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

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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 dermataxis (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. Overall, reduction and dehydration 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 wA, 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...
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, a bluish-green conidial form, and A. fumigatus B-5233/RGD12-8 is an alb1 disruptant which produces white conidia. RGD12-8/pPKS33-3, obtained by transforming B-5233/RGD12-8 with pPKS33, produces bluish-green conidia. Aspergillus minimal medium contains 1% glucose, 10 mM NaNO₃, and trace elements (20). Malt extract medium contains 1% glucose, 2% malt extract, and 0.1% peptone. Cultures were grown at 37°C. Asparaginase-succrose 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 flavinol and 2-hydroxyjuglone (2-HJ) production. Scytalone and tricyclazole (Eli Lilly Research Laboratories, Greenfield, Ind.) and an ABI automatic DNA sequencing system (Perkin-Elmer, Norwalk, Conn.) were used for blot analysis. DNA probes were formed according to standard protocols (43). A Hybond-N nylon membrane (Amersham, Arlington Heights, Ill.) was used for Southern blot analyses. DNA cloning and Southern blot analyses were performed as previously described (52). Cultures were harvested 14 h after incubation at 37°C. Cultures were grown on ASA medium with various modifications) were examined for the presence of flavinol, scytalone, 1,3,6,8-THN, 1,3,8-THN, and DHN by ethyl acetate extraction and TLC analysis. Cultures were grown on ASA medium with various modifications) were examined for the presence of flavinol, scytalone, 1,3,6,8-THN, 1,3,8-THN, and DHN by ethyl acetate extraction and TLC analysis. Cultures were grown on ASA medium with various modifications) were examined for the presence of flavinol, scytalone, 1,3,6,8-THN, 1,3,8-THN, and DHN by ethyl acetate extraction and TLC analysis.

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 (biphasic 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 (US Biochemical, Cleveland, Ohio) and an ABI automatic DNA sequencing system (Perkin-Elmer, Carlsbad, Calif.) and sequenced with primers DW16N and DW34 (5'). (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% agarose gel. The 5.5 kb AvrII DNA fragment was used as a probe. The size of the hybridizing signal is indicated by an arrow.

Transformation of A. fumigatus. A. fumigatus protoplasts were prepared with murinease (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 μg/ml) for pCosHX-based constructs or phleomycin (30 μg/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. Flaviloin and 2-HJ were made synthetically as previously described (60, 63). Scytalone was obtained from a Bem-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 x 10⁵ conidia in 0.25 ml of H₂O 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 flaviloin, 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/HFR (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, flaviloin, and 2-HJ were yellow to orange.
under visible light, and secaltochrome quenched 254-nm UV light and fluoresced yellow under 365-nm UV light. Flavilin, 2-HJ, and secaltochrome 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 secaltochrome by strains B-5233 and B-5233/RGD12-8 were visually compared by TLC. For comparison, 60-μl samples of ethyl acetate extracts from the different cultures were spotted on thin-layer plates with Kieselgel G (E. Merck, Darmstadt, Germany). Each 60-μl sample was representative of material from 2.4 ml of secaltochrome culture medium and was obtained from a 2.5-ml ethyl acetate sample, obtained from five secaltochrome 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 Maugel 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-μm-pore-size Nuclepore 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 postfixed 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⁺. Concomitant component C3 (Advanced Research Technologies, San Diego, Calif.) was labeled with 125I to a specific activity of 200,000 cpm/mg. C3 binding assays were performed with 2 × 10⁶ 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 × 10⁶ 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, which 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 × 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). pG1A8, 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 *p*AvrII 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 Alb1p 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 *Mini-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/PSKS3-3, was analyzed by Southern hybridization to confirm that pPKS33 had integrated into the *alb1* locus of genomic 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
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). Accumulation 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 accumulation 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.

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such a phenomenon, suggesting that disruption of \textit{alb1} altered the conidial surface properties which affect conidial dispersibility. Since we suspected that one of the changed properties was conidial hydrophobicity, the wettable 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, \textit{alb1} disruptant, and \textit{alb1}-complemented strains. This suggests that conidial hydrophobicity was not affected by the disruption of \textit{alb1}.

SEM studies, however, revealed a distinct change in the conidial surface morphology of the \textit{alb1} disruptant. While conidia of the wild type exhibited echinulate surfaces, the \textit{alb1} disruptant produced nearly smooth conidia (Fig. 6). Furthermore, the \textit{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 \textit{alb1} had altered not only conidial pigmentation but also conidial surface morphology.

**Significant influence of \textit{alb1} on fungal virulence.** Two different sets of animal studies were carried out to compare the degrees of virulence of the wild-type, \textit{alb1} disruptant, and \textit{alb1}-complemented strains. In these two experiments, mice injected with the \textit{alb1} disruptant showed a dramatic reduction in mortality compared to the rates of the wild-type and the \textit{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 \textit{alb1} disruptant showed only 0 and 10% mortality at day 21. However, the \textit{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 \textit{alb1} disruptant compared to that for B-5233 or the \textit{alb1}-complemented strain ($P < 0.05$, Kaplan-Meier analysis).

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

**\textit{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 \textit{alb1} disruptant was significantly greater than that for the wild-type strain and the \textit{alb1}-complemented strain. Neutrophils that were incubated with conidia of the \textit{alb1} disruptant became aggregated, suggesting activation. In contrast, neutrophils incubated with the wild-type or \textit{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 \textit{alb1} disruptant.

**DISCUSSION**

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

FIG. 7. Virulence studies. Each study included 10 mice per strain in two independent experiments. Mice were injected intravenously with \( 1.5 \times 10^5 \) 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).

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 mean \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).
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. lagenum 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. 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. lagenum (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.

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