Role of Aspergillus fumigatus DvrA in Host Cell Interactions and Virulence

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The transcription factors that regulate *Aspergillus fumigatus* interactions with host cells and virulence are incompletely defined. We investigated the role of the putative C2H2 transcription factor, DvrA in governing these processes. Although DvrA was identified by its limited homology to *Candida albicans* Bcr1, a $\Delta dvrA$ mutant strain of *A. fumigatus* had wild-type adherence to host constituents in vitro. However, it had increased capacity to damage both endothelial cells and a pulmonary epithelial cell line, as compared to the wild-type strain and a $\Delta dvra::dvra$ complemented strain. This increase in damage required direct contact between the mutant and host cells. The $\Delta dvra$ mutant also stimulated greater CCL20, interleukin 8, and tumor necrosis factor mRNA expression in a pulmonary epithelial cell line compared to the control strains. Also, it was resistant to nikkomycin Z, suggesting an altered cell wall composition. As predicted by these in vitro results, the $\Delta dvra$ mutant had increased virulence and stimulated a greater pulmonary inflammatory response compared to the wild-type strain and $\Delta dvra::dvra$ complemented strains in the non-neutropenic mouse model of invasive pulmonary aspergillosis. These results indicate that DvrA influences *A. fumigatus* virulence as well as its capacity to damage host cells and stimulate a pro-inflammatory response.
INTRODUCTION

Aspergillus fumigatus is the leading cause of invasive pulmonary aspergillosis, a disease that is still associated with a 25% to 35% mortality, even with current antifungal therapy (23, 41). Invasive aspergillosis is initiated by inhalation of conidia, which are deposited on and adhere to the epithelial cell lining of the alveoli. In susceptible hosts, these adherent conidia subsequently germinate, and then invade and damage the pulmonary cells. Host cells respond by synthesizing pro-inflammatory mediators, which recruit phagocytic leukocytes to the foci of infection. Although these phagocytes may kill some of the invading fungi, they can also cause significant tissue destruction and thereby contribute to the pathogenesis of invasive aspergillosis (22, 38).

The molecular mechanisms that mediate the interactions of A. fumigatus with host cells during invasive aspergillosis are incompletely defined. In particular, the regulatory factors that govern the capacity of A. fumigatus to adhere to and damage host cells have not been identified. In the pathogenic yeast Candida albicans, the C2H2 transcription factor, Bcr1 governs the expression of multiple cell surface proteins that mediate biofilm formation, as well as adherence to host cells (20, 21). These proteins include Hwp1, Als1, and Als3 (11, 25, 39, 43). Here, we identified a putative C2H2 transcription factor in A. fumigatus, which shares limited homology with Bcr1. We found that unlike Bcr1, this protein does not regulate adherence to host constituents. Instead, it appears to functions as a negative regulator of host cell damage and stimulation, as well as virulence during invasive pulmonary disease. Hence, we named it DvrA (Damage and Virulence Regulator).
MATERIALS AND METHODS

**Fungal strain and growth conditions.** *A. fumigatus* Af293, originally a clinical isolate, was used as the wild-type strain in these investigations. It was a generous gift from P. T. Magee, University of Minnesota, St. Paul, MN. Conidia were obtained by growing the organism on Sabouraud agar (Difco) at 37°C for 7 d. The conidia were harvested by rinsing with phosphate buffered saline (PBS) containing 0.1% Tween 80 (Sigma-Aldrich). Germlings were prepared as described previously (17). Briefly, freshly harvested conidia were suspended in Sabouraud broth and then added to 150 mm Petri dishes. The Petri dishes were incubated at 37°C in 5% CO₂ for 6 to 7 h until the majority of the conidia had begun to germinate and had produced germ tubes that were approximately 2 conidial diameters in length. The resulting germlings were rinsed with PBS, harvested with a cell scraper, and enumerated using a hemacytometer.

**Strain construction.** A split-marker approach was used to disrupt the first 1,679 bp of the protein coding sequence of *dvrA* (Afu3g09820) in *A. fumigatus* Af293 (5). Briefly, a DNA fragment encompassing 1,578 bp upstream of *dvrA* was PCR amplified from genomic DNA of strain Af293 using primers BCR-F1 and BCR-F2 (Table S1). Similarly, a fragment encompassing 1,451 bp downstream of *dvrA* was amplified using primers BCR-F3 and BCR-F4 (Table S1). Next, the 5’ region of the hygromycin resistance cassette was amplified from plasmid pAN7-1 (28) using primers HYG-F and HY, and the 3’ region of this cassette was amplified using primers HYG-R and YG. The *dvrA* upstream sequence was combined with the 3’ portion of the hygromycin resistance cassette by fusion PCR using primers BCR-F1 and YG. The *dvrA* downstream sequence was combined with the 5’ region of the resistance cassette using primers HY and BCR-F4. *A. fumigatus* Af293 was co-transformed with both fragments by protoplasting (4). Hygromycin-resistant clones were screened for disruption of *dvrA* by PCR.
using the primers BCR-VER1, BCR-VER2 and HY-VER (Table S1). Southern blotting with a 412 bp fragment of the hygromycin resistance cassette was used to confirm homologous integration of the disruption cassette and to assure that only single copy of a disruption cassette was integrated into the genome during transformation (data not shown).

The ΔdvrA mutant was complemented with a wild-type copy of dvrA as follows: The dvrA open reading frame plus 2,506 bp of upstream sequence and 67 bp of downstream sequence was cloned from genomic DNA of strain Af293 by high-fidelity PCR using primers BCRREV-F and BCRREV-R (Table S1). The resulting fragment was cloned into plasmid p402, which contains the phleomycin resistance gene (30). The resulting plasmid was linearized with SspI and used to transform the ΔdvrA mutant. Hygromycin and phleomycin-resistant transformants were selected and proper integration of the plasmid upstream of the disrupted dvrA locus was verified by PCR using primers BCR-VER1, BCR-VER2.

**Real-time PCR.** The time course of dvrA mRNA expression in the wild-type strain was analyzed by real-time PCR. This procedure was also used to verify that dvrA mRNA expression was restored to wild-type levels in the ΔdvrA::dvrA complemented strain, and to determine the transcript levels of ace2, asg3, and ecm33 in the various strains. The real-time PCR experiments were performed as described previously using the primers listed in Table S1 (9). Briefly, conidia were inoculated into Sabouraud broth and incubated at 37°C in a shaking incubator. At selected time points, an aliquot of the organisms was removed for RNA extraction and subsequent cDNA synthesis. Real-time PCR amplification was monitored using SYBR green. The relative gene expression levels were determined by the $2^{-\Delta\Delta CT}$ method using tefl as the reference gene (13, 33).
**Germination rate, radial growth rate, and conidial production.** To determine the germination rate, $10^5$ conidia of the different *A. fumigatus* strains were suspended in either RPMI 1640 medium containing 50% fetal bovine serum or Sabouraud broth. These conidia were added to wells of a 24-well tissue culture plate and incubated at 37°C in 5% CO$_2$. At various time points, the medium above the conidia was aspirated and the organisms were fixed in 3% paraformaldehyde in PBS. The wells were examined using an inverted microscope and 100 organisms per strain were evaluated for germination, which was defined as the production of a germ tube that was at least the one conidial diameter in length.

The radial growth rate and conidial production of the different *A. fumigatus* strains were measured as described previously (9). Briefly, $5 \times 10^3$ conidia were spotted onto Sabouraud, YPD (yeast extract, peptone, D-glucose), or Aspergillus minimal medium (27) agar and incubated at 37°C. The colony diameter was measured daily (4). After a 7 d incubation, the conidia were harvested in a standardized manner and then counted using a hemacytometer.

**Susceptibility to stressors.** The susceptibility of conidia and germlings of the different strains to various stressors was determined by the microdilution method as outlined previously (9). Susceptibility to cell wall, cell membrane, and osmotic stress was determined by adding $5 \times 10^3$ conidia or germlings in Sabouraud broth to separate wells of a 96-well microtiter plate containing 2-fold dilutions of Nikkomycin Z (Sigma-Aldrich; range 1 to 1000 µg/ml), Congo red (Sigma-Aldrich; range 5 to 5000 µg/ml) calcofluor white (Sigma-Aldrich; 5 to 5000 µg/ml), caspofungin (Merck; 0.004 to 4 µg/ml), SDS (0.0002 to 0.2%) or NaCl (0.002 to 2 M). After incubation at 37°C for 2 days, the wells were scored for visually for growth. The minimal inhibitory concentration was determined as the concentration of each compound that caused at least an 80% reduction in growth compared to organisms grown in the absence of the compound.
Susceptibility to nikkomycin Z and Congo red were also tested by the agar dilution approach. Serial 10-fold dilutions of conidia of the various strains were spotted onto YPD agar containing either 75 µg/ml nikkomycin Z or 200 µg/ml Congo red. The plates were incubated at 37°C for 24 h and then imaged. Susceptibility to each stressor was tested at least 3 times.

**Host cells.** The A549 type II pneumocyte cell line was obtained from the American Type Culture Collection and grown in F-12K medium containing 10% fetal bovine serum (Gemini Bio-Products) and penicillin and streptomycin. Endothelial cells were harvested from the veins of human umbilical cords and maintained in M199 medium (Gibco) containing 10% fetal bovine serum, 10% bovine calf serum (Gemini Bio-Products), and 2 mM L-glutamine with penicillin and streptomycin (Irvine Scientific) as previously described (15, 17). All host cells were grown in 5% CO₂ at 37°C.

**Adherence assay.** The capacity of the different strains to adhere to A549 epithelial cells, endothelial cells, gelatin, and fibronectin was determined as previously described method (14). When adherence to epithelial or endothelial cells was determined, the cells were grown to confluency in 6-well tissue culture plates. For adherence to extracellular matrix proteins, the 6-well plates were coated overnight with 1 ml of 0.01 mg/ml gelatin (Sigma) or fibronectin (Becton Dickinson). For adherence to all substrates, 200 conidia or germings in 1 ml of Hank’s balanced salt solution (HBSS; Irvine Scientific) were added to each well. After a 30 min incubation, the wells were rinsed three times with HBSS in a standardized manner and then overlaid with Sabouraud agar. The number of adherent organisms in each well was determined by colony counting after overnight incubation at 37°C. The adherence of each strain was expressed as a percentage of the original inoculum, which was measured by quantitative culture. Each experiment was performed in triplicate and repeated at least 3 times.
**Host cell damage.** The extent of damage to the A549 cells and endothelial cells caused by the various *A. fumigatus* strains was determined a 51 Cr release assay as described previously (9, 17). Briefly, A549 epithelial cells or endothelial cells were grown to confluency in 24-well tissue culture plates and loaded with $^{51}$Cr. After the unincorporated $^{51}$Cr was removed by rinsing, the epithelial cells were infected with $5 \times 10^5$ conidia per well and the endothelial cells were infected with $5 \times 10^5$ germlings per well in their respective culture media. After a 24 h incubation, the medium above the cells was collected and the residual $^{51}$Cr remaining in the host cells was collected by lysing the wells once with 6N NaOH and rinsing twice with RadiacWash (Biodex Medical Systems, Inc.). The rinses from each well were combined and the amount of $^{51}$Cr in the medium and rinses was determined by gamma counting. To measure the spontaneous release of $^{51}$Cr, uninfected host cells exposed to medium alone were processed in parallel. The percent specific release of $^{51}$Cr was calculated using the following formula: (experimental release – spontaneous release) / (total incorporation – spontaneous release) × 100. Each experiment was performed in triplicate three different times.

To determine the role of soluble factors produced by *A. fumigatus* in inducing host cell damage, the organisms were added to cell culture inserts (2 µm pore size; Nunc) that were suspended approximately 1 mm above $^{51}$Cr-loaded A549 pulmonary epithelial cells (17). The extent of epithelial cell damage was determined after 24 h, as described above.

**Epithelial cell stimulation.** The capacity of each strain of *A. fumigatus* to stimulate epithelial cell cytokine mRNA expression was determined by real-time PCR. The A549 cells were grown to 80% confluency in 6-well tissue culture plates and infected with $5 \times 10^5$ conidia of each strain in serum free F-12K medium. After a 24 h incubation, the epithelial cell RNA was harvested using TRIzol (Gibco-Invitrogen), followed by phenol chloroform extraction and
ethanol precipitation. Cytokine mRNA levels were analyzed by real-time RT-PCR using Taqman probes (Applied Biosystems) for CCL20 (H00171125_m1), tumor necrosis factor (TNF; Hs00174128_m1), and interleukin-8 (IL-8; Hs00174103_m1). Relative gene expression was determined by the $2^{-\Delta\Delta CT}$ method using 18s rRNA as the reference.

**Galleria melonella model of invasive aspergillosis.** The *A. fumigatus* strains were screened for alterations in virulence in *G. melonella* larvae (Magazoo) (19, 29). Larvae at the sixth instar of development were injected through the last proleg with $10^6$ conidia suspended in 10 µl PBS using a Hamilton syringe. The larvae were kept at 37°C in a humidified chamber and monitored daily for survival. The virulence of each strain was tested twice in total of 20 larvae, and 20 additional larvae were injected with PBS alone as a negative control.

**Mouse model of invasive pulmonary aspergillosis.** The virulence of the various strains was assessed in a mouse model of invasive aspergillosis using two different immunosuppressive regimens (7, 35, 36). In the non-neutopenic regimen, male Balb/c mice (Taconics Labs) were immunosuppressed with cortisone acetate (Sigma-Aldrich) administered at 500 mg/kg subcutaneously every other day, starting on day -4 relative to infection and finishing on day +4. In the neutropenic regimen, the mice were immunosuppressed with cortisone acetate administered subcutaneously at 250 mg/kg on days -2 and +3, and cyclophosphamide (Western Medical Supply) administered intraperitoneally at 250 mg/kg on day -2 and 200 mg/kg on day +3. In both models of infection, the mice received daily subcutaneous injections of 5 mg of ceftazidime while they were immunosuppressed to prevent bacterial infections. The immunosuppressed mice were infected by placing them for 1 h in an acrylic chamber into which $1.2 \times 10^{10}$ conidia were aerosolized. Control mice were immunosuppressed with either of the two regimens, but not infected.
In the survival studies, 11 to 13 mice were infected with each strain of *A. fumigatus*. Shortly after inoculation, 3 mice from each group were sacrificed, after which their lungs were homogenized and quantitatively cultured to verify conidial delivery to the lungs. In these experiments, a median of $4.1 \times 10^3$ conidia (interquartile range $1.6 \times 10^3$ to $4.9 \times 10^3$ conidia) was delivered to the lungs of each mouse, and the pulmonary conidial delivery was similar for all strains of *A. fumigatus*. The remaining mice were monitored for survival. The survival experiments were repeated twice and the results were combined. The animal studies were approved by the Institutional Animal Use and Care Committee, and performed according to the National Institutes of Health guidelines for animal housing and care.

**Pulmonary fungal burden and inflammatory response.** To determine the pulmonary fungal burden and host response to infection, the mice were immunosuppressed with cortisone acetate alone and 9 to 12 mice per strain were infected as above. After 4 days of infection, the mice were sacrificed and their lungs were harvested, weighed, and then homogenized in ice cold PBS containing protease inhibitor cocktail (Sigma-Aldrich). The homogenates were clarified by centrifugation and aliquots of the resulting supernatants were stored at -80°C for later analysis.

The pulmonary fungal burden was assessed by measuring the pulmonary galactomannan content using the Platelia *Aspergillus* enzyme immunoassay (BioRad). The assay was performed following our previously described method (9, 34). Briefly, each pulmonary homogenate was diluted 1:10 in ultra-pure water and processed according to the manufacturer's instructions. To determine the relative galactomannan content per gram of lung tissue, the resulting optical densities were compared with a standard curve, which was made using serial dilutions of a pool of lung homogenates from 5 heavily infected immunosuppressed mice (7 days after intranasal infection with strain Af293).
In preliminary experiments, we determined that the ΔdvrA mutant released a similar amount of galactomannan as did strain Af293 when grown in vitro (Supplementary Fig. S1). Conidia from each strain were inoculated into RPMI 1640 medium containing MOPS, to achieve a final concentration of \(10^5\) conidia per mL and then incubated in an orbital shaker at 37°C. After 24, 48 or 72 h, the conditioned medium was collected by filtration and stored at -80°C. The galactomannan content of each sample was determined as described above and normalized to the dry weight of the organisms in culture. The experiment was performed two times.

The pulmonary inflammatory response to the various \(A. fumigatus\) strains was assessed by measuring the myeloperoxidase (MPO) and cytokine content of the lungs. The MPO content was used to assess the accumulation of phagocytes during infection (12, 40). The MPO levels in the lung homogenates were measured by enzyme immunoassay (Cell Sciences). The pulmonary content of interleukin 6 (IL-6), IL-10, IL-12, interferon \(\gamma\), tumor necrosis factor (TNF), and monocyte chemoattractant protein 1 (MCP-1) was measured using the mouse inflammation cytometric bead array kit (BD Biosciences).

Statistical analyses. The results of the in vitro experiments were compared by Analysis of Variance followed by pair-wise comparisons using the student’s t-test. Differences in survival were analyzed using the Log-Rank test, and the pulmonary fungal burden, MPO, and cytokine data were analyzed with the Wilcoxon Rank Sum test. \(P\) values of \(\leq 0.05\) were considered to be significant.

RESULTS

Identification and disruption of \(A. fumigatus\) dvrA. The closest \(A. fumigatus\) homolog of \(C. albicans\) BCR1 was identified by BLAST searches of the \(A. fumigatus\) genome. Reciprocal
BLAST searches verified that the *A. fumigatus* gene *dvrA* (Afu3g09820; Damage and Virulence Regulator) shared the closest homology to *BCR1* within the *A. fumigatus* genome. The products of *dvrA* and *BCR1* share significant homology in their C2H2 zinc finger domains, whereas there are very limited regions of homology outside of this region (Fig. 1). Interestingly, orthologs of *dvrA* are present in other species of *Aspergillus*, as well as other filamentous fungal pathogens, such as *Penicillium marneffei*, and *Coccidioides immitis*. The products of these *dvrA* orthologs are predicted to have much greater similarity to DvrA than to Bcr1 (Fig. 1).

The time course of *dvrA* mRNA levels in *A. fumigatus* Af293 was analyzed by real-time RT-PCR. When the organism was grown in Sabouraud broth, relatively low *dvrA* mRNA levels were detected in swollen conidia (Fig. 2A). The level of *dvrA* expression increased as the conidia germinated and formed mature hyphae, peaking at 40 h of incubation. After 48 h, *dvrA* transcript levels declined, possibly due to depletion of nutrients from the medium.

To determine the function of *dvrA*, we constructed a Δ*dvrA* deletion mutant. We also constructed a Δ*dvrA::dvrA* complemented mutant to verify that any phenotype of the mutant was due to disruption of *dvrA*. Using real-time RT-PCR, we confirmed the absence of *dvrA* mRNA in the Δ*dvrA* mutant and restoration of *dvrA* mRNA to wild-type levels in the Δ*dvrA::dvrA* complemented strain (data not shown).

The Δ*dvrA* mutant had normal germination, growth, and conidiation. Next, we investigated whether *dvrA* influences the growth, development, and stress resistance of *A. fumigatus*. The Δ*dvrA* conidia germinated at a similar frequency to wild-type conidia in both RPMI 1640 medium containing 50% fetal bovine serum (Fig. 2B) or Sabouraud broth and produced hyphae that were morphologically similar to wild-type hyphae (data not shown). Also,
the ΔdvrA mutant had normal radial growth rate on three different media and these colonies produced similar number of conidia as did the wild-type strain (data not shown).

**Deletion of dvrA resulted in increased resistance to nikkomycin Z.** The susceptibility of the ΔdvrA mutant to environmental stress was examined. When this mutant was tested in a microbroth dilution assay using the endpoint of at least 80% growth inhibition at 48 h, it had wild-type susceptibility to SDS, NaCl, caspofungin, calcofluor white, Congo red, and nikkomycin Z (data not shown). However, we noticed that after exposure to nikkomycin Z for 24 h, the majority of the hyphae of the ΔdvrA mutant had normal morphology in all nikkomycin Z concentrations tested (up to 1000 µg/ml). In contrast, most of the hyphae of the wild-type strain had ballooning of the cell walls when exposed to 63 µg/ml nikkomycin Z, and hyphae of the ΔdvrA::dvrA complemented strain had abnormal morphology at 31 µg/ml. To verify these results, we assessed the nikkomycin Z susceptibility of the three strains in an agar dilution assay. As expected, the ΔdvrA mutant grew better in the presence of nikkomycin Z compared to the wild-type and ΔdvrA::dvrA complemented strains (Fig. 3). We also tested the growth of the ΔdvrA mutant in the presence of Congo red as a control in the agar dilution assay. This strain grew similarly to the control strains in this assay, as predicted by the microbroth dilution studies. The resistance to nikkomycin Z, which is a chitin synthase inhibitor, suggests that the ΔdvrA mutant has an altered cell wall, and possibly increased chitin content.

**Adherence of A. fumigatus was not influenced by deletion of dvrA.** In *C. albicans* and *Candida parapsilosis*, *BCR1* is required for the normal expression of multiple cell surface proteins, especially adhesins (8, 20, 21). Therefore, we investigated the adherence of the ΔdvrA mutant to biologically relevant host substrates. We found that the adherence of ΔdvrA conidia and germlings was similar to that of the wild-type strain for all substrates tested, including
vascular endothelial cells, pulmonary epithelial cells, fibronectin, and gelatin (data not shown). Therefore, DvrA does not appear to govern adherence in A. fumigatus.

The ΔdvrA mutant caused increased damage to pulmonary epithelial cells and vascular endothelial cells. To determine if DvrA influences other interactions of A. fumigatus with host cells, we measured the extent of damage to the A549 pulmonary epithelial cell line caused by conidia of the ΔdvrA mutant. We also determined the capacity of ΔdvrA germlings to damage human umbilical vein endothelial cells. Germlings were used instead of conidia in the endothelial cell experiments because hyphae are the main form of the organism that interacts with endothelial cells during invasive aspergillosis (10, 13). We found that the ΔdvrA mutant caused nearly 2-fold more damage to the pulmonary epithelial cells and 1.4-fold more damage to the endothelial cells compared to either the wild-type strain and the ΔdvrA::dvrA complemented strain (p < 0.0001) (Fig. 4). These results suggest that DvrA negatively regulates A. fumigatus factors that mediate host cell damage.

In both the epithelial and endothelial cell experiments, the extent of germination and hyphal elongation of the ΔdvrA mutant were similar to the wild-type strain, as determined by microscopic examination. Therefore, the increased host cell damage of the ΔdvrA mutant was not due to accelerated germination. We next investigated whether the increased host cell damage caused by the ΔdvrA mutant was due to the release of soluble factors, such as mycotoxins (2, 24). Conidia of the ΔdvrA mutant were incubated for 24 h in cell culture inserts that were suspended over the pulmonary epithelial cells. We found that when contact between the organisms and the epithelial cells was prevented, no detectable epithelial cell damage was induced even after 24 h of incubation (data not shown). Thus, the enhanced capacity of the
$\Delta dvrA$ mutant to induce epithelial cell damage does not appear to be due to increased release of toxic soluble factors.

**The $\Delta dvrA$ mutant stimulated increased pulmonary epithelial cell cytokine gene expression.** Although many of the manifestations of invasive aspergillosis are due to direct damage of host cells by *A. fumigatus*, it is probable that the host inflammatory response to the organism also contributes to the pathogenesis of this disease in some hosts. We have previously found that A549 pulmonary epithelial cells respond to infection with wild-type *A. fumigatus* by increasing mRNA levels of the chemokines CCL20 and IL-8, as well as the pro-inflammatory cytokine TNF (14). In current study, we found that the $\Delta dvrA$ mutant induced significantly greater CCL20, IL-8 and TNF transcript levels than did the wild type and $\Delta dvrA::dvrA$ complemented strains ($p < 0.001$) (Fig. 5). These findings indicate that the $\Delta dvrA$ mutant induces a very strong inflammatory response in vitro.

**The $\Delta dvrA$ mutant had increased virulence during invasive aspergillosis.** The increased capacity of the $\Delta dvrA$ mutant to damage and stimulate host cells in vitro suggested that this mutant might have increased virulence. To test this hypothesis, we first infected *G. melonella* larvae with the $\Delta dvrA$ mutant as a screening test for alterations in virulence. We found that the survival of larvae injected with the $\Delta dvrA$ mutant was significantly shorter than that of larvae injected with the wild-type or $\Delta dvrA::dvrA$ complemented strain ($p < 0.0001$) (Fig. 6).

To verify that deletion of *dvrA* resulted in increased virulence, we tested the $\Delta dvrA$ mutant in the mouse model of invasive pulmonary aspergillosis. The virulence of this mutant was analyzed in mice that were immunosuppressed with two different regimens. These different regimens were used because previous studies indicate that the type of immunosuppression
significantly alters the pathogenesis of invasive pulmonary aspergillosis in mice (3, 7, 37). In the first regimen, the mice were given high dose cortisone acetate, but they were not neutropenic. In the second regimen, the mice were administered cyclophosphamide to render them neutropenic, as well as a lower dose of cortisone acetate.

When the non-neutropenic mice were infected with an aerosol of conidia from the \( \Delta dvrA \) mutant their survival was significantly shorter than mice infected with either the wild-type or \( \Delta dvrA::dvrA \) complemented strains \( (p < 0.001) \) (Fig. 7A). In contrast, when neutropenic mice were infected with the \( \Delta dvrA \) mutant, their survival was similar to that of the control mice \( (p > 0.35) \) (Fig. 7B). Therefore, the effect of \( dvrA \) on virulence during invasive pulmonary aspergillosis is dependent on the immune status of the host; \( dvrA \) influences virulence in non-neutropenic mice, but not in neutropenic mice.

The \( \Delta dvrA \) mutant induced an exaggerated inflammatory response in non-neutropenic mice. The in vitro data indicated that the \( \Delta dvrA \) mutant induced an abnormally strong inflammatory response in A549 pulmonary epithelial cells. These results suggested that the increased mortality of non-neutropenic mice infected with this mutant may have been due in part to an overexuberant inflammatory response. To investigate this possibility, we determined the pulmonary fungal burden, as well as pulmonary levels of MPO (a marker of phagocyte accumulation (12, 40)) and pro-inflammatory cytokines in these mice. After 4 days of infection, the pulmonary galactomannan content of mice infected with the \( \Delta dvrA \) mutant was significantly higher than mice infected with the wild-type strain \( (p = 0.018) \), but not mice infected with the \( \Delta dvrA::dvrA \) complemented strain \( (p = 0.21) \) (Fig. 8). Also, the pulmonary content of MPO, IL-6, TNF, and MCP-1 were significantly greater in the mice infected with the \( \Delta dvrA \) mutant compared to both the wild-type and \( \Delta dvrA::dvrA \) complemented strain \( (p < 0.03 \) for all.
comparisons) (Fig. 8). The pulmonary IL-12 levels were similar in mice infected with all 3 strains of \textit{A. fumigatus} (Fig. 8), and the levels of IL-10 and interferon γ were undetectable (data not shown). Collectively these results suggest that the shortened survival of mice infected with the Δ\textit{dvrA} mutant was due to both increased pulmonary fungal burden and enhanced inflammatory response.

The increased virulence of the Δ\textit{dvrA} mutant was not due to reduced expression of genes previously known to inhibit virulence. Deletion of \textit{ace2}, \textit{ags3}, and \textit{ecm33} in \textit{A. fumigatus} is known to result in increased virulence in experimental models of invasive aspergillosis (9, 18, 31). Therefore, it was possible that the expression of one or more of these genes might be reduced in the Δ\textit{dvrA} mutant and thereby contribute to the increased virulence of this strain. To investigate this possibility, we compared the relative transcript levels of \textit{ace2}, \textit{ags3}, and \textit{ecm33} in the Δ\textit{dvrA} mutant with the wild-type and Δ\textit{dvrA}::\textit{dvrA} complemented strains. All three genes were expressed at similar levels in all three strains of \textit{A. fumigatus} (Fig. 9), indicating that the increased virulence of the Δ\textit{dvrA} mutant is due to a unique mechanism that is unrelated to decreased expression of \textit{ace2}, \textit{ags3}, or \textit{ecm33}.

DISCUSSION

The results of these investigations demonstrate that deletion of \textit{A. fumigatus dvrA} results in enhanced virulence in the non-neutropenic mouse model of invasive pulmonary disease. Based on the homology between \textit{A. fumigatus DvrA} and \textit{C. albicans Bcr1}, we had expected that the Δ\textit{dvrA} mutant would have reduced adherence. However, we could find no evidence that DvrA governs the adherence properties of either conidia or germlings. While DvrA and Bcr1 share significant homology in their predicted DNA binding domains, the other regions of these
proteins have only scattered areas of homology. Thus, the phenotypic data and sequence data both suggest that DvrA has a different function than Bcr1. Interestingly, close orthologs of DvrA are present in other *Aspergillus* spp. as well as other medically important filamentous fungi, including *Penicillium marneffei* and *Coccidioides immitis*. This close homology suggests that the DvrA orthologs of these organisms may have similar function to *A. fumigatus* DvrA.

The enhanced virulence of the ∆dvrA mutant was likely due in part to its increased capacity to damage host cells. We found that this mutant caused greater damage to both endothelial and pulmonary epithelial cells in vitro. The results of experiments with cell culture inserts demonstrated that the increased capacity of the ∆dvrA mutant to damage pulmonary epithelial cells was not due to the enhanced release of soluble toxins. We have found previously that *A. fumigatus* must be in direct contact with host cells to damage them (16, 17). Therefore, it is probable that the enhanced capacity of the ∆dvrA mutant to damage host cells was due to an alteration in its cell surface or cell wall. Consistent with this hypothesis, we found that the ∆dvrA mutant was resistant to the chitin synthase inhibitor, nikkomycin Z, which suggests that DvrA influences the composition of the *A. fumigatus* cell wall.

The capacity of different *C. albicans* strains to damage endothelial cell in vitro is a fairly good predictor of their virulence in the mouse model of disseminated candidiasis (32). Our recent data suggest a similar relationship exists between host cell damage and virulence for *A. fumigatus*. Both ∆dvrA and ∆ace2 mutants cause increased damage to endothelial cells in vitro and both of these mutants have increased virulence in the non-neutopenic mouse model of invasive pulmonary aspergillosis (9). Also, we have found that a ∆medA mutant has reduced capacity to damage pulmonary epithelial cells in vitro and attenuated virulence in non-neutopenic mice (14). These results suggest that determining the capacity of an *A. fumigatus*
mutant to damage endothelial or pulmonary epithelial cells in vitro could be used as a screening test for alterations in virulence. These data further suggest that the ability of *A. fumigatus* to damage host cells is an important virulence determinant of this organism.

In non-neutropenic hosts with invasive pulmonary aspergillosis, significant tissue destruction is caused not only by the fungus, but also by toxic products released by activated neutrophils (3, 7, 37),(3, 37, 38). It was thus notable that the Δ*dvrA* mutant induced exaggerated pro-inflammatory cytokine production in the lungs of non-neutropenic mice. This excessive inflammatory response likely contributed to the accelerated mortality of mice infected with the Δ*dvrA* mutant. The higher pulmonary fungal burden of mice infected with the Δ*dvrA* mutant likely contributed to their augmented inflammatory response. However, this mutant also stimulated a stronger pro-inflammatory response in pulmonary epithelial cells in vitro than did the wild-type strain, even at the same inoculum. Therefore, the Δ*dvrA* mutant appears to have an intrinsic capacity to stimulate a supra-normal inflammatory response in host cells.

The resistance of the Δ*dvrA* mutant to nikkomycin Z suggests that it may have an increased amount of chitin in its cell wall. In *C. albicans*, chitin induces an inflammatory response by stimulating monocytes to synthesize IL-1β (42). Recently, it has been reported that a Δ*pmrA* mutant of *A. fumigatus* has increased cell wall chitin content, yet normal virulence in the mouse model of invasive pulmonary aspergillosis (26). However, the virulence of this mutant was only tested in neutropenic mice. While our Δ*dvrA* mutant also had normal virulence in neutropenic mice, it had increased virulence in non-neutropenic mice. Thus, it is possible that the relationship between increased chitin content and enhanced *A. fumigatus* virulence may only be detectable in non-neutropenic hosts, where the inflammatory response induced by the fungus plays a key role in the pathogenesis of the infection.
Only a limited number of genes have been described that functioned as negative regulators of *A. fumigatus* virulence. Deletion of *ace2*, *ags3*, and *ecm33* is associated with enhance virulence in murine models of invasive pulmonary aspergillosis (9, 18, 31). Recently we reported that an *A. fumigatus* double mutant lacking the trehalose biosynthesis genes *tpsA* and *tpsB* was also hypervirulent in mice (1). This mutant had reduced expression of *ags3*, suggesting that a reduction in cell wall α(1-3)glucans contributed to its hypervirulent phenotype. Our finding that the ∆dvrA mutant expressed *ace2*, *ags3*, and *ecm33* at wild-type levels, makes it unlikely that these genes contribute to increased virulence of this strain. However, a common finding with the ∆dvrA, ∆ace2, ∆ags3, ∆ecm33, and ∆tpsAB mutants is an alteration in the fungal cell wall (1, 6, 9, 18, 31). Furthermore, the ∆dvrA, ∆ace2, ∆ags3, ∆tpsAB mutants all induced an exaggerated host inflammatory response (1, 9, 18). Collectively, these results suggesting that the composition of the cell wall plays a key role in determining the host inflammatory response to and virulence of *A. fumigatus*.

In summary, *A. fumigatus* DvrA has negative influence on virulence during invasive pulmonary disease, and functions independently of the three known virulence modulating genes, *ace2*, *ags3*, and *ecm33*. Although DvrA does not govern the adherence of *A. fumigatus*, it likely influences virulence by governing the capacity of the organism to damage host cells and stimulate a pro-inflammatory response. Studies to identify DvrA target genes that are responsible for these host cell interactions are currently in progress.
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Figures Legends

FIG. 1. Alignment of DvrA orthologs from *Aspergillus* spp., *Penicillium marneffei*, and *Coccidioides immitis* with *Candida albicans* Bcr1. Shaded areas indicate amino acids that are conserved among all of the indicated fungi. Boxed areas indicate amino acids that are conserved in ≥ 75% of the organisms. Thick horizontal lines indicate the predicted zinc cluster DNA binding domains.

FIG. 2. Time course of dvrA mRNA levels in wild-type *A. fumigatus* (A) and the effect of dvrA disruption on germination (B). (A) Conidia of strain Af293 were incubated in Sabouraud broth at 37°C in a shaking incubator. At the indicated time points, an aliquot of the organisms was removed for RNA extraction. The dvrA transcript levels were determined by real-time PCR using *tefl* as the endogenous control gene. Results are mean ± SD of two biological replicates, each measured in duplicate. (B) Conidia of the three strains of *A. fumigatus* were incubated in RPMI 1640 medium containing 50% fetal bovine serum at 37°C in 5% CO₂. At the indicated time points, the organisms were fixed and the percentage of cells that had germinated was determined. Results are mean ± SD of 3 independent experiments.

FIG. 3. Reduced susceptibility to nikkomycin Z of the ΔdvrA mutant. Serial 10-fold dilutions of conidia of the indicated strains were spotted onto YPD agar containing either 75 µg/ml nikkomycin Z or 200 µg/ml Congo red. Images of the plates after a 24 h incubation at 37°C.
FIG. 4. The ΔdvrA mutant causes increased damage to pulmonary epithelial cells and vascular endothelial cells. (A) The A549 pulmonary epithelial cell line was infected with conidia of the indicated strains of *A. fumigatus* for 24 h and the extent of epithelial cell damage was measured using a ^51^Cr release assay. (B) Extent of endothelial cell damage following a 24 h incubation with germlings of the indicated strains. Results are the mean ± SD of three experiments, each performed in triplicate. *P < 0.02 compared to Af293 or the ΔdvrA::dvrA complemented strain.

FIG. 5. Increased pulmonary epithelial cell stimulation by the ΔdvrA mutant. A549 pulmonary epithelial cells were incubated with conidia of the indicated *A. fumigatus* strains for 12 h, after which the mRNA levels of CCL20, IL-8 and TNF were determined by real-time PCR. Results are the mean ± SD of three experiments, each performed in duplicate. *P < 0.001 compared to Af293 or the ΔdvrA::dvrA complemented strain.

FIG. 6. Increased virulence of the ΔdvrA mutant in *G. melonella*. Survival of *G. melonella* larvae following inoculation with conidia of each the indicated strains of *A. fumigatus*. Results are from 20 larvae per strain of *A. fumigatus*. *P < 0.0001 compared to Af293 or the ΔdvrA::dvrA complemented strain.
FIG. 7. Influence of DvrA on the survival of mice with invasive pulmonary aspergillosis. Mice were immunosuppressed with either cortisone acetate alone (A) or cortisone acetate and cyclophosphamide (B) and then infected with the indicated strains of *A. fumigatus* in an aerosol chamber. Results are the combined data from two independent experiments, for a total of 16 mice per strain in (A) and 20 mice per strain in (B). *P < 0.001 compared to Af293 or the ΔdvrA::dvrA complemented strain.

FIG. 8. Effects of DvrA on pulmonary fungal burden, phagocyte accumulation, and cytokine content in mice with invasive aspergillosis. Mice were immunosuppressed with cortisone acetate and then infected with the indicated *A. fumigatus* strains in an aerosol chamber. After 4 d of infection, the mice were sacrificed, after which the amount of galactomannan, myeloperoxidase, IL-6, TNF, MCP-1, and IL-2 in lung homogenates was measured. Results are the median ± the interquartile range of 9 to 12 mice per strain of *A. fumigatus*. *P = 0.018 compared to Af293 and \( p = 0.21 \) compared to the ΔdvrA::dvrA complemented strain; **\( p < 0.03 \) compared to Af293 or the ΔdvrA::dvrA complemented strain. Abbreviations: GM, galactomannan; MPO, myeloperoxidase.

FIG. 9. Transcript levels of *ace2*, *ags3*, and *ecm33*. Conidia of the indicated strains were incubated in Sabouraud broth at 37°C in a shaking incubator. After 24 h, an aliquot of the organisms was removed for RNA extraction. The transcript levels of the indicated genes were determined by real-time PCR using *tef1* as the endogenous control gene. Results are mean ± SD of two biological replicates, each measured in triplicate.