LaeA, a Regulator of Morphogenetic Fungal Virulence Factors†

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Opportunistic animal and plant pathogens, well represented by the genus Aspergillus, have evolved unique mechanisms to adapt to and avoid host defenses. Aspergillus fumigatus, an increasingly serious pathogen owing to expanding numbers of immunocompromised patients, causes the majority of human infections; however, an inability to identify bona fide virulence factors has impeded therapeutic advances. We show that an A. fumigatus mutation in a developmentally expressed transcriptional regulator (ΔlaeA) coordinating morphological and chemical differentiation reduces virulence in a murine model; impaired virulence is associated with decreased levels of pulmonary gliotoxin and multiple changes in conidial and hyphal susceptibility to host phagocytes ex vivo. LaeA, a conserved protein in filamentous fungi, is a developmental regulator of virulence genes and, possibly, the first antimicrobial target specific to filamentous fungi that are pathogenic to plants and animals.

For opportunistic pathogens, host infection may not be necessary, or even advantageous, for species survival. True “virulence” factors are typically missing in these organisms; instead, species that have adapted mechanisms to persist in hostile environments (including in vivo) become selected as the most common causes of disease. Identification of specific products or pathways critical to in vivo adaptation would potentially enable development of the most-targeted therapeutics, but the discovery of single-gene disease determinants in opportunists is hampered by redundancy both in the organism and in the host’s response to infection. This is one factor impeding development of effective treatments for opportunistic fungal plant and animal pathogens; in this case, the delay has been particularly detrimental because drugs targeting fungal cell wall and fungal membrane components confer toxicities to eukaryotic hosts.

All pathogenic fungi that sporulate or form fruiting bodies have developed mechanisms to mediate coordinate expression of biologically active secondary metabolites and spore-related products during development (7). The hydrophobins, conidial cell wall proteins that have been adopted by different plant and animal pathogens to serve diverse functions necessary for fungal pathogenicity, are an example. Despite the diversity of amino acid sequence and function, hydrophobins from disparate fungi complement each other in a variety of environmental and pathological niches (16). Thus, mechanisms of developmental regulation which more broadly control fungal pathogenicity may prove to be the pivotal virulence factors and drug targets for filamentous fungi that cause disease in plants and animals.

Members of the genus Aspergillus are common causes of disease in plants and animals worldwide. Currently, invasive aspergillosis is a leading cause of infection-related mortality in immunocompromised patients, occurring in people with hematological malignancies and AIDS, and limiting the success of hematopoietic stem cell and organ transplantation (8, 23, 24). Mortality is high and in the setting of disseminated infection approaches 100% even with the use of newer antifungal agents (10, 20). Aspergillus species cause additional environmental concern and morbidity in nonimmunosuppressed people and are implicated as a cause of fungal sinusitis, asthma, and allergic bronchopulmonary aspergillosis (17). Plant-pathogenic aspergilli are infamous for their elaboration of several mycotoxins, including aflatoxin, the most potent natural carcinogen known to date (38).

Despite the multitude of Aspergillus species that are ubiquitous in our environment, one species, Aspergillus fumigatus, accounts for at least 90% of invasive infections by Aspergillus spp. in people (5, 17). The clinical significance of this species justified prioritization for genome sequencing, which was recently completed (www.tigr.org). However, few null mutants have demonstrated avirulent phenotypes. For example, single- and multiple-gene disruption in a pigment biosynthesis gene cluster has been associated with decreased virulence in mouse models without inducing a concomitant alteration in microbial fitness; this defect is associated with increased microbial susceptibility to oxidative killing by monocytes and polymorphonuclear neutrophils (PMNs) (13, 34, 37).

Recently, we identified a nuclear protein, LaeA, that acts as a regulator of secondary metabolism in the aspergilli, including the model organism Aspergillus nidulans, the industrial agent Aspergillus terreus, and A. fumigatus (3). Here we show that the loss of laeA (ΔlaeA) in A. fumigatus, while not producing gross changes in growth or sporulation in media, results in reduced virulence in a murine model. Impairment is associated with the loss of detectable gliotoxin, an immunotoxin putatively involved in virulence (18, 30), increased conidial susceptibility to...
TABLE 1. Strains used in this study

| Fungal strain | Genotype | Source |
|---------------|----------|--------|
| AF293         | Wild type| 39     |
| AF293.1       | pyrG1    | 39     |
| TJW54.1       | ΔlaeA::A. parasiticus pyrG; pyrG1 | This study |
| TJW54.2       | ΔlaeA::A. parasiticus pyrG; pyrG1 | This study |
| TJW55.2       | pyrG1; A. parasiticus pyrG | 3     |
| TJW68.6       | ΔlaeA::A. parasiticus pyrG; pyrG1; laeA::hygB | This study |
| TJW69.2       | ΔlaeA::A. parasiticus pyrG; pyrG1; laeA::hygB | This study |

macrophage phagocytosis, and decreased ability of hyphae to kill neutrophil cells.

MATERIALS AND METHODS

Media and reagents. All fungal strains (Table 1) were maintained as glycerol stocks and were cultured at 25°C or 37°C on glucose minimal medium (GMM) with appropriate supplements (32). Sabouraud’s dextrose agar (Becton Dickinson, Sparks, MD), or RPMI 1640 (plus 10 mM HEPES, pH 7.4, without phenol red; Sigma, St. Louis, MO). Acetoxymethyl ester of calcein (calcein AM), calcein AM, and fluorescein isothiocyanate (FITC) were purchased from Molecular Probes (Eugene, OR). The complete medium used for macrophage culture included Iscove’s modified Dulbecco’s medium (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated human AB serum, 0.3 mg/ml l-glutamine, and 200 U/ml penicillin-streptomycin. Recombinant macrophage colony-stimulating factor was obtained from R&D Systems (Minneapolis, MN).

Fungal strains and nucleic acid analysis. The strains used are listed in Table 1. Aspergillus fumigatus laeA was previously cloned, sequenced, and disrupted in wild-type (WT) strain AF293.1 (a pyrG auxotroph) by the replacement of laeA with pyrG (3). Briefly, the disruption vector pJW58 was constructed by flanking the Aspergillus parasiticus pyrG marker gene with DNA fragments from upstream of the A. fumigatus laeA start codon (0.9 kb) and downstream of the laeA stop codon (1.0 kb). Fungal protoplasts were transformed as previously described (3). Transformant TW54.2 was documented to have single-gene replacement of laeA by Southern analysis and PCR. pJW72.1, containing a wild-type copy of the 3-kb laeA gene, including a 1-kb promoter, was used to complement the ΔlaeA strain TWJ54.2 to make TJW68.6 and TJW69.2. The 3-kb laeA gene was amplified by primers FUM1 and FUM4 (3) and was inserted in the Zero Blunt TOPO vector (Invitrogen) to produce pJW57.2. pJW72.1 was created by ligating the 3.4-kb NotI-SpeI fragment from pJW57.2 into the NotI-SpeI site of pUC82-8 (1). pUC82-8 contains the gene encoding hygromycin B phosphotransferase, which was the selectable marker used for transformation of Aspergillus.

The plasmid pBZ5, containing a wild-type copy of the A. parasiticus pyrG gene, was used to complement AF293.1, creating the reconstituted strain TJW55.2 (3). Extraction of fungal DNA, restriction enzyme digestion, gel electrophoresis, Southern and Northern blotting, hybridization, and probe preparation were performed using standard methods (21, 31).

RNA blots were hybridized with a 3-kb PCR fragment of laeA using primers FUM1 (5′GCCGACCTCTGGTTGTTCCT3′) and FUM4 (5′CATGACGCTATACTAGGTTG3′), a 1-kb ab1 PCR product using primers ab1f (5′AATTCACTACTCTGGAGATCAGCGG3′) and ab1r (5′GAGGCACCTTTAAGGATGTTTCG3′), a 1-kb rodA PCR product using primers rodAF (5′TGTCATACTACATTCTGTACGG3′) and rodAR (5′ACCCTGTTCCCATCAGCCA3′), and a 1-kb rodB PCR product using primers rodBF (5′AATCGAGACGCCAAGTTG3′) and rodBR (5′TAAAGGCTTTCATCAATAGCC3′).

Phenotypic characterization and secondary metabolite analysis. Mycelia were cultured overnight in liquid GMM or RPMI 1640 (37°C), and the fungal mats were lyophilized and weighed. Colony growth radius, morphology, spore germination, and spore production were measured as previously described (15). Published procedures were used to extract compounds from conidial spores of wild-type A. fumigatus and the ΔlaeA mutant (26). Glutathione production was measured by modification of a previously published method (3).

metabolites were measured from organic extracts of 7-day-old agar cultures (GMM) analyzed by liquid chromatography–photo diode array detection–high-resolution mass spectrometry (28) using the column and solvent system described by Bruhn et al. (6). Gliotoxin and other metabolites were identified by their retention times, UV spectra, and positive electrospray spectra, which should match both the relative intensities of ions and their respective accurate masses (less than ±0.01 Da of deviation). Metabolites were measured in murine lung homogenate after infection (methods detailed below).

Animal model of Aspergillus infection. The virulence of isogenic wild-type, pyrG2, and ΔlaeA strains was studied in a lung infection model, with approval of the University of Wisconsin Animal Care Committee. Conidia were harvested by flooding fungal colonies with 0.85% NaCl with Tween 80, enumerated with a hemocytometer, and adjusted to a final concentration of 6.2 log10 CFU/ml. Counts and the viability of the inocula were verified by duplicate plating serial dilutions on GMM plates. Six-week-old, outbred Swiss ICR mice (Harlan Sprague Dawley), weighing 24 to 27 g, were immunosuppressed by intraperitoneal injection of cyclophosphamide (150 mg/kg of body weight) on days −4, −1, and 3 and with a single dose of cortisone acetate (200 mg/kg). The mice were anesthetized via halothane inhalation in a bell jar on day 0. Sedated mice (10 mice/fungal strain) were infected by nasal instillation of 50 μl of the inoculum (day 1) and monitored three times daily for 7 days postinfection. All surviving mice were sacrificed on day 7. The tissue fungal burden in whole-lung homogenate was quantified by serial dilution and enumeration of CFU (number of CFU/two lungs). The duration of survival in days after inoculation was recorded for each animal. Moribund animals were sacrificed, and cumulative survival was recorded. Survival and clearance of residual fungal burden in tissue (number of CFU/two lungs) were used as the outcome variables to assess the relative virulence levels of fungal strains.

Human macrophage phagocytosis and killing of conidia. Peripheral blood mononuclear cells (PBMCs) from healthy adult human donors were isolated by centrifugation of heparinized blood over a Ficoll-Hypaque density gradient at 800 × g for 30 min. Isolated PBMCs were washed and resuspended to a concentration of 2 × 10^6 PBMCs/ml in complete medium. Monocytes were isolated by adherence using Falcon Primaria-coated (24-well) tissue culture plates (Becton Dickinson, Franklin Lakes, NJ). The cells that adhered to the plates were then trypsinized and separated and resuspended in 1.5 ml of complete medium containing 10% human serum and 40 ng/ml macrophage colony-stimulating factor. After 7 to 10 days in culture, macrophages were documented to be >95% CD14+ by flow cytometry. For isolation of murine thioglucololate-elicited peritoneal macrophages, 1 ml of 4% Brewer-modified thioglycollate medium (Becton Dickinson, Irvine, CA) was injected intraperitoneally into 12-week-old C57BL/6 mice. Five days later, peritoneal exudate cells, consisting of mostly macrophages (~90%), were collected in 12 ml of phosphate-buffered saline (PBS). Macrophages were washed with complete medium and resuspended to a concentration of 5 × 10^6 cells/ml. Cells were plated in Falcon Primaria-coated (24-well) tissue culture plates and incubated overnight at 37°C with 5% CO2. Nonadherent cells were removed, and the purity of adherent macrophages was assayed by flow cytometry using both FITC-conjugated rat anti-mouse Mac-3 monoclonal antibody and anti-FITC-conjugated rat anti-mouse CD14 monoclonal antibody (BD Biosciences). For phagocytosis experiments, WT and ΔlaeA conidia were prelabeled with FITC by gentle shaking (30°C) in the dark for 1 h. Labeled conidia (conidiaFITC) were washed twice with PBS, resuspended in RPMI 1640-HEPES, and used immediately. Macrophages were incubated with conidiaFITC at a ratio of 1:1, and plates were centrifuged briefly (500 × g) to ensure particle exposure. Macrophages were allowed to ingest conidiaFITC for 5, 10, and 30 min at 37°C, and adherent cells were gently washed with PBS, counterstained with calcein white (25 μM), and reincubated for 15 min at 4°C in the dark. The cells were then washed, fixed with 4% paraformaldehyde, and viewed with a Deltavision wide-field fluorescence microscope (Applied Precision Inc., Issaquah, WA) that was fitted with an FITC and rhodamine filter set. The number of macrophages containing FITC-positive inclusions was recorded as the percentage of phagocytic macrophages multiplied by the mean number of organisms internalized per macrophage. The percentage of phagocytosis was defined as the ratio of phagocytizing macrophages that ingested one or more conidia. Macrophage viability after conidial exposure was assessed by propidium iodide (PI; final concentration, 1 μg/ml) uptake after a 15-min incubation using a fluorescence microscope. Experiments were performed with triplicate wells and repeated at least three times. The percentage of conidia internalized per dilution as previously described (22). Monocyte-derived macrophages were allowed to ingest conidia for 1 h, wells were washed with warm PBS, and cells were incubated for another 6 h before intracellular conidia were harvested by exposure to isotonic saline with rapid freezing-thawing (~70°C to 37°C). Cellular lysis

This study
was confirmed by microscopic visualization, and the serial dilutions of superna-
tant were plated on yeast extract agar containing Triton (10 g yeast extract, 20 g
peptide, 20 g dextrose, 20 g agar, and 50 ml Triton X-100 per liter). Colonies
were counted after 24 h of growth at 37°C.

PMN killing and fungal cytotoxicity. PMNs were purified from whole blood of
healthy adult human donors by Ficoll centrifugation, following the leukocyte
separation protocol (Sigma). Briefly, red blood cells were lysed in hypotonic
solution, and PMNs were separated by centrifugation (800 × g, 10 min) and
resuspended in RPMI 1640-HEPES. Cellular viability was assessed by trypan
blue exclusion. PMN killing of Aspergillus hyphae was measured by a modifica-
tion of a previously described XTT [2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-
2H-tetrazolium-5-carboxanilide] assay (25). Briefly, conidial suspensions were
prepared by flooding the surface of the colonies with RPMI 1640-HEPES buffer
and filtered through gauze to separate the larger hyphal fragments. Early hyphal
elements were generated from conidia (106/ml) in 96-well flat-bottom plates
and filtered through gauze to separate the larger hyphal fragments. Early hyphal

| Colony diam (mm) | GMM agar | 37°C (6 days) | 25°C (12 days) |
|-----------------|----------|--------------|---------------|
|                  |          | 72.3 ± 0.3   | 63.7 ± 1.6    |
|                  |          | 52.3 ± 0.4   | 59.3 ± 0.4    |

% Germination NA

* From results of three experiments.

# NA, not applicable.

**RESULTS**

Phenotypic characterization. Despite the decreased production of mycelial metabolites, the ΔlaeA mutant demonstrated normal conidial pigmentation (3). Filamentous growth rates in liquid and colony cultures were similar between ΔlaeA mutant and WT isolates, although the A. fumigatus ΔlaeA mutant pro-
duced substantially more mass than the WT did under the conditions tested (Table 2 and Fig. 1). The A. fumigatus ΔlaeA mutant was impaired in production of conidiophores and

| Table 2. Growth of the A. fumigatus ΔlaeA mutant
|-----------------|----------|--------------|---------------|
| Parameter       | Growth conditions | A. fumigatus WT | ΔlaeA mutant |
| Dry wt (mg)     | GMM, liquid shake | 133.3 ± 4.4 | 200 ± 0   |
|                 | 37°C (40 h) | 60 ± 0        | 180 ± 0     |
|                 | 25°C (4 days) | 43.3 ± 4.4 | 63.3 ± 4.4 |
|                 | RPMI 1640, liquid shake | 29.5 ± 2.8 | 48.8 ± 2.8 |

| Colony diam (mm) | GMM agar | 37°C (6 days) | 25°C (12 days) |
|-----------------|----------|--------------|---------------|
|                  |          | 72.3 ± 0.3   | 63.7 ± 1.6    |
|                  |          | 52.3 ± 0.4   | 59.3 ± 0.4    |

% Germination NA

* From results of three experiments.

# NA, not applicable.

conidia in liquid shake culture, although the mutant’s germina-
tion of conidia was equivalent to that of the WT. Conidial
production levels were similar on solid medium (3). The A.
fumigatus ΔlaeA mutant-complemented strains presented the
wild-type phenotype in all aspects of physiological growth (Fig. 1
and data not shown). Neither the A. nidulans WT nor the
ΔlaeA mutant sporulated in liquid shake culture (data not
shown).

Animal model of Aspergillus infection and secondary metab-
olite analysis. The relative virulence levels of the WT and the
A. fumigatus ΔlaeA mutant were evaluated in a murine pulmo-
nary infection model (Fig. 2 and see Fig. S1 in the supple-
mental material). The null mutant caused less infection than WT
strains carrying ΔlaeA (WT and the pyrG strain) (Fig. 2) or the
complemented strain (see Fig. S1 in the supplemental mate-
rial). Fewer ΔlaeA fungal CFU were recovered from the lungs
of mice that were sacrificed 3 days after infection (Fig. 2A and
B). The isolated fungal colonies from these lungs were con-
firmed to be the ΔlaeA mutant or WT by phenotype and Sou-
thern analysis (data not shown).

The results of liquid chromatography–high-resolution mass spectrometry showed that gliotoxin was present in lungs infected with WT and pyrG A. fumigatus isolates but
not in ΔlaeA mutant-infected or noninfected lungs (Fig. 2C).
No other gliotoxin analogues or secondary metabolites nor-
manly produced during in vitro culture of A. fumigatus (e.g.,
helvolic acids, fumigacavines A to C, tryptoquinovilines A to
H, fumiquinazolines A to E, pseurotin A to F2, fumagillin,
fumitremorgins A to C, verruculogen, or TR-2) could be
detected in the WT- or ΔlaeA mutant-infected lung tissues
(data not shown). ΔlaeA mutant-complemented strains were
restored in metabolite production (see Fig. S2 in the sup-
plemental material).

Macrophage phagocytosis. We reasoned that the decrease in
pulmonary infection might be reflected by aberrancies in the
fungus-macrophage interaction. Toward this end, the ability of
macrophages to ingest ΔlaeA conidia was measured. Human monocyte-derived macrophages were exposed to FITC-labeled conidia for different time periods, and fungal cells were counterstained with calcofluor white to distinguish between ingested and adherent cells. The number of macrophages containing FITC green (internal) and calcofluor white blue (external adhered) organisms was quantified microscopically. Figure 3A and B show that macrophages internalized more ΔlaeA conidia than WT conidia, with phagocytosis indices separating within 10 min of exposure. Similar results were observed with murine-derived macrophages (data not shown). Altered phagocytosis was not associated with differences in viability of exposed macrophages, as measured by propidium iodide uptake (data not shown). Altered phagocytosis was not associated with differences in viability of exposed macrophages, as measured by propidium iodide uptake (data not shown). No differences in intracellular killing of ΔlaeA and WT conidia were measured by either serial dilution plating or intracellular germination (data not shown); however, the large differences in the numbers of organisms ingested per macrophage likely limited our ability to detect small differences in intracellular killing.

Conidial phenotype. To define the nature of the differences in macrophage phagocytosis, we more closely examined spore metabolites and architecture. Conidia harvested from the ΔlaeA mutant produced diminished the amounts of at least one metabolite in vitro (Fig. 4A). Surface features of conidia harvested from the ΔlaeA mutant demonstrated overall losses of prominent protrusions, consistent with defective rodlet synthesis (Fig. 4B). Accordingly, ΔlaeA conidia were less adherent to latex microspheres than the WT conidia were (Fig. 4C), and Northern profiling revealed delayed expression of genes encoding rodlets (rodA and rodB) and conidial pigments (alb1), which was correlated with sporulation patterns (Fig. 4D). These data indicate that LaeA regulates the expression of genes involved in conidial biosynthesis.

PMN killing and fungal cytotoxicity. We next measured the ability of PMNs to kill *A. fumigatus* hyphae as well as the ability of hyphae to kill neutrophils. *A. fumigatus* invasive growth is via hyphal proliferation, where neutrophils play a role in host defense. We found that human PMNs were equally effective in

FIG. 1. Filamentous growth and conidiophore development of *A. fumigatus* WT, ΔlaeA mutant, and ΔlaeA mutant-complemented strains in liquid medium. Images obtained by microscopy of colonies developed after 48 h of incubation in shaking (300 rpm) culture at 25°C.
killing WT and ΔlaeA hyphae 1 h after exposure, as measured by conversion of the viability dye 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (data not shown). Speculating that differences in PMN viability may become apparent due to alterations in secondary metabolite elaboration during hyphal growth of the ΔlaeA mutant, we measured PMN death 3 h after exposure to live hyphae by using an assay that measures the release of intracellular calcein AM (27). ΔlaeA hyphae induced less PMN cellular death than did hyphae of the WT or the pyrG strain (Fig. 5A). During this late time period, it is not possible to accurately measure hyphal viability or PMN killing of fungi due to fungal overgrowth. Differences in PMN death are associated at least in part with Aspergillus products secreted during later stages of growth, as the same differences in cellular viability were observed after PMN were exposed to concentrated WT and ΔlaeA mutant culture supernatants (Fig. 5B). Collectively, these data suggest that multiple phenotypes are associated with laeA deletion; decreased PMN death is consistent with altered secondary metabolite production by hyphae.

DISCUSSION

In its environmental niche, Aspergillus species propagate in a multicellular mycelium; at a critical cell density, the organism
FIG. 3. Conidial and hyphal interactions with human phagocytic cells. (A and B) Human CD14⁺ monocyte-derived macrophages ingested higher numbers of ΔlaeA conidia than of WT conidia (internal conidia are stained green). The phagocytosis index was calculated as the percentage of phagocytic macrophages multiplied by the mean number of organisms internalized per cell. Experiments were performed with triplicate wells and repeated at least three times.

FIG. 4. Conidial properties. (A) Spore metabolites analyzed by thin-layer chromatography. Spore diffusate was obtained by incubation of 10⁸ cells/ml in Hanks balanced salt solution for 1 h; products were filter sterilized, chloroform extracted, and dried under vacuum suction overnight. Products were redisolved with 100 μl chloroform for thin-layer chromatography as previously described (26). Metabolite produced by the WT and not the ΔlaeA mutant is indicated with an arrow. (B) ΔlaeA conidia (right panel) have decreased rodlet protrusions compared to WT conidia. (C) ΔlaeA conidia are less adhesive, as measured by adhesion of latex microspheres (9). Conidia (1 × 10⁸/μl) were suspended in 0.1 mol/liter KNO₃ solution (pH 6.5) and incubated at a 20:1 ratio with latex particles (0.6 μm); the percentage of conidia with adhered particles was calculated from triplicate measurements in two experiments. The two-tailed P value from Student’s t test comparing results for the ΔlaeA mutant with those of the WT and the pyrG strain was <0.0001. The P value comparing results for the WT and the pyrG strain was >0.05. (D) Northern blots demonstrating laeA, alb1, rodA, and rodB gene expression in A. fumigatus ΔlaeA mutant and WT strains after growth in GMM from 24 to 72 h. Ethidium bromide-stained rRNA is indicated for loading controls.
generates aerial stalks with terminal conidia produced on conidiophores. Conidia are readily aerosolized and are the infective form of the organism; in the lungs, conidia that escape killing by resident phagocytes germinate into hyphae, which can cause local pneumonia and disseminate hematogenously (17). What distinguishes *Aspergillus fumigatus* from the remaining hundreds of thousands of filamentous molds is not clear, as few single-gene “virulence factors” have been identified (4). Microbial disease attributes include a combination of factors secreted from growing mycelia and terminal hyphal cells and cell wall structural components, including hydrophobins and pigments that confer resistance to phagocytic killing (11–13, 17, 34–37). The impaired virulence of the \( \Delta \text{laeA} \) strain correlates with the loss or alteration of several of these factors of pathogenicity.

Our findings are the first genetic studies to draw potential significance to the role of *A. fumigatus* secondary metabolites in the establishment of intrapulmonary infection (Fig. 2; see Fig. S1 in the supplemental material). We hypothesized that secreted cytotoxic molecules may impact the relative ability of

![FIG. 5. PMN killing and fungal cytotoxicity. (A) Damage to PMN (calcein release) after 3 h of incubation with live WT and \( \Delta \text{laeA} \) mutant germ tubes was measured by the release of calcein AM (25). Shown are mean fluorescent units (MFU) of triplicate measurements for six healthy donors (standard deviations), representative of results obtained for nine different experiments. The two-tailed \( P \) value from Student’s *t* test comparing the results for the WT and the \( \text{pyrG} \) strain was \( >0.05 \). The \( P \) value for comparisons of the results for the \( \Delta \text{laeA} \) mutant with the WT and the \( \text{pyrG} \) strain was \( <0.001 \). (B) PMN death after exposure to concentrated culture supernatant from the WT and the \( \Delta \text{laeA} \) mutant. The percentage of total cells that were PI positive is shown. Controls (not shown) included Triton X-treated PMNs and untreated PMNs, which were \( >90\% \) and \( <10\% \) dead, respectively, with the assay. Results are means of triplicate measurements ± standard deviations from two experiments with different donors.](image1)

![FIG. 6. Proposed model of \( \text{LaeA} \) regulation of *A. fumigatus* phenotypes that confer virulence. \( \text{LaeA} \) regulation by the protein kinase A pathway was previously reported (13). Virulence factors purported but not yet identified by genetic analyses (spore diffusible metabolite) are shown with dashed lines.](image2)
host phagocytic cells to ingest and kill fungal conidial and hyphal elements ex vivo. The increased macrophage phagocytosis of \( \Delta laeA \) conidia was correlated with the loss of at least one secreted factor (Fig. 4A). Although not well defined, factors diffused from spores have been reported to impact macrophage handling of \( A. fumigatus \) conidia (26), including inhibition of phagocytosis (2). Characterization of the metabolites missing from \( \Delta laeA \) conidia may provide insight into the factors governing phagocytosis.

Another factor possibly contributing to the increased phagocytosis of \( \Delta laeA \) conidia was the altered architecture of the conidial hyphal elements ex vivo. An interesting observation is that adherence is not an important factor in phagocytosis, as the \( \Delta laeA \) conidia, although more easily ingested by macrophages, adhered less well to microspheres (Fig. 4C).

Whereas macrophage-mounted defense is most important for clearing conidial burden, invasive growth of \( A. fumigatus \) hyphal morphology is countered through host production of neutrophils. Here, our experiment strongly implicated the role of secreted metabolites in the killing of these host cells (Fig. 5B) with emphasis on the potential role of gliotoxin (Fig. 2C) in this process. Access to the \( A. fumigatus \) genome (www.tigr.org) indicates the potential for the presence of up to 40 novel metabolites from polyketide and nonribosomal peptides alone, and the characterization of a putative gliotoxin gene cluster may shortly answer the question of how much this hyphal toxin contributes to the phenotype described herein.

Genes encoding conidial components such as pigments and hydrophobins are positively regulated during sporulation to allow for the production of conidia that are resistant to environmental stresses (33, 36); secondary metabolites are considered part of the chemical arsenal required for niche specialization (7). The data presented within show that LaeA acts as a regulator of gene expression during vegetative growth and sporulation in \( A. fumigatus \); disruption of this positive regulator yields a mutant with reduced ability to establish pulmonary disease under the conditions described. This relatively avirulent phenotype is associated with altered expression of multiple microbial determinants critical for conferring both conidial and hyphal cellular resistance to host phagocytic attack. Given the opportunistic nature of these organisms, it is most likely that virulence is dependent on coordinate expression of multiple gene products that mediate host-fungus interactions. As \( A. fumigatus \) full-genome arrays will soon be available, it may be possible to identify additional conidial and hyphal genes involved in conferring microbial resistance to host defenses and the ability to cause invasive and allergic disease in \( \Delta laeA \) mutant-profiling experiments. Figure 6 depicts our current understanding of LaeA regulation of \( A. fumigatus \) virulence attributes.

The implications of these findings extend well beyond the field of medical mycology. Data suggest that LaeA serves as a regulator for pathogenicity genes during fungal sporulation and hyphal elongation, critical morphological events shared by, but specific to, filamentous plant and animal pathogens. In \( A. nidulans \), LaeA was shown to be a regulator for secondary metabolite production, including products with pharmaceutical and toxic properties (3). Genes homologous to laeA are found in other pathogens that exhibit a filamentous cellular state, including \( Coccidioides immitis \) (GenBank), \( Fusarium sporotrichioides \) (http://www.genome.ou.edu/fsporo.html), and \( Magnaporthe grisea \) (http://www-genome.wi.mit.edu/annotation/fungi/magnapartho.html). These findings indicate that LaeA is a critical developmental regulator of genes involved in fungal environmental propagation and disease in plants and animals.

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