DNA fragments. DNA cloning and Southern blot analyses were performed according to standard protocols (43). A Hybond-N nylon membrane (Amersham, Arlington Heights, Ill.) was used for blot analysis. DNA probes were labeled with [α -³²P]dCTP (Amersham) by using a Prime It kit (Stratagene, La Jolla, Calif.).

Partial-length cDNA clones were obtained by screening a cDNA library of *A. fumigatus* B-5233, using the 5-kb *AvrII* DNA fragment as a probe (54). To confirm the presence of two introns at the 5'-end region of *alb1*, double-stranded cDNA was obtained by reverse transcription (RT)-PCR (Life Technologies, Gaithersburg, Md.), using poly(A)⁺ RNA of strain B-5233 harvested 14 h after induction of conidiation as the template and DW10 (5'-ACTCGGTGACTTTG TCCC-3') and DW16N (5'-TTGCAGGCGAAGAACCAT-3') as primers. The

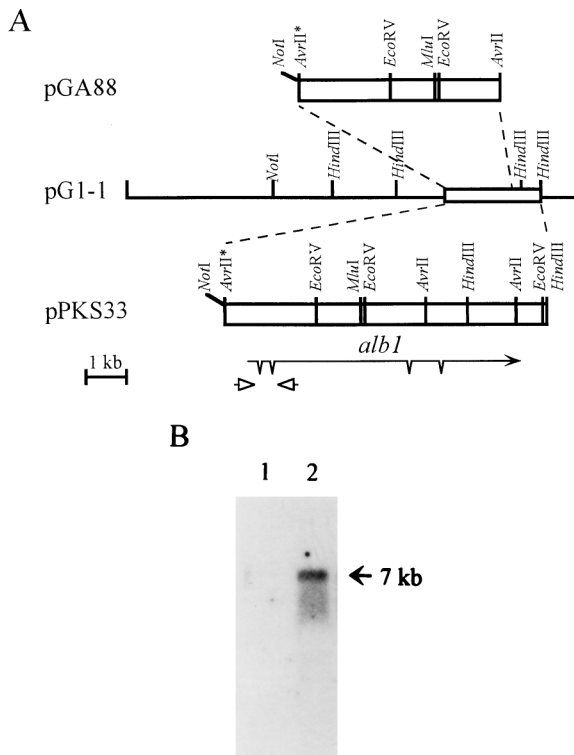


FIG. 2. Structure and expression of *alb1*. (A) Restriction enzyme map and structure of *alb1*. The asterisk indicates an *AvrII* site destroyed during cloning. The *alb1* transcript is represented by an arrow, and introns are marked with spikes. Arrowheads represent the two oligonucleotides used to amplify cDNA in the RT-PCR. (B) Northern analysis of *alb1* expression at different developmental stages. Twelve micrograms of total RNA from strain B-5233 harvested at 0 h (lane 1) or 14 h (lane 2) after induction of conidiation was fractionated on a 1% formaldehyde-agarose gel. The 5-kb *AvrII* DNA fragment was used as a probe. The size of the hybridizing signal is indicated by an arrow.

amplified double-stranded cDNA was cloned into pCR2.1TOPO (Invitrogen, Carlsbad, Calif.) and sequenced with primers DW16N and DW34 (5'-ATTGACATCCACCTCGG-3').

Plasmids. Cosmid pG1-1 (Fig. 2A) was rescued from a restored conidial-color mutant, RP3/G1-1 (54), and contained a 44-kb insert. Plasmid pGA88 was derived from pG1-1 and carried a 5-kb *AvrII* DNA fragment containing a truncated *alb1* gene in pCosHX (obtained from J. Hamer, Purdue University, West Lafayette, Ind.). Plasmid pPKS33 carried a 8.2-kb *AvrII*-*HindIII* fragment which encompassed the *alb1* gene on the pBC-phleo vector (44). To create an *alb1* gene disruption construct, pRGD12, a 6.2-kb *AvrII*-*HindIII* *alb1* DNA fragment was cloned into *SpeI*- and *HindIII*-digested pBC KS+ vector (Stratagene); subsequently, a 1.5-kb *MluI*-*AvrII* fragment was replaced with a 2.8-kb *hph* cassette from pAN7-1 (39) (see Fig. 4A).

Transformation of *A. fumigatus*. *A. fumigatus* protoplasts were prepared with mureinase (Amersham) and transformed by the polyethylene glycol method described by Yelton et al. (66). Transformants were selected on *Aspergillus* minimal medium containing hygromycin B (200 μ g/ml) for pCosHX-based constructs or phleomycin (30 μ g/ml) for pPKS33.

TLC analysis. Technical-grade tricyclazole (5-methyl-1,2,4-triazole[3,4-*b*]benzothiazole) was obtained from Eli Lilly Research Laboratories. Flaviolin and 2-HJ were made synthetically as previously described (60, 63). Scytalone was obtained from a *Brm-1* mutant strain of *Verticillium dahliae* (60).

For thin-layer chromatography (TLC) analysis, cultures were grown on ASA medium at 24°C. Inoculation of ASA medium was achieved by spreading 5×10^5 conidia in 0.25 ml of H_2O containing 0.05% Tween 80 over the agar plate. Eight-day-old cultures of strains B-5233 and B-5233/RGD12-8 (grown on ASA medium with various modifications) were examined for the presence of flaviolin, 2-HJ, scytalone, 1,3,6,8-THN, 1,3,8-THN, and DHN by ethyl acetate extraction and TLC procedures as previously described (62). In these studies, the crude extracts were spotted on TLC plates coated with silica gel G/HR (J. T. Baker Inc., Phillipsburg, N.J.) containing 2.5% zinc silicate phosphor (Sigma Chemical Co., St. Louis, Mo.), a fluorescent indicator. The TLC plates were developed in diethyl ether-hexanes (boiling point, 68 to 70°C)-formic acid (70:29:1, vol/vol/vol). Standards of naphthoquinones, flaviolin, and 2-HJ were yellow to orange

under visible light, and scytalone quenched 254-nm UV light and fluoresced yellow under 365-nm UV light. Flavivolin, 2-HJ, and scytalone also gave characteristic colors after the plates were sprayed with 1% FeCl₃. These three compounds also were identified by high-performance liquid chromatography as described previously (62).

The inhibitory effects of tricyclazole on the metabolism of scytalone by strains B-5233 and B-5233/RGD12-8 were visually compared by TLC. For comparison, 60- μ l samples of ethyl acetate extracts from the different cultures were spotted on TLC plates with a 50- μ l syringe (Hamilton Co., Reno, Nev.). Each 60- μ l sample was representative of material from 2.4 ml of ASA culture medium and was obtained from a larger, 2.5-ml ethyl acetate sample, obtained from five ASA culture plates, that collectively contained approximately 100 ml of medium and fungal material. The results were confirmed by additional repetitions.

SEM study. The scanning electron microscopy (SEM) studies were performed by Tim Mangel at the University of Maryland, College Park. Conidia from 7-day-old cultures were fixed in 2% glutaraldehyde for 60 min. One milliliter of the fixative solution was collected onto a 0.6- μ m-pore-size Nucleopore filter, and fixation was continued for an additional 60 min. The filters were subsequently washed three times in double-distilled water over a period of 10 min. Samples were then postfixated with 1% osmium tetroxide for 50 min and washed three times as described above. The filters were subsequently dehydrated, mounted on aluminum stubs, and coated with gold-palladium alloy. An Amray 1820D scanning electron microscope was operated at an accelerating potential of 25 kV to view the conidia.

Complement component C3 binding assay. Opsonization of resting conidia was performed as described previously (54). Briefly, conidia were harvested from 10-day-old cultures in phosphate-buffered saline (PBS), filtered through sterile cheesecloth to remove mycelial fragments, washed three times with PBS, and finally resuspended in Hanks' balanced salt solution (HBSS) with Ca²⁺ and Mg²⁺. Complement component C3 (Advanced Research Technologies, San Diego, Calif.) was labeled with ¹²⁵I to a specific activity of 200,000 cpm/ μ g. C3 binding assays were performed with 2 \times 10⁷ resting conidia and an opsonization system containing 5% nonimmune human serum, ¹²⁵I-labeled C3 (200,000 cpm), and HBSS with Ca²⁺ and Mg²⁺ (30 min, 37°C). Covalently bound ¹²⁵I-C3 was measured with a gamma counter (Packard Instrument Co., Downer's Grove, Ill.). Data were expressed as ¹²⁵I-C3 bound to 2 \times 10⁷ conidia. Heat-inactivated serum served as a negative control.

Phagocytosis assay. Phagocytosis was measured as described previously (58). Human neutrophils were purified by Ficoll-Hypaque density gradient centrifugation and mixed with resting conidia at an effector/target ratio of 1:1 (10⁶ neutrophils: 10⁶ conidia in 1 ml of HBSS with Ca²⁺ and Mg²⁺). Fresh nonimmune autologous serum served as a source of fresh complement (5% by volume). Incubation mixtures were tumbled for 30 min at 37°C; this was followed by cytospin preparation, Wright-Giemsa staining, and visual scoring of phagocytosis. The phagocytic index was defined as the mean number of intracellular conidia per 100 randomly visualized neutrophils. Neutrophil viability was consistently >95% as judged by trypan blue exclusion.

Animal study. Conidia were harvested from 10-day-old cultures grown on *Aspergillus* minimal medium at 37°C with PBS containing 0.1% Tween 20. Seven-week-old BALB/c mice were injected with 1.5 \times 10⁶ conidia in 0.2 ml of PBS containing 0.002% Tween 20 via the tail vein. Mouse mortality was monitored daily for 21 days.

Nucleotide sequence accession number. Sequence data reported in this paper have been submitted to the GenBank database under accession no. AF025541.

RESULTS

Cloning and analysis of the *alb1* gene from *A. fumigatus*.

Previous analysis of a reddish-pink (RP3) conidial-color mutant of *A. fumigatus* led to identification of a complementing cosmid, pG1-1 (54). pGA88, a subclone of pG1-1 carrying a 5-kb *AvrII* DNA fragment (Fig. 2A), transformed strain RP3 to produce white conidia at a high frequency (49 of 58 transformants). A Blast search revealed that sequences of this 5-kb DNA were highly similar to the sequences of the *wA* gene of *A. nidulans*, which encodes a putative polyketide synthase (29). This 5-kb DNA contains a 3'-truncated gene which we designated *alb1* (for albino). The complete *alb1* gene was cloned from cosmid pG1-1 (Fig. 2A, pPKS33) and sequenced. Several cDNA clones were obtained after screening a cDNA library of wild-type strain B-5233, using the 5-kb *AvrII* DNA fragment as a probe. Comparison of genomic and cDNA sequences revealed the presence of two introns at the 3'-end region of the *alb1* gene. Due to the large size (7 kb) of the *alb1* transcript, a full-length cDNA clone was never obtained. Comparison of the *alb1* genomic sequences with the *A. nidulans wA* cDNA sequence suggested the possible presence of two additional

introns at the 5'-end region of the *alb1* gene. The presence of these two introns at the 5' end was confirmed by RT-PCR and DNA sequencing (see Materials and Methods) (Fig. 2A). The sizes of the four introns ranged from 47 to 73 nucleotides. The DNA sequences of *alb1* and *wA* were well conserved downstream of the proposed ATG translation initiation codon (A as base 1) but not upstream of this codon. A stop codon was located 132 bp upstream of the proposed translation initiation codon. These observations render the ATG as the most likely initiation codon for *alb1*. The cDNA sequences of *alb1* suggest that there is a poly(A) addition site at base 6840. The *alb1* gene encodes a putative protein of 2,146 amino acids.

Northern blot analysis using the 5-kb *AvrII* DNA as a probe revealed that *alb1* encodes a 7-kb transcript. This 7-kb transcript was observed only during conidiation and not in the hyphal stage (Fig. 2B).

***alb1* encodes a putative polyketide synthase.** The dot plot matrix of the deduced Alb1 protein (Alb1p) and the putative polyketide synthase encoded by *wA* of *A. nidulans* showed a high degree of similarity existed throughout the complete open reading frame (Fig. 3A). Alb1p exhibits 66% identity and 79% similarity to the WA protein based on Gap analysis (9). Alb1p contains three motifs conserved among polyketide synthases: a β -ketoacyl synthase motif, an acetyltransferase motif, and an acyl carrier protein motif (Fig. 3B) (4, 6, 12, 29, 49, 67). Alb1p, however, lacks a β -ketoacyl reductase motif, which is conserved among fatty acid synthases in addition to the three previously described motifs. Collectively, that information suggests that Alb1p is a polyketide synthase rather than a fatty acid synthase (16, 18).

***alb1* is required for conidial pigmentation.** Gene disruption was carried out to delineate the role of *alb1* in conidial pigmentation of *A. fumigatus*. A gene disruption construct, pRGD12, was used to transform strain B-5233, and transformants were selected with hygromycin B. Transformants producing white conidia were observed at a frequency of 30% among 27 hygromycin B-resistant transformants. Southern analysis was used to determine whether the wild-type *alb1* copy was deleted from these white transformants. Genomic DNA of B-5233 and a white transformant, B-5233/RGD12-8, was hybridized with the 1.5-kb *MluI-AvrII* DNA fragment which was replaced with the *hph* gene in the disruption cassette. B-5233 gave a hybridizing signal of 5.0 kb, while B-5233/RGD12-8 did not reveal any hybridizing fragment (Fig. 4B, panel I). The blot was stripped and rehybridized with the entire disruption cassette, pRGD12. B-5233 gave two hybridizing signals, of 2.5 and 5.0 kb, while B-5233/RGD12-8 also showed two hybridizing fragments, of 2.9 and 5.4 kb (Fig. 4B, panel II). These results indicate that the wild-type copy of *alb1* in B-5233/RGD12-8 was replaced by the disrupted copy of *alb1* from pRGD12 via a double-crossover event. Thus, the albino phenotype of strain B-5233/RGD12-8 was the result of *alb1* disruption.

To confirm that the albino phenotype was not due to a secondary, hidden mutation, the albino strain, B-5233/RGD12-8, was transformed with pPKS33 (Fig. 2A) which lacks a portion of the 5' flanking region of *alb1*. In the resulting analysis, 8 of 27 phleomycin-resistant transformants were restored to bluish-green pigment synthesis. One of these transformants, RGD12-8/PKS33-3, was analyzed by Southern hybridization to confirm that pPKS33 had integrated into the *alb1* locus in B-5233/RGD12-8 and restored the functional copy of *alb1* (data not shown).

Involvement of *alb1* in the DHN-melanin pathway. Previously we reported that *A. fumigatus* uses a DHN-melanin pathway to synthesize its conidial pigments; thus, *alb1* is likely to be involved in DHN-melanin biosynthesis. Growth of strain

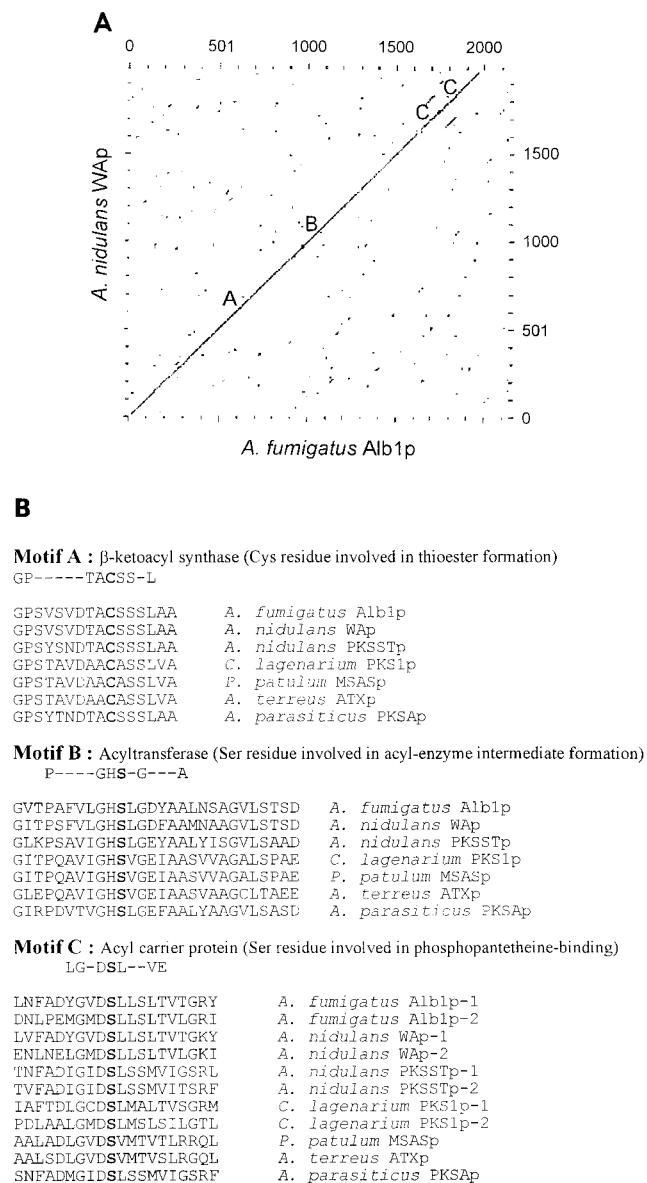


FIG. 3. Comparison of Alb1p and other polyketide synthases. (A) Dot plot matrix of Alb1p and the *A. nidulans* WAp (EMBL accession no. X65866) (29). The locations of the conserved active sites among polyketide synthases are labeled as follows: A, β -ketoacyl synthase; B, acyltransferase; and C, acyl carrier protein. Parameters used for the comparison were a window size of 10 and a stringency of 16. (B) Alignment of Alb1p with active sites of other polyketide synthases. GenBank accession numbers for the corresponding genes are as follows: *A. nidulans* pksST, L39121 (67); *Aspergillus terreus* atx, D85860 (12); *Aspergillus parasiticus* pksA, Z47198 (6); *C. lagenarium* pks1, D83643 (49); and *Penicillium patulum* msas, X55776 (4). Conserved active-site residues important for enzyme function are in boldfaced letters, and their functions are indicated.

B-5233 on ASA medium containing tricyclazole resulted in the accumulation of flaviolin, an autoxidative product of 1,3,6,8-THN, while controls grown without tricyclazole did not accumulate flaviolin (Fig. 5A). Accumulation of flaviolin is due to blockage of the first reduction step (conversion of 1,3,6,8-THN to scytalone [Fig. 1]). Simultaneous feeding of scytalone and tricyclazole to strain B-5233 led to accumulation of flaviolin and 2-HJ (Fig. 5B). 2-HJ is an autoxidative product derived from 1,3,8-THN, which is derived from scytalone (Fig. 1). Ac-

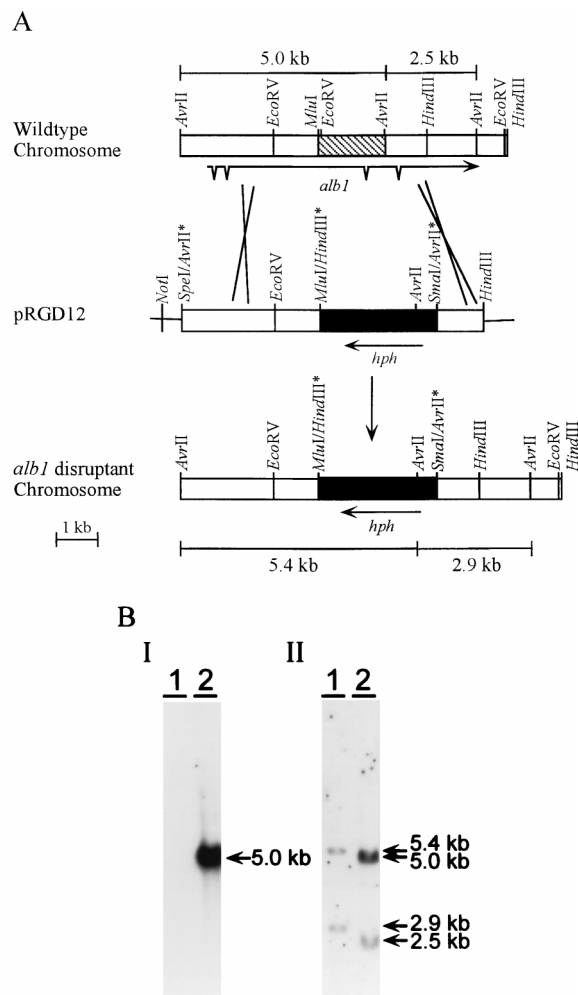


FIG. 4. Disruption of *alb1* in strain B-5233. (A) Diagram of gene replacement via double-crossover recombination. White boxes represent *A. fumigatus* DNA; black boxes represent *hph* genes. The *alb1* region deleted during construction of pRGD12 is shown as a hatched box. Arrows aligned with boxes represent transcripts, with spikes indicating introns. Asterisks indicate the destroyer/restriction enzyme sites. (B) Southern blot analysis. Total DNA from strain B-5233 (lane 1) or strain B-5233 (lane 2) was digested with *Avr*II and fractionated on a 0.8% agarose gel. The membrane was hybridized with the 1.5-kb *Mlu*I-*Avr*II fragment probe (hatched box, panel A) (I) or pRGD12 (II). The sizes of hybridizing signals are indicated by arrows.

cumulation of 2-HJ is due to blockage of the reduction step of 1,3,8-THN. In contrast, the *alb1* disruptant (B-5233/RGD12-8) showed no accumulation of flaviolin while growing on ASA medium containing tricyclazole. This indicates that the production of 1,3,6,8-THN was blocked and therefore no flaviolin was produced. Accumulation of 2-HJ was observed when the *alb1* disruptant was fed scytalone and tricyclazole simultaneously, indicating that the *alb1* disruptant retained the ability to convert scytalone to 1,3,8-THN. These results suggest that the *alb1* disruptant had lost the ability to produce 1,3,6,8-THN without changing the machinery involved in the downstream DHN-melanin pathway.

Disruption of *alb1* resulted in altered conidial surface morphology. Wild-type conidia are readily released from their conidiophores; conidia of cultures more than 5 days of age were often observed to be deposited on the inner side of the petri dish lids. In contrast, the *alb1* disruptant culture rarely showed

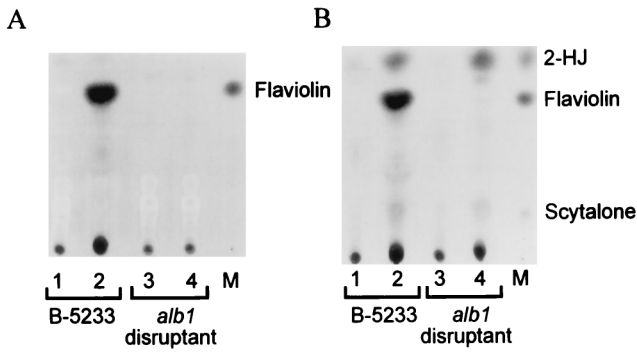


FIG. 5. TLC analysis of scytalone metabolism. *A. fumigatus* was grown on ASA medium without (A) or with (B) scytalone supplementation. The extract samples were obtained from a mixture of mycelia and agar medium by an ethyl acetate extraction procedure. Lanes: 1 and 3, extract samples from cultures without tricyclazole; 2 and 4, extract samples from cultures with tricyclazole; M, standards.

such a phenomenon, suggesting that disruption of *alb1* altered the conidial surface properties which affect conidial dispersibility. Since we suspected that one of the changed properties was conidial hydrophobicity, the wettability phenotype of conidia was tested with water or various concentrations of sodium dodecyl sulfate, Tween 20, or Tween 80 with or without EDTA (47). We did not observe any difference among the wild-type, *alb1* disruptant, and *alb1*-complemented strains. This suggests that conidial hydrophobicity was not affected by the disruption of *alb1*.

SEM studies, however, revealed a distinct change in the conidial surface morphology of the *alb1* disruptant. While conidia of the wild type exhibited echinulate surfaces, the *alb1* disruptant produced nearly smooth conidia (Fig. 6). Furthermore, the *alb1*-complemented strain, RGD12-8/PKS33-3, produced bluish-green conidia with the echinulate surface of wild-type conidia (Fig. 6). These findings indicate that mutation of *alb1* had altered not only conidial pigmentation but also conidial surface morphology.

Significant influence of *alb1* on fungal virulence. Two different sets of animal studies were carried out to compare the degrees of virulence of the wild-type, *alb1* disruptant, and

alb1-complemented strains. In these two experiments, mice injected with the *alb1* disruptant showed a dramatic reduction in mortality compared to the rates of the wild-type and the *alb1*-complemented strains. Lethality in mice infected with strain B-5233 began on days 9 and 10, with mortality rates of 70 and 80% by the end of experiment, day 21 (Fig. 7). In contrast, mice injected with the *alb1* disruptant showed only 0 and 10% mortality at day 21. However, the *alb1*-complemented strain recovered full virulence; mortality started on day 7 and reached 80 to 100% by the termination date. Statistical analysis showed a significantly reduced lethality for the *alb1* disruptant compared to that for B-5233 or the *alb1*-complemented strain ($P < 0.05$, Kaplan-Meier analysis).

***alb1* modulates C3 deposition on conidia.** Our previous study showed that conidial pigmentation affects the deposition of complement component C3 on *A. fumigatus* conidia (54). Since disruption of *alb1* resulted in an albino conidial phenotype, C3 binding assays were performed to delineate the effect of *alb1* on C3 binding. The level of quantitative binding of complement component C3 to bluish-green conidia, produced by the wild-type and the *alb1*-complemented strains, was significantly lower than that to conidia of the *alb1* disruptant (Fig. 8). These results suggest that, similar to the *arp1* gene, *alb1* modulates C3 deposition on conidia.

***alb1* affects neutrophil-mediated phagocytosis.** Results for neutrophil-mediated phagocytosis of resting conidia paralleled those for quantitative C3 deposition. As shown in Fig. 9, quantitative phagocytosis for the *alb1* disruptant was significantly greater than that for the wild-type strain and the *alb1*-complemented strain. Neutrophils that were incubated with conidia of the *alb1* disruptant became aggregated, suggesting activation. In contrast, neutrophils incubated with the wild-type or *alb1*-complemented strain remained individually suspended (data not shown). These results suggest that enhanced phagocytosis is an important mechanism accounting for the reduced virulence of the *alb1* disruptant.

DISCUSSION

Only 16 of over 130 known *Aspergillus* spp. have been reported from clinical cases of infection in humans (25). Among these aspergilli, *A. fumigatus* is the leading cause of both non-invasive and invasive aspergillosis. This fungus is responsible

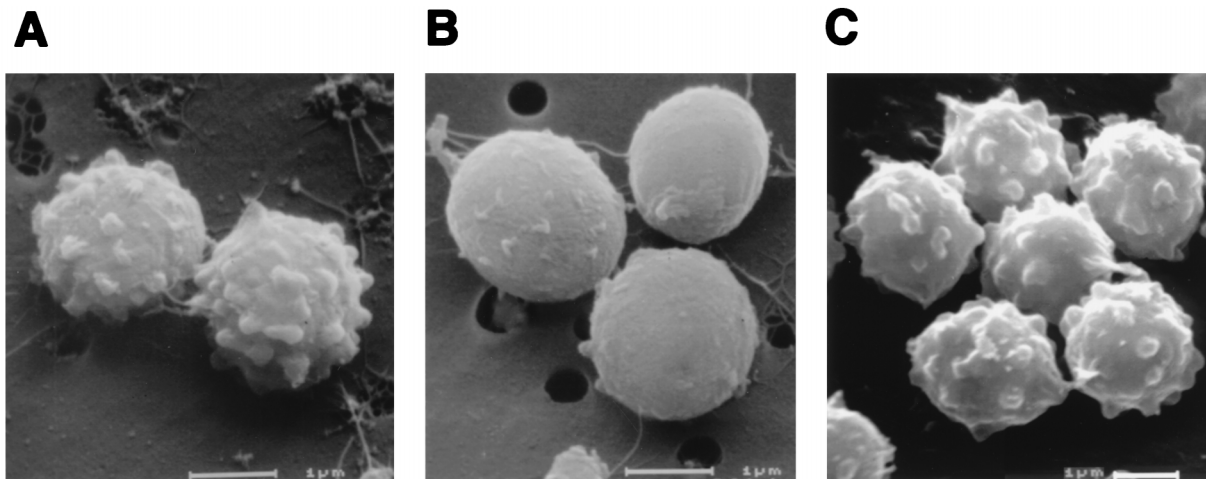


FIG. 6. SEM study of conidial surface structure. Conidia were from 7-day-old cultures. (A) B-5233, the wild-type strain; (B) B-5233/RGD12-8, the *alb1* gene disruptant; (C) RGD12-8/PKS33-3, the *alb1*-complemented strain. Bars, 1 µm.

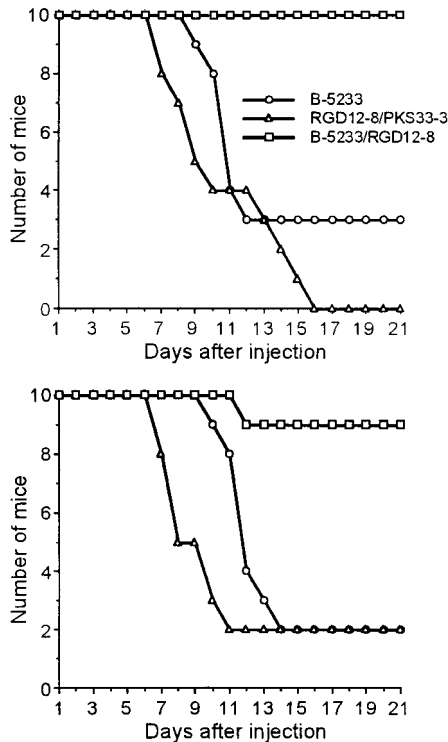


FIG. 7. Virulence studies. Each study included 10 mice per strain in two independent experiments. Mice were injected intravenously with 1.5×10^5 conidia. Mouse mortality was monitored daily for 21 days. Strains: B-5233, the wild-type strain; B-5233/RGD12-8, the *alb1* disruptant; RGD12-8/PKS33-3, the *alb1*-complemented strain. $P < 0.05$ for comparison of the *alb1* disruptant to B-5233 or the *alb1*-complemented strain (Kaplan-Meier analysis).

for more than 60% of invasive aspergillosis cases in immunosuppressed patients. Identification of the determinants rendering *A. fumigatus* more virulent than the other aspergilli has been a research interest. Recently, several putative virulence factors have been analyzed using molecular genetic tools. These factors included alkaline proteases, elastase, catalase, chitin synthase, hydrophobin, cytotoxin ASPFI, and restrictocin. However, disruption of those genes individually or in var-

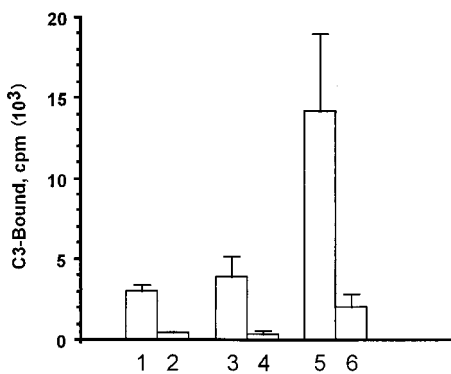


FIG. 8. Complement component C3 binding analysis. Strains are as follows: lanes 1 and 2, wild-type strain B-5233; lanes 3 and 4, the *alb1*-complemented strain, RGD12-8/PKS33-3; and lanes 5 and 6, the *alb1* disruptant strain, B-5233/RGD12-8. Lanes 1, 3, and 5 were with 5% fresh serum, while lanes 2, 4, and 6 were with heat-inactivated serum. Data represent means \pm standard deviations ($n = 4$). $P < 0.05$ by Student's *t* test for comparison of the *alb1* disruptant to strain B-5233 or the *alb1*-complemented strain.

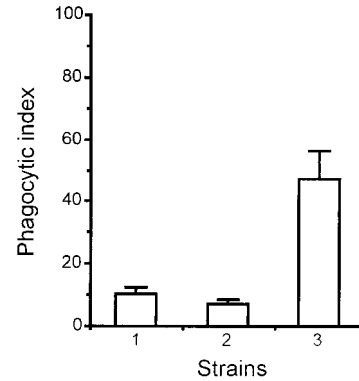


FIG. 9. Phagocytosis assay. Strains are as follows: lane 1, wild-type strain B-5233; lane 2, the *alb1*-complemented strain, RGD12-8/PKS33-3; and lane 3, the *alb1* disruptant strain, B-5233/RGD12-8. Data represent phagocytic index means \pm standard deviations for three experiments performed in duplicate. $P < 0.05$ for comparison of the *alb1* disruptant to strain B-5233 or the *alb1*-complemented strain (Student's *t* test).

ious combinations in *A. fumigatus* failed to yield significant changes in fungal virulence (3, 31, 35, 40, 45, 46, 50, 51).

A. fumigatus propagates by conidia, which are encountered by the human host chiefly through inhalation. Inhaled conidia face challenges from the host immune system, and evasion of these challenges is a pivotal step in establishing an invasive infection. In this context, conidial surface components are likely to play important roles because of their initial contact with host defenses. We have focused our attention on molecular aspects of conidial pigment biosynthesis since the pigments are among the visible components of the conidial wall that protects the conidium.

The *alb1* gene is developmentally regulated, being expressed only during sporulation. The gene is not essential in *A. fumigatus*, since disruption of the gene did not cause any visible change in fungal growth in vitro. The pleiotropic effects on conidial morphology caused by disruption of *alb1* underscore its importance for *A. fumigatus*. The *alb1*-disrupted strain exhibited an albino conidial phenotype and did not accumulate flavin, a hallmark shunt product of the DHN-melanin pathway. These results support our previous hypothesis that *A. fumigatus* synthesizes its conidial pigments via a DHN-melanin pathway, based on the observations that *arp1* encodes a scytalone dehydratase and that conidial pigmentation is sensitive to tricyclazole (54). Although *alb1* is apparently a homolog of the *wA* gene of *A. nidulans*, we did not detect any *arp1* homolog in *A. nidulans* even under low-stringency hybridization conditions (53a). In addition, tricyclazole did not inhibit conidial pigmentation of *A. nidulans* (62). Together, these observations suggest that the polyketide pathways for conidial pigmentation in *A. fumigatus* and *A. nidulans* may share the upstream steps but diverge at the downstream steps.

One of the phenotypes caused by disruption of the *alb1* gene was the loss of echinulation on the conidial surface. Changes in conidial surface structure may have been secondary to the defect in conidial pigment biosynthesis, because conidial pigment is one of the main conidial surface components. Whether the pigment is independently formed and deposited on the outer surface or is codeposited with other molecules remains to be elucidated. If the protrusions formed on conidial surfaces were solely composed of pigments, the loss of the pigment layer could produce smooth conidial surfaces. Alternatively, if pigments are codeposited with other molecules (e.g., proteins

or polysaccharides), pigment deposition may serve a crucial function for the formation of the echinulate surface.

The most intriguing phenotype of the *alb1* disruptant was its significant loss of virulence. Reduced virulence in the murine model was also observed with a white conidial mutant obtained by UV mutagenesis (19). This genetically uncharacterized mutant also exhibited smooth conidial surfaces. It is likely that the reduced virulence in the present study was due to altered conidial pigmentation and surface morphology induced by the *alb1* disruption. In the plant pathogenic fungi *C. lagenarium* and *M. grisea*, deficient DHN-melanin biosynthesis resulted in nonmelanized appressoria (24, 28, 37). Those mutants lost their ability to penetrate plant leaf tissue and became avirulent, possibly due to the fact that melanin is important for the rigidity of appressoria. These reports indicate an association between melanin biosynthesis and both cell wall rigidity and virulence. Similarly, melanin biosynthesis in *A. fumigatus* may be associated with conidial surface characteristics that protect these structures from environmental and immunological challenges, including C3 deposition and oxidative killing (19).

Disruption of *alb1* not only altered conidial morphology but also led to efficient C3 binding on conidial surfaces. It is tempting to speculate that the alteration of the conidial surface topography is the major factor leading to enhanced C3 binding. Previous observations about complement activation by *A. fumigatus* include inefficient C3 deposition on resting conidia compared to that of swollen conidia (22) and identification of a 54- to 58-kDa C3 binding protein (48). Deposition of pigments on conidial surfaces may mask the C3 binding sites and reduce the C3 binding capacity, while loss of conidial pigments due to swelling or *alb1* disruption may result in elevated C3 binding due to exposure of more binding sites. Alternatively, extracellular C3 binding inhibitors derived from the DHN-melanin pathway may directly downregulate C3 deposition on wild-type conidia. In fact, extracellular compounds which inhibit complement activation have been isolated from *A. fumigatus* and found to possess properties consistent with phenolic compounds (57, 59). Since the DHN-melanin pathway produces various phenolic compounds, it is possible that some of these compounds interfere with complement activation. The absence of these phenolic compounds due to defects in DHN-melanin biosynthesis may enhance C3 deposition on conidial surfaces. Finally, Alb1p may be involved in spore wall assembly, raising the possibility that *alb1* disruption produces structural changes in conidial walls that enhance the surface exposure of C3 binding sites.

The present studies showed that the bluish-green pigmentation of wild-type conidia contributes to virulence in an animal model and modulates human complement-mediated opsonization and neutrophil-mediated phagocytosis. Thus, bluish-green pigmentation at least partially thwarts the complement-phagocyte axis of the mammalian host defense against invasive aspergillosis. To our knowledge, this represents the first demonstration of such an effect for *A. fumigatus* at the molecular genetic level. Of interest, these findings fit into a pattern of pigment-mediated virulence that has been emerging in recent literature for both mammalian and plant fungal pathogens, including *C. neoformans* and dematiaceous molds such as *M. grisea* and *C. lagenarium* (11, 17, 19, 38, 55). Several different virulence mechanisms have been proposed for those fungal pigments, but to our knowledge the present study provides the first definitive evidence that fungal pigmentation is a factor interfering with complement-mediated opsonization and phagocytosis. Thus, the bluish-green conidial pigmentation of *A. fumigatus* provides a novel mechanism of escape from mammalian defenses.

In summary, *alb1* is an important virulence determinant for the human pathogenic fungus *A. fumigatus*. This novel discovery offers an impetus to further explore interactions between fungal pathogens and the human host. Since the *alb1* gene plays an important role in modulating conidial morphology and fungal virulence in vivo, a strong association between conidial morphology and fungal virulence is apparent. Further investigation into the interactions between conidia and the host immune system will now lead to a better understanding of the mechanisms involved in fungal pathogenesis.

ACKNOWLEDGMENTS

We thank Herman Edskes for critical reviews and helpful suggestions, Tim Maugel for assistance in the SEM study, Lisa Penoyer for assistance in DNA sequencing, Amy Rye and Sandeep Kumar for technical assistance, and P. Silar for providing plasmids pBC-hygro and pBC-phleo.

R.G.W. was supported by NIH grant AI-01036 and Pfizer, U.S. Pharmaceuticals.

REFERENCES

1. Agrios, G. N. 1997. Plant pathology, 4th ed. Academic Press, London, United Kingdom.
2. Aramayo, R., and W. E. Timberlake. 1990. Sequence and molecular structure of the *Aspergillus nidulans* *yA* (laccase I) gene. *Nucleic Acids Res.* **18**:3415.
3. Aufauvre-Brown, A., E. Mellado, N. A. R. Gow, and D. W. Holdren. 1997. *Aspergillus fumigatus* *chsE*: a gene related to *CHS3* of *Saccharomyces cerevisiae* and important for hyphal growth and conidiophore development but not pathogenicity. *Fungal Genet. Biol.* **21**:141–152.
4. Beck, J., S. Ripka, A. Siegner, E. Schiltz, and E. Schweizer. 1990. The multifunctional 6-methylsalicylic acid synthase gene of *Penicillium patulum*—its gene structure relative to that of other polyketide synthases. *Eur. J. Biochem.* **192**:487–498.
5. Bell, A. A., and M. H. Wheeler. 1986. Biosynthesis and functions of fungal melanins. *Annu. Rev. Phytopathol.* **24**:411–451.
6. Chang, P.-K., J. W. Cary, J. Yu, D. Bhatnagar, and T. E. Cleveland. 1995. The *Aspergillus parasiticus* polyketide synthase gene *pksA*, a homolog of *Aspergillus nidulans* *wA*, is required for aflatoxin B₁ biosynthesis. *Mol. Gen. Genet.* **248**:270–277.
7. Cohen, M. S., R. E. Isturiz, H. L. Malech, R. K. Root, C. M. Wilfert, L. Gutman, and R. H. Buckley. 1981. Fungal infection in chronic granulomatous disease: the importance of the phagocyte in defense against fungi. *Am. J. Med.* **71**:59–66.
8. Denning, D. W. 1996. Therapeutic outcome in invasive aspergillosis. *Clin. Infect. Dis.* **23**:608–615.
9. Devereux, J., P. Haeblerli, and O. Smithies. 1984. A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res.* **12**:387–395.
10. Dixon, D. M., A. Polak, and P. J. Szanislo. 1987. Pathogenicity and virulence of wild-type and melanin-deficient *Wangiella dermatitidis*. *J. Med. Vet. Mycol.* **25**:97–106.
11. Emery, H. S., C. P. Shelburne, J. P. Bowman, P. G. Fallon, C. A. Schulz, and E. S. Jacobson. 1994. Genetic study of oxygen resistance and melanization in *Cryptococcus neoformans*. *Infect. Immun.* **62**:5694–5697.
12. Fujii, I., Y. Ono, H. Tada, K. Gomi, Y. Ebizuka, and U. Sankawa. 1996. Cloning of the polyketide synthase gene *atX* from *Aspergillus terreus* and its identification as the 6-methylsalicylic acid synthase gene by heterologous expression. *Mol. Gen. Genet.* **253**:1–10.
13. Gerson, S. T., G. H. Talbot, S. Hurwitz, B. L. Strom, E. J. Lusk, and P. A. Cassileth. 1984. Prolonged granulocytopenia: the major risk factor for invasive pulmonary aspergillosis in patients with acute leukemia. *Ann. Intern. Med.* **100**:345–351.
14. Gustafson, T. L., W. Schaffner, G. B. Lavelly, C. W. Stratton, H. K. Johnson, and R. H. Hutcheson, Jr. 1983. Invasive aspergillosis in renal transplant recipients: correlation with corticosteroid therapy. *J. Infect. Dis.* **148**:230–238.
15. Hopwood, D. A., and C. Khosla. 1992. Genes for polyketide secondary metabolic pathways in microorganisms and plants. *Ciba Found. Symp.* **171**: 88–112.
16. Hopwood, D. A., and D. H. Sherman. 1990. Molecular genetics of polyketides and its comparison to fatty acid biosynthesis. *Annu. Rev. Genet.* **24**:37–66.
17. Huffnagle, G. B., G.-H. Chen, J. L. Curtis, R. A. McDonald, R. M. Strieter, and G. B. Toews. 1995. Down-regulation of the afferent phase of T cell-mediated pulmonary inflammation and immunity by a high melanin-producing strain of *Cryptococcus neoformans*. *J. Immunol.* **155**:3507–3516.
18. Hutchinson, C. R., and I. Fujii. 1995. Polyketide synthase gene manipulation: a structure-function approach in engineering novel antibiotics. *Annu. Rev. Microbiol.* **49**:201–238.

19. Jahn, B., A. Koch, A. Schmidt, G. Wanner, H. Gehringer, S. Bhakdi, and A. A. Brakhage. 1997. Isolation and characterization of a pigmentless-conidium mutant of *Aspergillus fumigatus* with altered conidial surface and reduced virulence. *Infect. Immun.* **65**:5110–5117.
20. Kaefer, E. 1977. Meiotic and mitotic recombination in *Aspergillus* and its chromosomal aberrations. *Adv. Genet.* **19**:33–131.
21. Keller, N. P., and T. M. Hohn. 1997. Metabolic pathway gene clusters in filamentous fungi. *Fungal Genet. Biol.* **21**:17–29.
22. Kozel, T. R., M. A. Wilson, T. P. Farrell, and S. M. Levitz. 1989. Activation of C3 and binding to *Aspergillus fumigatus* conidia and hyphae. *Infect. Immun.* **57**:3412–3417.
23. Kubo, Y., Y. Takano, N. Endo, N. Yasuda, S. Tajima, and I. Furusawa. 1996. Cloning and structural analysis of the melanin biosynthesis gene *SCD1* encoding scytalone dehydratase in *Colletotrichum lagenarium*. *Appl. Environ. Microbiol.* **62**:4340–4344.
24. Kubo, Y., Y. Takano, and I. Furusawa. 1996. Molecular genetic analysis of melanin biosynthetic genes essential for appressorium function in *Colletotrichum lagenarium*, p. 73–82. *In* D. Mills, H. Kunoh, N. Keen, and S. Mayama (ed.), *Molecular aspects of pathogenicity: requirements for signal transduction*. APS Press, St. Paul, Minn.
25. Kwon-Chung, K. J., and J. E. Bennett. 1992. Aspergillosis, p. 201–247. *In* *Medical mycology*. Williams & Wilkins, Baltimore, Md.
26. Kwon-Chung, K. J., I. Polacheck, and T. J. Popkin. 1982. Melanin-lacking mutants of *Cryptococcus neoformans* and their virulence for mice. *J. Bacteriol.* **150**:1414–1421.
27. Kwon-Chung, K. J., and J. C. Rhodes. 1986. Encapsulation and melanin formation as indicators of virulence in *Cryptococcus neoformans*. *Infect. Immun.* **51**:218–223.
28. Lundqvist, T., J. Rice, C. N. Hodge, G. S. Basarab, J. Pierce, and Y. Lundqvist. 1994. Crystal structure of scytalone dehydratase—the disease determinant of a rice pathogen, *Magnaporthe grisea*. *Structure* **15**:937–944.
29. Mayorga, M. E., and W. E. Timberlake. 1992. The developmentally regulated *Aspergillus nidulans* *wA* gene encodes a polypeptide homologous to polyketide and fatty acid synthases. *Mol. Gen. Genet.* **235**:205–212.
30. Mayorga, M. E., and W. E. Timberlake. 1990. Isolation and molecular characterization of the *Aspergillus nidulans* *wA* gene. *Genetics* **126**:73–79.
31. Monod, M., S. Paris, J. Sarfati, K. Jatton-Ogay, P. Ave, and J.-P. Latgé. 1993. Virulence of alkaline protease-deficient mutants of *Aspergillus fumigatus*. *FEMS Microbiol. Lett.* **106**:39–46.
32. Mullins, J., R. Harvey, and A. Seaton. 1976. Sources and incidences of airborne *Aspergillus fumigatus*. *Clin. Allergy* **6**:209–217.
33. O'Hagan, D. 1992. Biosynthesis of polyketide metabolites. *Nat. Prod. Rep.* **9**:447–479.
34. O'Hara, E. B., and W. E. Timberlake. 1989. Molecular characterization of the *Aspergillus nidulans* *yA* locus. *Genetics* **121**:249–254. (Erratum, **124**:791, 1990.)
35. Paris, S., M. Monod, M. Diaquin, B. Lamy, L. K. Arruda, P. J. Punt, and J.-P. Latgé. 1993. A transformant of *Aspergillus fumigatus* deficient in the antigenic cytotoxin ASPFI. *FEMS Microbiol. Lett.* **111**:31–36.
36. Parta, M., Y. Chang, S. Rulong, P. Pinto-DaSilva, and K. J. Kwon-Chung. 1994. *HYP1*, a hydrophobin gene from *Aspergillus fumigatus*, complements the *rodletless* phenotype in *Aspergillus nidulans*. *Infect. Immun.* **62**:4389–4395.
37. Perpetua, N. S., Y. Kubo, N. Yasuda, Y. Takano, and I. Furusawa. 1996. Cloning and characterization of a melanin biosynthetic *THR1* reductase gene essential for appressorial penetration of *Colletotrichum lagenarium*. *Mol. Plant-Microbe Interact.* **9**:323–329.
38. Polacheck, I., Y. Platt, and J. Aronovitch. 1990. Catecholamines and virulence of *Cryptococcus neoformans*. *Infect. Immun.* **58**:2919–2922.
39. Punt, P. J., R. P. Oliver, M. A. Dingemase, P. H. Pouwel, and C. A. van den Hondel. 1987. Transformation of *Aspergillus* based on the hygromycin B resistance marker from *Escherichia coli*. *Gene* **56**:117–124.
40. Ramesh, M. V., T. Sirakova, and P. E. Kolattukudy. 1994. Isolation, characterization, and cloning of cDNA and the gene for an elastinolytic serine proteinase from *Aspergillus flavus*. *Infect. Immun.* **62**:79–85.
41. Raper, K. B., and D. I. Fennell. 1965. *Aspergillus fumigatus* group, p. 238–268. *In* K. B. Raper and D. I. Fennell (ed.), *The genus Aspergillus*. Williams & Wilkins, Baltimore, Md.
42. Salas, S. D., J. E. Bennett, K. J. Kwon-Chung, J. R. Perfect, and P. R. Williamson. 1996. Effect of the laccase gene, *CNLAC1*, on virulence of *Cryptococcus neoformans*. *J. Exp. Med.* **184**:377–386.
43. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
44. Silar, P. 1995. Two new easy to use vectors for transformation. *Fungal Genet. Newsletter* **42**:73.
45. Smith, J. M., J. E. Davies, and D. W. Holden. 1993. Construction and pathogenicity of *Aspergillus fumigatus* mutants that do not produce the ribotoxin restrictocin. *Mol. Microbiol.* **9**:1071–1077.
46. Smith, J. M., C. M. Tang, S. Van Noorden, and D. W. Holden. 1994. Virulence of *Aspergillus fumigatus* double mutants lacking restrictocin and an alkaline protease in a low-dose model of invasive pulmonary aspergillosis. *Infect. Immun.* **62**:5247–5254.
47. Stringer, M. A., and W. E. Timberlake. 1995. *dewaA* encodes a fungal hydrophobin component of the *Aspergillus* spore wall. *Mol. Microbiol.* **16**:33–44.
48. Sturtevant, J. E., and J.-P. Latgé. 1992. Interactions between conidia of *Aspergillus fumigatus* and human complement component C3. *Infect. Immun.* **60**:1913–1918.
49. Takano, Y., Y. Kubo, K. Shimizu, K. Mise, T. Okuno, and I. Furusawa. 1995. Structural analysis of *PKS1*, a polyketide synthase gene involved in melanin biosynthesis in *Colletotrichum lagenarium*. *Mol. Gen. Genet.* **249**:162–167.
50. Tang, C. M., J. Cohen, T. Krausz, S. Van Noorden, and D. W. Holden. 1993. The alkaline protease of *Aspergillus fumigatus* is not a virulence determinant in two murine models of invasive pulmonary aspergillosis. *Infect. Immun.* **61**:1650–1656.
51. Thau, N., M. Monod, B. Crestani, C. Rolland, G. Tronchin, J.-P. Latgé, and S. Paris. 1994. *rodletless* mutants of *Aspergillus fumigatus*. *Infect. Immun.* **62**:4380–4388.
52. Timberlake, W. E. 1986. Molecular genetics of *Aspergillus* development. *Annu. Rev. Genet.* **24**:5–36.
53. Tokousbalides, M. C., and H. D. Sisler. 1979. Site of inhibition by tricyclazole in the melanin biosynthetic pathway of *Verticillium dahliae*. *Pestic. Biochem. Physiol.* **11**:64–73.
- 53a. Tsai, H.-F. Unpublished data.
54. Tsai, H.-F., R. G. Washburn, Y. C. Chang, and K. J. Kwon-Chung. 1997. *Aspergillus fumigatus* *arp1* modulates conidial pigmentation and complement deposition. *Mol. Microbiol.* **26**:175–183.
55. Wang, Y., P. Aisen, and A. Casadevall. 1995. *Cryptococcus neoformans* melanin and virulence: mechanism of action. *Infect. Immun.* **63**:3131–3136.
56. Washburn, R. G. 1996. Opportunistic mold infections, p. 147–158. *In* L. Esser and P. A. Lemke (ed.), *The Mycota*, vol. VI. Springer-Verlag, Heidelberg, Germany.
57. Washburn, R. G., D. J. DeHart, D. E. Agwu, B. J. Bryant-Varela, and N. C. Julian. 1990. *Aspergillus fumigatus* complement inhibitor: production, characterization, and purification by hydrophobic interaction and thin-layer chromatography. *Infect. Immun.* **58**:3508–3515.
58. Washburn, R. G., J. I. Gallin, and J. E. Bennett. 1987. Oxidative killing of *Aspergillus fumigatus* proceeds by parallel myeloperoxidase-dependent and -independent pathways. *Infect. Immun.* **55**:2088–2092.
59. Washburn, R. G., C. H. Hammer, and J. E. Bennett. 1986. Inhibition of complement by culture supernatants of *Aspergillus fumigatus*. *J. Infect. Dis.* **154**:944–951.
60. Wheeler, M. H. 1982. Melanin biosynthesis in *Verticillium dahliae*: dehydration and reduction reactions in cell-free homogenates. *Exp. Mycol.* **6**:171–179.
61. Wheeler, M. H., and A. A. Bell. 1988. Melanins and their importance in pathogenic fungi, p. 338–387. *In* M. R. McGinnis (ed.), *Current topics in medical mycology*, vol. 2. Springer-Verlag, New York, N.Y.
62. Wheeler, M. H., and M. A. Klich. 1995. The effects of tricyclazole, pyroquilon, phthalide, and related fungicides on the production of conidial wall pigments by *Penicillium* and *Aspergillus* species. *Pestic. Biochem. Physiol.* **52**:125–136.
63. Wheeler, M. H., and R. D. Stipanovic. 1985. Melanin biosynthesis and the metabolism of flaviolin and 2-hydroxyjuglone in *Wangiella dermatitidis*. *Arch. Microbiol.* **142**:234–241.
64. Wheeler, M. H., and R. D. Stipanovic. 1979. Melanin biosynthesis in *Thielaviopsis basicola*. *Exp. Mycol.* **3**:340–350.
65. Yang, G., M. S. Rose, B. G. Turgeon, and O. C. Yoder. 1996. A polyketide synthase is required for fungal virulence and production of the polyketide T-toxin. *Plant Cell* **8**:2139–2150.
66. Yelton, M. M., J. E. Hamer, and W. E. Timberlake. 1984. Transformation of *Aspergillus nidulans* by using a *trpC* plasmid. *Proc. Natl. Acad. Sci. USA* **81**:1470–1474.
67. Yu, J.-H., and T. J. Leonard. 1995. Sterigmatocystin biosynthesis in *Aspergillus nidulans* requires a novel type I polyketide synthase. *J. Bacteriol.* **177**:4792–4800.