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Bayry, J. ; Beaussart, Audrey ; Dufrêne, Yves ; Sharma, M. ; Bansal, K. ; Kniemeyer, O. ; Amanianda, V. ; Brakhage, A.A. ; Kaveri, S.V. ; Kwon-Chung, K.J. ; Latgé, J.-P. ; Beauvais, A.

Abstract
In Aspergillus fumigatus, the conidial surface contains dihydroxynaphthalene (DHN)-melanin. Six-clustered gene products have been identified that mediate sequential catalysis of DHN-melanin biosynthesis. Melanin thus produced is known to be a virulence factor, protecting the fungus from the host defense mechanisms. In the present study, individual deletion of the genes involved in the initial three steps of melanin biosynthesis resulted in an altered conidial surface with masked surface rodlet layer, leaky cell wall allowing the deposition of proteins on the cell surface and exposing the otherwise-masked cell wall polysaccharides at the surface. Melanin as such was immunologically inert; however, deletion mutant conidia with modified surfaces could activate human dendritic cells and the subsequent cytokine production in contrast to the wild-type conidia. Cell surface defects were rectified in the conidia mutated in downstream melanin biosynthetic pathway, and maximum immune inertness...

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Surface Structure Characterization of Aspergillus fumigatus Conidia Mutated in the Melanin Synthesis Pathway and Their Human Cellular Immune Response

Jagadeesh Bayry, Audrey Beaussart, Yves F. Dufrène, Meenu Sharma, Kushagra Bansal, Olaf Kniemeyer, Vishukumar Amanianda, Axel A. Brakhage, Srini V. Kaveri, Kyung J. Kwon-Chung, Jean-Paul Latgé and Anne Beauvais


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Surface Structure Characterization of Aspergillus fumigatus Conidia Mutated in the Melanin Synthesis Pathway and Their Human Cellular Immune Response

Jagadeesh Bayry,a,b Audrey Beauvais,a Kyung J. Kwon-Chung,a Srini V. Kaveri,b Kushagra Bansal,a,b Jean-Paul Latgé,a Anne Beauvaisf

Institut National de la Santé et de la Recherche Médicale, Unité 1138, Paris, France; Centre de Recherche des Cordeliers, Université Pierre et Marie Curie–Paris 6, Université Paris Descartes, Paris, France; Université Catholique de Louvain, Institute of Life Sciences, Louvain-la-Neuve, Belgium; Molecular and Applied Microbiology, Leibniz-Institute for Natural Product Research and Infection Biology, University of Jena, Jena, Germany; Integrated Research and Treatment Center, Center for Sepsis Control and Care Jena, University Hospital, Jena, Germany; Unite des Aspergillus, Institut Pasteur, Paris, France; Molecular Microbiology Section, Laboratory of Clinical Infectious Diseases, National Institute of Allergy and Infectious Disease, National Institutes of Health, Bethesda, Maryland, USA

In Aspergillus fumigatus, the conidial surface contains dihydroxynaphthalene (DHN)-melanin. Six-clustered gene products have been identified that mediate sequential catalysis of DHN-melanin biosynthesis. Melanin thus produced is known to be a virulence factor, protecting the fungus from the host defense mechanisms. In the present study, individual deletion of the genes involved in the initial three steps of melanin biosynthesis resulted in an altered conidial surface with masked surface rodlet layer, leaky cell wall allowing the deposition of proteins on the cell surface and exposing the otherwise-masked cell wall polysaccharides at the surface. Melanin as such was immunologically inert; however, deletion mutant conidia with modified surfaces could activate human dendritic cells and the subsequent cytokine production in contrast to the wild-type conidia. Cell surface defects were rectified in the conidia mutated in downstream melanin biosynthetic pathway, and maximum immune inertness was observed upon synthesis of vermelone onward. These observations suggest that although melanin as such is an immunologically inert material, it confers virulence by facilitating proper formation of the A. fumigatus conidial surface.

Melanin is a pigment that exists from humans to plants and has several functions, including resistance against environmental stress, such as UV light and oxidizing agents (1, 2). In airborne fungal spores, melanin helps in invasion of the host (3, 4) and contributes to the virulence of fungal pathogens (5, 6). Fungi produce different types of melanin: dihydroxynaphthalene (DHN)-melanin, pyomelanin, and DOPA-melanin. Aspergillus fumigatus produces the pigment DHN-melanin, responsible for the characteristic gray-green color of conidia. A. fumigatus is also able to produce a brownish pigment, pyomelanin, as an alternative melanin (7). Pyomelanin is produced via degradation of L-tyrosine with homogenetic acid as the main intermediate. On the other hand, Cryptococcus neoformans and Paracoccidioides brasiliensis synthesize DOPA-melanin (8). The production of melanin has been associated with the survival of the fungal species in the host (8, 9). DHN-melanin is hydrophobic and negatively charged, which modulates the binding capacity of conidia to host fibronectin and laminin present in the lungs (10). DHN-melanin is also essential for the proper assembly of cell wall layers in A. fumigatus. Pyomelanin was shown to protect the fungus from host defense mechanisms, i.e., reactive oxygen intermediates and hence considered to be protecting the fungus against immune effector cells during infection (11). DOPA-melanin contributes to host death, fungal burden, and dissemination (8).

Genes responsible for the synthesis of DHN-melanin in A. fumigatus belong to a 19-kb cluster located on chromosome 2. Six genes have been identified in this cluster, and their functions were elucidated (Fig. 1) (4–6, 12–14). PKS1 (ALB1; AFUA_2G17600) is the first gene of the pathway and codes for a polyketide synthase, which is responsible for catalyzing the synthesis of the heptaketide naphthopyrone from acetyl coenzyme A (acetyl-CoA) and malonyl-CoA. The heptaketide is then shortened by hydrolysis, reduction and dehydration by Ayg1p (AFUA_2G17550), Arp2p (AFUA_2G17560), and Arp1p (AFUA_2G17580), respectively. The generated product 1,3,6,8-tetrahydroxynaphthalene is reduced again by Arp2p and the resulting vermelone is oxidized by the copper oxidase Abr1p (AFUA_2G17540) to form the 1,8-DHN-melanin, which is polymerized by the laccase Abr2p (AFUA_2G17530) (12, 15).

The role of the conidial melanin in the A. fumigatus virulence has been studied by using either melanin ghosts or the pigment-less mutant, wherein the PKS1 gene, which encodes a protein involved in the first step of melanin biosynthesis, has been deleted (4, 9, 16–19). These reports demonstrated that melanin protects the conidia against reactive oxygen species, masks the recognition of various A. fumigatus pattern-associated molecular patterns, inhibits macrophage apoptosis and phagolysosome fusion, and attenuates the host immune response. All of these functions of melanin contribute to the increased survival of conidia in macrophages and promote the dissemination of A. fumigatus within the host.

However, the importance of melanin in the organization of the A. fumigatus conidial cell wall, the structural organization of the
MATERIALS AND METHODS
Fungal strains and culture conditions. The melanin precursor $\Delta pksP$, $\Delta ayg1$, $\Delta arp2$, $\Delta arp1$, $\Delta abr1$, and $\Delta abr2$ mutant strains and the WT strain B5233 have been maintained in silica gel at J. Kwon-Chung’s laboratory at the National Institutes of Health until use (6, 14, 15). All strains were cultivated on malt-agar (2%) medium at ambient temperature for at least 15 days before collecting the resting conidia. Conidia were harvested from the culture medium using 0.05% Tween 20 in water. Conidial suspensions were filtered using BD Falcon filters (BD Biosciences) to remove any mycelium. For immunolabeling and DC experiments, resting conidia were fixed with paraformaldehyde (PFA)-fixed (2.5% [vol/vol] PFA in phosphate-buffered saline [PBS]) overnight at 4°C. The fixed conidia were subsequently washed three times with 0.1 M NH$_4$Cl and once with PBS–0.1% Tween 20.

Melanin extraction. The isolation of melanin from the WT conidia was performed as previously described (20, 21). After the fungi were grown on malt-agar medium for 15 days at ambient temperature, the conidia of each strain were collected in 0.05% Tween-water. Briefly, conidia were treated with a combination of proteolytic (proteinase K; Sigma) and glycohydrolitic (Glucanex; Novo) enzymes, denaturant (guanidine thiocyanate), and hot, concentrated HCl (6 M). This treatment resulted in an electron-dense layer similar in size and shape to the original conidial melanin layer without underlying cell components, for which reason these electron-dense materials were called “melanin ghosts” (20).

Extraction of the AS polysaccharide fraction from conidia. Conidia were disrupted with 0.5-mm-diameter glass beads in a FastPrep (MP Biomedicals). The conidial cell wall fraction was recovered by centrifugation, washed with water, and then freeze-dried. The dried cell wall fraction was boiled in 50 mM Tris-HCl (pH 7.4) containing 50 mM EDTA, 2% sodium dodecyl sulfate (SDS), and 40 mM $\beta$-mercaptoethanol (10 min, twice) to remove proteins and extensively washed with water to obtain cell wall polysaccharides. From the latter, the alkali-soluble (AS) fraction was extracted as described earlier (22).

Extraction of conidial surface RodA protein (RodAp) involved in rodlet formation. RodAp was extracted from the spore surface by incubating 10$^9$ dry conidia with 48% (vol/vol) hydrofluoric acid (HF) for 72 h at 4°C (23). The contents were centrifuged (10,000 $\times$ g, 10 min), and the supernatant obtained was dried under N$_2$. The dried material was reconstituted in H$_2$O, and an aliquot was subjected to SDS-PAGE (15% [wt/vol]) analysis and visualized by silver nitrate staining.

Analysis of proteins on the conidial surface. Conidia were incubated in 0.5 M NaCl solution for 2 h at room temperature at a ratio of 10$^{10}$ conidia per ml. The NaCl supernatant was recovered after centrifugation and directly subjected to SDS-PAGE (10% [wt/vol]). Two-dimensional (2D) gel electrophoretic analysis of the NaCl extract was carried out as described previously with slight modifications (24, 25). A 50- to 100-$\mu$g portion of protein was loaded onto IPG strips (11 cm, pH 3 to 7; GE Healthcare Life Sciences) by in-gel rehydration. After equilibration of the IPG strips, SDS-gel electrophoresis was carried out using Criterion AnyKD TGX Stain-Free precast gels (Bio-Rad). Proteins were visualized by UV light and colloidal Coomassie blue staining (Candiano2004). After...
scanning, the gel images were analyzed with the software Delta 2D 4.3 (Decodon). Protein spots were excised and analyzed by mass spectrometry using an ultraliftXtreme MALDI-TOF/TOF device (Bruker Daltonics) as described previously (26).

Analysis of carbohydrate on the conidial surface by fluorescence microscopy. The mannose moieties of glycoproteins on the resting conidial surface were labeled with concanavalin-fluorescein isothiocyanate (ConA-FITC; Sigma) at 0.1 mg/ml after incubating the resting conidia for 1 h at 37°C in 0.1 M carbonate buffer (pH 9.6) containing 0.1% Tween 20. The hexosamines were labeled with wheat germ agglutinin (WGA)-FITC at 0.1 mg/ml upon incubating the resting conidia for 1 h at the room temperature in PBS containing 0.1% Tween 20. For immunolabeling, PFA-fixed conidia were incubated with different antibodies as described previously (27). β-(1,3)-Glucan was labeled with dectin-1 (Fc-dectin1, 6 μg/ml), followed by FITC-conjugated GaHu-Fab2-human IgG (5 μg/ml; kindly provided by G. Brown, University of Aberdeen, Aberdeen, United Kingdom) (28).

Antibodies and reagents for human immunology. Recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4) were from Millenyi Biotec (France). Fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies (MAbs) to CD80 and phycocerythrin (PE)-conjugated MAbs to CD83 and CD86 were from BD Biosciences (France), and PE-conjugated MAb to CD40 was from Becton Dickinson (France). Anti-Thr202/Tyr204 phospho-extracellular signal-related kinase 1/2 (ERK1/2) and anti-Thr180/Tyr182 phospho-p38 mitogen-activated protein (MAPK) antibodies were purchased from Cell Signaling Technology (USA). Anti-β-actin antibody (AC-15) was from Sigma-Aldrich (USA).

Generation and culture of human DCs. Monoctye-derived DCs were generated as previously described (29, 30). Immature DCs (0.5 × 10^6 cells/well/ml) were cultured in the presence of GM-CSF and IL-4 (cytokines) alone, with cytokines and PFA-fixed conidia of the wild type or melanin mutants (1:1 ratio), with cytokines and 1 μg of melanin extracts, with cytokines and 1 μg of AS polysaccharide fraction of A. fumigatus cell wall (positive control), or with cytokines and NaCl extracts from 0.75 × 10^3 conidia for 48 h. Cells were harvested, and cell-free supernatants were stored at -80ºC for cytokine analysis. Cells were labeled with fluorochrome-conjugated MAbs for surface marker analysis by using LSR II flow cytometry (BD Biosciences). Five thousand events were recorded for each sample, and data were analyzed by BD FACS DIVA software (BD Biosciences).

Mixed lymphocyte reaction. CD4+ T cells were isolated from peripheral blood mononuclear cells of healthy donors using CD4 microbeads (Miltenyi Biotec, France). DCs were washed extensively and cocultured with 10^3 responder CD4+ T cells at DC/T cell ratios of 1:10, 1:20, 1:40, and 1:80. After 4 days, either cells were harvested and cell-free supernatants were stored at -80ºC for cytokine analysis, or cells were pulsed for 16 to 18 h with 0.5 μCi of [3H]thymidine. Radioactive incorporation was measured by standard liquid scintillation counting. The proliferation of cells was measured as mean counts per minute (cpm) ± the standard error of the mean (SEM) of quadruplicate values after subtracting the values of responder T cell cultures alone.

Measurement of cytokines. Cytokines were quantitated in cell-free culture supernatants using CBA human inflammation and human Th1/Th2 kits (BD Biosciences).

Statistical analysis. A two-sided, Student-t test was used for statistical analysis. A P value of <0.05 was considered significant (*, P < 0.05; **, P < 0.01).

Immunoblotting. Immunoblotting was performed as described previously (31). DCs were washed with ice-cold PBS in radioimmuno precipitation assay lysis buffer (50 mM Tris-HCl [pH 7.4]; 1% NP-40; 0.25% sodium deoxycholate; 150 mM NaCl; 1 mM EDTA; 1 mM phenylmethylsulfonyl fluoride; 1 μg of aprotinin, leupeptin, and pepstatin/ml; 1 mM NaVO_4; 1 mM NaF). Equal amounts of proteins from the total cell lysates were subjected to SDS-PAGE, followed by transfer of proteins to polyvinylidene difluoride membranes. Membranes were blocked in TBST buffer (0.02 M Tris-HCl [pH 7.5], 0.15 M NaCl, 0.1% Tween 20) containing 5% nonfat dried milk and investigated using a primary antibody overnight at 4°C. After washing with TBST, membranes were incubated with horseradish peroxidase-conjugated secondary antibody (Jackson Immunologica lls, USA). The blots were then developed with an enhanced chemiluminescence detection system (Perkin-Elmer, USA) according to the manufacturer’s instructions.

Analysis of the conidial surface by AFM. Conidial surfaces were analyzed by atomic force microscopy (AFM) using a Multimode VIII instrument (Bruker, Santa Barbara, CA). Sample immobilization was achieved by mechanically trapping living conidia into porous polycarbonate membranes (0.4μp Sa; Belgium). After a concentrated suspension of cells was filtered, the membrane was rinsed with deionized water, carefully cut, and attached to a metallic puck using double-sided tape. The mounted sample was then transferred to the AFM liquid cell, while avoiding dewetting. Imaging was performed in contact mode under minimal applied force using oxide-sharpened microfabricated Si3N4 cantilevers (MSCT; Bruker) with a nominal spring constant of 0.01 N/m. Force measurements were carried out by chemical-force microscopy (32, 33) using gold tips (OMCL-TR4; Olympus, Tokyo, Japan) coated with hydrophobic thiol.

RESULTS A. fumigatus conidia from ΔpksP, Δayg1, and Δarp2 mutants induce maturation and activation of human DCs. Conidia from individual melanin biosynthetic pathways gene deletion mutants were used to study the maturation and activation of DCs. Melanin-mutant conidia deficient in the early steps of the biosynthetic pathway (ΔpksP, Δayg1, and Δarp2) induced the maturation of DCs, as demonstrated by the significantly enhanced expression of CD83 and costimulatory molecules CD86, CD80, and CD40 (Fig. 2) (34, 35). The ΔpksP, Δayg1, and Δarp2 conidia also stimulated a panel of DC-cytokines such as tumor necrosis factor alpha (TNF-α), IL-1β, IL-6, and IL-10 (Fig. 3). Upon vermelome biosynthesis, the subsequent downstream melanin biosynthetic pathway mutant conidia (Δabr1 and Δabr2) behaved like the WT strain, becoming immunologically inert (Fig. 2 and 3). The Δarp2 conidia presented an intermediate phenotype, since they induced only modest changes in the expression of costimulatory molecules of DCs and the secretion of DC-cytokines (Fig. 2 and 3).

Melanin does not stimulate DC maturation. As shown in Fig. 4, melanin ghosts from WT conidia did not induce DC maturation (Fig. 4A). We further verified the lack of activation of DCs by analyzing the intracellular signaling pathways and the ability of DCs to induce T cell proliferation and cytokines (Fig. 4B to D). We show that melanin ghost failed to phosphorylate p38 MAPK and ERK, thus confirming the immunological inert nature of melanin (Fig. 4B). The lack of activation of DCs by WT ghost extract was also reflected in the inability of WT ghost extract-treated DCs to promote T cell proliferation (Fig. 4C) and the T cell cytokines IL-2, interferon gamma (IFN-γ), and IL-5 (Fig. 4D). In contrast, the AS polysaccharide fraction [rich in α-(1,3)-glucan] of the A. fumigatus cell wall, used as a positive control, induced maturation

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of DCs, activated intracellular signaling pathways, and promoted DC-mediated T cell proliferation and cytokine production (Fig. 4). Taken together, these data indicated that conidial surface melanin, either in situ or in the extracted form (melanin ghosts), failed to stimulate DC activation and cytokine production.

The surface rodlet layer is masked by an amorphous hydrophilic layer. To investigate whether the structural modifications in the ΔpksP, Δayg1, Δarp2, and Δarp1 conidia could be responsible for stimulating the DCs, conidial surfaces were imaged by AFM (36, 37) (Fig. 5A to O). Rather than using an incident beam as in classical microscopy, AFM probes the small forces acting on the sample surface (37). Three-dimensional images are generated in buffer by scanning a sharp tip over the cell surface while sensing the interaction force between the tip and the surface. Originally invented for topographic imaging, AFM has evolved into a multifunctional molecular toolkit, enabling researchers not only to observe structural details of cells to near molecular resolution (36, 38) but also to measure their biophysical properties and interac-

FIG 2 Effect of melanin biosynthetic pathways mutant conidia on the maturation of human DCs. Immature DCs (0.5 × 10^6 cells/ml) were cultured in the presence of cytokines GM-CSF and IL-4 (Ctr DC) or with cytokines and WT conidia or melanin biosynthetic pathway mutant conidia (ΔpksP, Δayg1, Δarp1, Δarb1, and Δarb2) at a 1:1 ratio for 48 h. The percent expression of CD83 (A) and CD86 (B) and the mean fluorescence intensities (MFI) of CD80 (C) and CD40 (D) were analyzed by flow cytometry. The data (means ± the SEM) are from four to five donors. The level of statistical significance is indicated (*, P < 0.05; **, P < 0.01).

FIG 3 Induction of DC cytokines by the melanin biosynthetic pathways mutant conidia. Immature DCs (0.5 × 10^6 cells/ml) were cultured in the presence of cytokines GM-CSF and IL-4 (Ctr DC) or with cytokines and WT conidia or melanin biosynthetic pathway mutant conidia (ΔpksP, Δayg1, Δarp2, Δarp1, Δarb1, and Δarb2) at a 1:1 ratio for 48 h. Cell-free culture supernatants were analyzed for the secretion of TNF-α (A), IL-1β (B), IL-6 (C), and IL-10 (D). The data (means ± the SEM) are from four donors and are presented as pg/ml. The level of statistical significance is indicated (*, P < 0.05; **, P < 0.01).
In contrast to the WT conidia that are covered with rodlet structure (30, 42) (Fig. 5M to O), the \( \text{pksP} \) mutant conidial surface was amorphous, without organized rodlet structure (Fig. 5A to C). Some patches of organized rodlet layers were observed on the \( \text{ayg1} \) conidial surface (Fig. 5D to F), and their percentage increased on \( \text{arp2} \) conidia (Fig. 5G to I) to finally cover almost the entire surface of the \( \text{arp1} \) conidia (Fig. 5J to L). However, the rodlet layer on \( \text{arp1} \) conidia appeared to be less compact and less organized than that of the WT conidia (Fig. 5M and O).
pathway genes (Δabr1 and Δabr2) presented organized and compact rods on the entire surface of their conidia, similar to WT conidia (data not shown).

To investigate whether RodAp (responsible for the formation of rodlet layer on the conidial surface) is still present on the ΔpksP mutant conidial surface, all of the mutant conidia, as well as the WT conidia, were treated with HF (30). RodAp could be extracted from the ΔpksP mutant, other melanin mutants, and WT conidia. Their SDS-PAGE profiles (Fig. 6) showed that two bands of RodAp classically seen in the HF extracts of conidia were present in all of the melanin pathway mutants and WT conidia (30). A band at 18 kDa could also be observed in ΔpksP, Δayg1, and Δarp2 HF extracts (Fig. 6). Mass spectrometry (MS) and tandem MS (MS/MS) analyses showed that this 18-kDa protein corresponds to the Aspf1 antigen, suggesting the loose architecture of rodlets in these mutants. These data confirmed AFM observations that the RodAp were present but hidden by an amorphous material on the surfaces of ΔpksP, Δayg1, and Δarp2 mutant conidia.

Because the presence of this amorphous material covers the hydrophobic rods, we sought to determine whether the observed surface changes correlated with differences in the conidial hydrophobic adhesive properties. To understand this, we mapped and quantified the nanoscale adhesion properties of WT and ΔpksP mutant conidia by AFM with hydrophobic tips. The pres-

FIG 5 AFM imaging reveals that the loss of melanin correlates with the lack of exposed rodlet layer. AFM deflection images of the surfaces of ΔpksP (A to C), Δayg1 (D to F), Δarp2 (G to I), Δarp1 (J to L), and WT (M to O) conidia recorded in deionized water at low (A, D, G, J, and M), medium (B, E, H, K, and N), and high (C, F, I, L, and O) resolutions. Black labels “R” and “A” indicate regions made of rodlets and amorphous materials, respectively.
presence of this unorganized material on the $\Delta$pksP mutant conidial surface was associated with a dramatic reduction in their conidial surface hydrophobicity (Fig. 7). For the WT, force-distance curves recorded across the cell surface revealed large adhesion forces, ranging from 0.2 to 6 nN (Fig. 7M to O). In contrast, structural changes in $\Delta$pksP conidia caused profound modifications in the cell surface physicochemical properties (Fig. 7A to C). Force-distance curves and force maps showed the absence of adhesion forces over the entire surface of the $\Delta$pksP mutant conidia, indicating that this mutant is hydrophilic. The adhesion force of the other mutants of the melanin pathway increased with the rank of mutants in the pathway, from a low adhesion with the other mutants of the melanin pathway increased with the rank of mutants in the pathway, from a low adhesion with the other melanin mutants, in-cluding ConA was either low or negative in other melanin mutants, in-

$\Delta$pksP, $\Delta$ayg1, and $\Delta$arp2 mutant conidia. However, labeling with ConA was observed only with the $\Delta$ayg1 mutant conidia (Fig. 7A to F) to a maximal adhesion with WT conidia (Fig. 7M to O). The low adhesion capacities of the $\Delta$pksP, $\Delta$ayg1, and $\Delta$arp2 mutants indicated a modification of the cell surface hydrophobicity that could have influenced conidial recognition by DCs.

**Proteins are present in the amorphous hydrophilic layer of $\Delta$pksP and $\Delta$ayg1 mutant conidia.** We then investigated the chemical nature of the amorphous layer present on the surfaces of $\Delta$pksP, $\Delta$ayg1, and $\Delta$arp2 mutant conidia. A strong labeling with ConA was observed only with the $\Delta$pksP conidia, suggesting that its surface layer is rich in glycoconjugates. However, labeling with ConA was either low or negative in other melanin mutants, including $\Delta$ayg1 and $\Delta$arp2 mutants, and WT conidia (Fig. 8; data not shown). The surface amorphous material could be extracted by incubating mutant conidia (mutants for the initial steps of melanin biosynthesis) with 0.5 M NaCl for 2 h, and they were positive for protein test, suggesting the presence of glycoproteins in this conidial surface amorphous material. As shown in Fig. 9, the amount of proteins present in the extract was very high in $\Delta$pksP mutant, followed by $\Delta$ayg1 mutant. Extract from $\Delta$pksP mutant contained 53 $\mu$g of protein per 3 X $10^8$ conidia, whereas $\Delta$ayg1 conidial extract contained 12 $\mu$g of protein. The surface extracts of other mutants conidia, i.e., $\Delta$arp2 and $\Delta$arp1 mutants contained 3.8 $\mu$g of protein, while in $\Delta$abr1, $\Delta$abr2 and WT extracts the amount of proteins was too low to detect. These results thus indicate that smaller amounts of proteins on the surface layers of $\Delta$ayg1, $\Delta$arp2, and $\Delta$arp1 conidia reflected in the low or negative ConA-FITC staining of these conidia.

Extracted protein mixture of $\Delta$pksP mutant was then subjected to proteomic analysis. Forty-one proteins were identified in the extract, and in silico analysis of these proteins by SigPred (http://www.cbs.dtu.dk/services/SignalP/) and CADRE (http://www.cadre-genomes.org.uk/Aspergillus_fumigatus/) revealed that all of them had a signal peptide (Table 1). Extracellular proteins, normally secreted during the vegetative growth of *A. fumigatus*, such as Cat1p, Exog1p, ExoG2p, Asp1p, and ChiB1, were identified in the NaCl extract of $\Delta$pksP resting conidia (43–46). Of the 41 proteins, nine were glycosylhydrolases. RodAp was also identified in the NaCl extract. Other proteins, such as proteasome components, translation elongation factors, pyruvate dehydrogenases, adenosine deaminase, and protein disulfide isomerase, normally found in intracellular compartments were present in very small amounts since they were identified only once or twice in the proteomic survey.

In order to determine whether the proteins present on the surface of $\Delta$pksP and $\Delta$ayg1 conidia are responsible for the activation of DC by these mutant conidia, we incubated DCs with the NaCl extracts of $\Delta$pksP, $\Delta$ayg1, $\Delta$arp2, and $\Delta$arp1 mutant and WT conidia. As expected, NaCl extract of $\Delta$pksP and $\Delta$ayg1 mutants induced the maturation of DCs, whereas NaCl extracts of $\Delta$arp2, $\Delta$arp1, and WT strains did not (Fig. 10). These results demonstrated that the surface protein layer was responsible at least in part for the induction of DC maturation following incubation of cells with resting conidia of $\Delta$pksP and $\Delta$ayg1 mutants. However, the amount of proteins present on the surfaces of $\Delta$arp2 and $\Delta$arp1 conidia was too low to stimulate DCs (Fig. 10). Although NaCl extracts from $\Delta$pksP and $\Delta$ayg1 mutant conidial surfaces induced maturation of the DCs based on the phenotype analysis of cells (Fig. 10), they did not induce the production of cytokines such as IL-1$\beta$, IL-10, or IL-6 (data not shown). The level of production of above cytokines was on par with that of control DCs. These data suggest that signals provided by NaCl extracts of $\Delta$pksP and $\Delta$ayg1 mutant conidia were not sufficient to induce the functional activation of the DCs. On the other hand, $\Delta$pksP, $\Delta$ayg1, and $\Delta$arp2 mutant conidia induced various DC cytokines, which could be due to the exposure of cell wall polysaccharides on their conidial surfaces.

**Glucosamine-containing components are exposed at the $\Delta$pksP, $\Delta$ayg1, and $\Delta$arp2 conidial surfaces.** To check whether any structural cell wall modification occurred and was responsible for DC activation by $\Delta$pksP, $\Delta$ayg1, and $\Delta$arp2 conidia, we labeled mutant and WT conidia with the $\beta$-(1,3)-glucan receptor dectin-1 and with the glucosamine (GlcN) recognizing lectin WGA. Mutants and WT conidia did not bind to dectin-1 (data not shown), suggesting that $\beta$-(1,3)-glucans were not exposed at the conidial surfaces. However, $\Delta$pksP, $\Delta$ayg1, and $\Delta$arp2 conidia were positive for WGA-FITC (Fig. 11), whereas $\Delta$abr1, $\Delta$abr2, and WT conidia were positive for WGA-FITC (Fig. 11). In line with the partial stimulation of DCs, $\Delta$arp1 mutant presented an intermediate phenotype: ca. 40% of the conidia were WGA positive. These results suggested that conidia with uniform exposure of glucosamine-containing components on the surface (in cases of $\Delta$pksP, $\Delta$ayg1, and $\Delta$arp2 mutant conidia) were able to induce DC activation, whereas conidia with low levels of exposure of GlcN content on the surface did not stimulate DCs ($\Delta$abr1, $\Delta$abr2, and WT). When GlcN exposure was intermediate as in $\Delta$arp1 conidia, such conidia were able to induce partial activation of DCs.

Consequently, the absence of melanin or at least the intermediate scytalone increased the permeability of the conidia to

![FIG 6 SDS-PAGE (15% gel) profile of the RodAp extracted from the WT and melanin biosynthetic pathway mutant conidial surfaces using hydrofluoric acid (HF). Protein bands were revealed by silver staining. RodAp*, degraded form of RodAp due to HF treatment.](image-url)
FIG 7 Structural modifications influence the conidial surface hydrophobicity. AFM deflection images (A, D, G, J, and M) recorded in deionized water, together with adhesion force maps (x-y, 1 by 1 μm; z-range, 3 nN) (B, E, H, K, and N) and corresponding adhesion histograms (n = 1,024) (C, F, I, L, and O) recorded with hydrophobic tips on the surfaces of ΔpksP (A to C), Δayg1 (D to F), Δarp2 (G to I), Δarp1 (J to L), and WT (M to O) conidia.
secreted proteins, which otherwise secreted normally during conidial germination, and exposed the GlcN polymers on the conidial surfaces. Proteins and GlcN polymers were responsible for the DC activation after incubation of cells with ΔpksP, Δayg1, and Δarp2 conidia. The Δabr1 and Δabr2 conidia were immunologically inert like their WT counterparts, whereas Δarp1 conidia presented an intermediate phenotype.

### TABLE 1 Identification of proteins extracted from the ΔpksP mutant conidial surface

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* Identification was accomplished using MS/MS and MS with a mascot score above a threshold of 54.

** A. fumigatus locus tag (www.aspergillusgenome.org).

### DISCUSSION

In fungal biology, melanin pigments are attributed with a variety of beneficial functions, including protection against exogenous stress, UV irradiation, and host defense mechanisms, including reactive oxygen species, lytic enzymes, and antimicrobial peptides (47), and hence considered to be one of the fungal virulence factors (48). Melanin, being extracellular, also contributes to the fungal spore structure (5). In A. fumigatus, melanin biosynthesis is reported to require a cluster of six genes (6, 13, 14). In the present study, we show for the first time the effect of the deletion of individual genes from the melanin biosynthetic gene cluster on the respective conidial surface morphology and the consequent immune responses to the mutant conidia.

The mutant conidia with a deletion in one of the first three genes of the melanin pathway (ΔpksP, Δayg1, and Δarp2) stimu-
The maturation of DCs.

Rodlets by an amorphous and hydrophilic layer enabled resting conidia were present in the above mutant conidia, the masking of the surface. Although the rodlets that immune-silence the resting conidia were the absence or very few patches of rodlets at the presence of the cytokines GM-CSF and IL-4 (Ctr DC) or cytokines and NaCl-conidia on the maturation of DCs. Immature DCs were cultured in the presence of cytokines and elicited the production of IL-6, IL-1β, TNF-α, and IL-10 cytokines by DCs, whereas WT conidia were not immunogenic. The Δarp1 mutant presented an intermediate phenotype since it induced partial activation of the DCs. Melanins as such were immunologically inert since the melanin ghost extracted from the WT conidia failed to activate DCs. These results suggested that the surfaces of ΔpksP, Δayg1, and Δarp2 resting conidia are covered with specific common compounds that are rare in Δarp1 conidia and are responsible for the maturation of DCs.

The major obvious phenotypes of ΔpksP, Δayg1, and Δarp2 resting conidia were the absence or very few patches of rodlets at the surface. Although the rodlets that immune-silence the resting conidia were present in the above mutant conidia, the masking of rodlets by an amorphous and hydrophilic layer enabled resting conidia to be immunogenic (24, 49). A few patches of amorphous and hydrophilic layers could also be observed on the Δarp1 conidial surface. When downstream genes of the DHN-melanin pathway were deleted, the appearance of the rodlets and the hydrophobicity of the conidia increased. Previous studies demonstrated that deletion of PKSP and ARP2 correlated with a decreased ability to bind laminin on the conidial surface (10). This result is explained by the low hydrophobicity of the conidia, which reduced the electronegative charge required for laminin binding.

The amorphous and hydrophilic layer is composed of GlcN-containing components and deposited ConA-positive proteins mostly in ΔpksP conidia. These proteins were analyzed in the ΔpksP mutant, and their identification showed that they are usually secreted during vegetative growth. Most hydrolyases (such as β-1,3-glucosidases, β-N-acetylgalactosaminidase, and mannosidase), catalase, Asp1, Asp-hemolysin, and chitinase found in the amorphous surface layer of the resting ΔpksP conidia were universally identified during mycelial growth in a protein-based medium (43–46). Their presence on the surfaces of resting conidia of ΔpksP and Δayg1 mutants is explained by modifications of the ionic strength of the hydrophobin layer resulting from the absence of melanin or at least the YWA1, a melanin biosynthetic intermediate that is synthesized by the combined activities of PksPp and Ayg1p. The easy removal of these hydrophilic glycoproteins by 0.5 M NaCl suggested that they adhered to the conidial cell wall through electrostatic binding. These surface proteins in the conidial amorphous layers were responsible for the DC maturation and cytokine production. Interestingly, cell wall structural modifications resulting from the absence of α-(1,3)-glucan, as in A. fumigatus Δags1 Δags2 Δags3 (Δags) mutant, also gave the similar conidial phenotype, a hydrophilic protein layer on the surface of the conidia that stimulated host defense reactions (24). However, the composition of proteins in this amorphous layer in triple Δags and ΔpksP deletion mutants was not the same, suggested defining cell wall permeability defects due to the deletion of different cell wall component biosynthetic genes. When the melanin synthesis pathway was blocked farther downstream by gene deletion, fewer proteins were able to cross the conidial cell wall. Of note, DC cytokine production was less for ΔpksP mutant than for Δayg1 and Δarp2 mutant conidia (Fig. 3). This result suggests that glycoproteins were less stimulatory than GlcN residues present on the surfaces of the ΔpksP, Δayg1, and Δarp2 mutant conidia and that these GlcN residues are less exposed on ΔpksP mutant surfaces due to the larger amount of glycoproteins in this mutant.

The presence of GlcN residues on the surfaces of the ΔpksP, Δayg1, and Δarp2 mutant conidia could be explained by the unmasking of cell wall chitin due to the absence of melanin. The hexosamines present in the conidial cell wall were composed of long chains of fibrillar water insoluble chitin, amorphous and soluble chitin oligosaccharides (containing 10 to 15 N-acetylgalactosamines), and deacetylated chitin (chitosan) (A. Beauvais et al., unpublished results). Previous studies on different-sized chitin polymers have shown that >70-μm chitin polymers were immunologically inert with murine macrophages, whereas both intermediate-sized (40- to 70-μm) and small (<40-μm) chitin polymers stimulated TNF elaboration by macrophages (50), and only <40-μm chitin polymers induced IL-10 production. Small particles of chitosan were even better macrophage immune stimulators. Chitosan of <20 μm elicited the most IL-1β from bone marrow-derived macrophages (50). Although several chitin-binding
proteins have been identified in mammalian cells, no chitin receptor had thus far been identified thus far. A recent study on the recognition of innate immune cells by *Candida albicans* chitin revealed that although there was no direct dectin-1 and chitin binding, chitin was capable of blocking dectin-1-mediated immune responses (51). Similarly, these small and/or deacetylated chitins were likely unmasked on the surfaces of the first three melanin mutants and were responsible for the DC maturation. Such an unmasking phenomenon was reported in chitin synthase ΔcsmA and ΔcsmB mutants, wherein the deletion of two of the

![Image of WGA-FITC labeling of mutant and WT resting conidia. Note the decreasing numbers of ConA-positive conidia from the first mutants of the melanin pathway to the last one. Scale bar, 10 μm.](http://iai.asm.org/)

**FIG 11** WGA-FITC labeling of mutant and WT resting conidia. Note the decreasing numbers of ConA-positive conidia from the first mutants of the melanin pathway to the last one. Scale bar, 10 μm.
chitin synthase genes (CSMA and CSMB) in A. fumigatus resulted in the increased exposure of WGA-positive components on the conidial surfaces (42). The Δcsma conidial surface was also amorphous and ConA positive; however, ConA-positive materials were not glycoproteins but rather due to the exposure of mannan-containing polymers. This further confirms the differential permeability defects due to the deletion of specific cell wall component biosynthetic genes.

Chai et al. (9) also demonstrated that melanin purified from WT conidia was also poorly immunogenic for the stimulation of cytokines by peripheral blood mononuclear cells. On the other hand, as seen in our study, ΔpksP conidia elicited significantly higher cytokine production, including IL-10, IL-6, and TNF-α. These researchers found that the blockage of dectin-1 with laminarin reduced cytokine production in response to ΔpksP conidia, which could be correlated with WGA-FITC positivity of ΔpksP, Δayg1, and Δarp2 mutant conidia and the observations that chitin can influence dectin-1-mediated immune responses (51). Jahn et al. (52) observed that the extent of macrophage phagocytosis and intracellular killing was significantly greater with ΔpksP conidia than with WT conidia. Thywissen et al. (18) showed that inside the phagolysosome, WT conidial DHN-melanin was responsible for the inhibition of the phagolysosomal acidification of mouse and human macrophages and neutrophils. The ΔpksP conidia, in contrast, were located in an acidic environment in the phagolysosome, which coincides with more effective killing of these conidia. The percentages of Δayg1, Δarp2, and Δabr2 conidia were lower in acidified phagolysosomes than those of ΔpksP conidia but higher than those of WT conidia. These results show that melanin intermediates formed by the downstream biosynthetic pathway increase conidial protection. However, the final product, the DHN-melanin, was important for the maximal protection since it facilitates the formation of a complete surface rodlet layer that hides conidia from their immediate recognition by the immune system.

**Conclusion.** The absence of at least the scytalone intermediate of DHN-melanin is responsible for structural and chemical modifications of the cell surface, which will have an obvious impact on the immune response of the host toward the corresponding mutant. Our results also show that melanin is essential to acquire the right surface properties with precise charge and hydrophobicity that are necessary to have immunologically inert conidia due to an exposed rodlet layer.

**ACKNOWLEDGMENTS**

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**REFERENCES**


