Deletion of the Regulatory Subunit of Protein Kinase A in Aspergillus fumigatus Alters Morphology, Sensitivity to Oxidative Damage, and Virulence

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Aspergillus fumigatus is an important opportunistic fungal pathogen. The cAMP-dependent protein kinase (PKA) signaling pathway plays an important role in regulating morphology, growth, and virulence in a number of fungal pathogens of plants and animals. We have constructed a mutant of A. fumigatus that lacks the regulatory subunit of PKA, pkaR, and analyzed the growth and development, sensitivity to oxidative damage, and virulence of the mutant, along with those of the wild type and a complemented mutant. Both growth and germination rates of the mutant are reduced, and there are morphological abnormalities in conidiophores, leading to reduced conidiation. Conidia from the ApkaR mutant are more sensitive to killing by hydrogen peroxide, menadione, paraquat, and diamide. However, the hyphae of the mutant are killed to a greater extent only by paraquat and diamide, whereas they are less susceptible to the effects of hydrogen peroxide. In an immunosuppressed mouse model, intranasally administered conidia of the mutant are significantly less virulent than those of the wild type or a complemented mutant. Unregulated PKA signaling is detrimental to the virulence of A. fumigatus, perhaps through the reduced susceptibility of the mutant to damage by oxidizing agents and reduced growth kinetics.

Aspergillus fumigatus is an important fungal pathogen of immunocompromised hosts (23, 34). Despite the recent introduction of newer antifungals with anti-Aspergillus activity, the morbidity and mortality of invasive aspergillosis (IA) remains high, especially once the infection has disseminated (7). In nature, the fungus plays a key role in the compost cycle by recycling carbon and nitrogen from plant material (24, 42). In this environment, A. fumigatus is likely to be exposed to broad fluctuations in pH, temperatures up to 50°C, and reactive oxygen species (24, 33, 45). These stressors are not unlike the kinds of factors an organism might encounter in vivo (13). Therefore, adaptive mechanisms that confer resistance to environmental stress may contribute to the efficient colonization and persistence of the organism in the human host.

The cyclic AMP-dependent protein kinase (PKA) is a well-known regulator of the stress response in eukaryotes. PKA is a heterotetramer, made up of a dimer of regulatory subunits and two catalytic subunits. Fungal regulatory subunits are homologues of mammalian type II subunits, based on the autoinhibition site (29). When cAMP binds to the regulatory subunits, a conformational change occurs, which releases the catalytic subunits to autophosphorylate and to phosphorylate downstream targets. PKA signaling in Saccharomyces cerevisiae regulates the general stress control pathway (11, 26). Mutants lacking BCYI, the gene encoding the regulatory subunit of PKA in S. cerevisiae, have unregulated PKA activity; these mutants are pseudohyphal in morphology and hypersensitive to killing with hydrogen peroxide (15, 44).

The PKA pathway also regulates morphology and virulence in a number of fungal pathogens of humans and plants. PKA signaling in Candida albicans has been shown to regulate efg1-controlled morphological responses (22). Deletion of the regulatory subunit of PKA in C. albicans, in a strain also lacking the tpk2 catalytic subunit, results in defective hyphal formation (5). Because efg1 regulates transcription of a number of hypha-specific, virulence-associated traits, it is not surprising that mutations in components of PKA would have reduced virulence (19). The PKA pathway regulates mating, virulence factor production, and virulence in serotype A of Cryptococcus neoformans. In serotype D, PKA regulates mating and some virulence factors but not virulence itself. Indeed, mutants of serotype A that lack the regulatory subunit of PKA overproduce capsule and are hypervirulent, whereas those same mutants in serotype D have wild-type (WT) virulence (16). In the plant pathogen Ustilago maydis, deletion of the regulatory subunit of PKA leads to a multiple-budding phenotype and the inability to form galls in colonized plants, which was reflected in reduced disease scores (14).

Within the genus Aspergillus, analysis of PKA signaling has been limited to Aspergillus niger, Aspergillus nidulans, and Aspergillus fumigatus (2, 21, 27, 40). Deletion of one or more catalytic subunits in each of these fungi has been shown to decrease growth, to reduce the tolerance to oxidative stress, and, in A. fumigatus, to reduce virulence, perhaps by modulation of the expression of polyketide synthase (18, 20). Interestingly, in A. nidulans, deletion of pkaA, one of the catalytic subunits, or overexpression of pkaB, the second catalytic subunit, leads to increased sensitivity to oxidative stress (27). We have previously shown with A. fumigatus that transcripts for the regulatory subunit of PKA, pkaR, are up-regulated when the
fungus is grown in the presence of endothelial cells or pulmonary epithelial cells (29, 36). In this study, we have used a mutant of A. fumigatus that lacks pkaR to test the hypothesis that tightly controlled PKA activity is required for the oxidative stress response and virulence of the organism. Here we report that a ΔpkaR mutant has reduced growth and germination rates, increased susceptibility to oxidative stress, and reduced virulence in an immunosuppressed mouse model of IA.

MATERIALS AND METHODS
Organism and growth conditions. Aspergillus fumigatus WT strain H237 used in this study is a clinical isolate. All strains were grown in Aspergillus minimal medium (AMM), modified to contain 10 mM ammonium tartrate as the nitrogen source (11). To grow hyphae, a 1% agarose gel and transferred to a nylon mem-

by phenol-chloroform extraction. Restriction digests of genomic DNA were fractionated on a 1% agarose gel and transferred to a nylon mem-

mycelial pellets by phenol-chloroform extraction. Restriction digests of genomic DNA were fractionated on a 1% agarose gel and transferred to a nylon mem-

fect the region of digestion with XhoI and XbaI, and the overhangs were filled in (46). The coding region of pkaR was digested with SpeI and filled in, and the phleomycin marker was removed from plasmid pMAD9 by digestion with XhoI and XbaI, and the overhags were filled in (46). The coding region of pkaR was digested from pSL1180-pkaR by digestion with SmaI and SacI, the SacI site was blunted, and the hygromycin cassette was cloned into the modified Sma-SacI-cut DNA. This plasmid was digested with EcoRI to release the hygromycin resistance gene and cloned into pCR 2.1-TOPO (Invitrogen, Carlsbad, CA). The PCR product was moved as a SpeI-XhoI frag-

imens were treated with a phosphorimager (Storm; GE Healthcare Bioscience). Activity was measured by kemptide phosphorylation (PepTag assay for nonradioactive de-

tincted with undigested pSL1180-phleo, and a phleomycin-resistant colony that showed homologous reconstitution of the pkaR gene by Southern blotting was designated pkaR C. Southern blotting analysis. Genomic DNA was extracted from the crushed mycelial pellets by phenol-chloroform extraction. Restriction digests of genomic DNA were fractionated on a 1% agarose gel and transferred to a nylon mem-

ity was measured with the bicinchoninic acid method (Pierce Chemicals, Rockford, IL) as described by the manufacturer. PKA enzyme activity was measured by kemptide phosphorylation (PepTag assay for nonradioactive de-

 RESULTS
Construction of the pkaR isogenic set. In order to create a strain of A. fumigatus that lacked pkaR, a disruption plasmid was constructed by replacing approximately 85% of the PkaR coding region with a hygromycin resistance cassette and using it to transform WT protoplasts (Fig. 1A and B). Monoclonal isolates of hygromycin-resistant colonies were screened for homologous recombination by PCR (data not shown) and confirmed by Southern blotting (Fig. 1D). Single-copy, homolo-

gous integration was confirmed by reprobing the blots with a DNA probe and washed under stringent con-

Developmental analysis of isogenic set. Cultures of each member of the isogenic set were grown for analysis of asexual development as previously described (29). Preparations enriched for the developmental stages were photographed using differential interference contrast microscopy.

PKA activity assay. A. fumigatus strains were grown in liquid AMM at 37°C and harvested at the same growth stage, 8 h for the WT and pkaR C strains and 16 h for the ΔpkaR strain. Mycelium was harvested and washed with ice-cold water twice, dried on filter paper, frozen in liquid nitrogen, and ground with a mortar and pestle. Crushed hyphae were suspended in extraction buffer (25 mM Tris-HCl, pH 7.4, 1 mM dithiothreitol, 1 mM EDTA) and centrifuged at 20,000 × g for 15 min in the cold. Protein was measured with the bicinchoninic acid method (Pierce Chemicals, Rockford, IL) as described by the manufacturer. PKA enzyme activity was measured by kemptide phosphorylation (PepTag assay for nonradioactive de-

posure was expressed as U per milligram of protein. Values were compared using a paired t test (SigmaStat).

Analysis of sensitivity to oxidative stress. Conidia from WT, ΔpkaR and pkaR C+ strains were harvested with sterile water from 5-day-old AMM plates, filtered through two layers of Miracloth (EMD Biosciences, San Diego, CA), and counted with a hemacytometer. Conidia were put on ice until treatment. The sensitivity of conidia and hyphae to oxidative stress was assayed separately, as previously described (32). For conidia, 105 were mixed with an oxidizing agent, incubated at 37°C for 30 min, and then diluted and plated on AMM plates, followed by incubation at 30°C. Colonies arising from surviving conidia were counted for up to 4 days. The sensitivity of hyphae to oxidative stress was measured by allowing conidia to form germ tubes at 30°C on AMM plates (24 h for WT and pkaR C strains and 30 h for the ΔpkaR strain). All plates were overlaid with 10 ml of the different stressors, incubated for 10 min at 30°C, washed twice with water, and then incubated at 30°C for up to 3 days. Control plates were overlaid with water, and the colony counts from these plates were set as 100%. Other treatment groups were compared to the water control for that strain. Each assay was performed in triplicate. Statistical significance was assessed by analysis of variance on square-root transformed proportional data, followed by post hoc, pairwise analysis using the Tukey Test (Instat; San Diego, CA). Differences were considered significant when P values were <0.05.

Animal infection model. Charles River CF-1 female mice weighing 20 to 24 g were immunosuppressed by intraperitoneal injection of cyclophosphamide (150 mg kg−1), Cytosan (Baxter Healthcare Corporation, Deerfield, IL) to determine rates of dissemination, and the genotypes of isolates were confirmed by drug resistance phenotype analysis. The statistical significance of the survival data was assessed by Kruskal-Wallis with pairwise analysis performed post hoc by using Dunn’s procedure (SigmaStat).

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PKA activity is not regulated by cAMP in the ΔpkaR mutant. Deletion of the regulatory subunit of PKA would be expected to result in loss of regulation of the PKA activity by cAMP. In order to test this prediction, hyphal extracts were prepared from each member of the isogenic set. Kemptide phosphorylation was used to measure PKA activity in each extract, with and without the addition of exogenous cAMP. As shown in Table 1, both the WT strain and the pkaR C′ complemented strain showed a significant increase in PKA activity following the addition of cAMP, whereas the activity in the mutant lacking the regulatory subunit did not change when cAMP was added. These findings confirm that the isogenic set has the expected phenotype with respect to cAMP responsiveness and that catalytic activity remains intact in the ΔpkaR mutant.

The ΔpkaR mutant has abnormal conidiation and pigmentation. When the three members of the isogenic set were grown on AMM, there were several obvious differences between the ΔpkaR mutant and the WT and pkaR C′ strains beyond the colony size. The surface of the mutant colony was less powdery than the other two, and there was a delay in conidiation in the mutant compared with the WT and pkaR C′ (Fig. 2A). The reduced conidiation phenotype was consistent with the microscopic morphology showing that the size of the terminal vesicle, the number of phialides, and the number of conidia per chain were all decreased in the mutant compared with the wild type (Fig. 2C). In addition, clavate vesicles were often formed at the apex of septate hyphae, rather than on a specialized hyphal branch, the nonseptate conidiophore. Although the hyphal wall of the ΔpkaR mutant appeared thicker and more pigmented than that of the WT (Fig. 2D), the more striking difference was seen on the reverse of the colonies; the reverse side of the ΔpkaR mutant was highly pigmented (Fig. 2B). The intensity of the pigmentation was accentuated when the carbon source of the AMM was changed from glucose to fructose or glycerol (data not shown).

Germination and growth rates of the ΔpkaR mutant are decreased. Point inoculation of each strain on AMM plates, followed by incubation at 37°C, showed that the growth rate of the mutant was impaired relative to those of the other two members of the isogenic set (Fig. 2A). The small-colony phenotype could be due to slower or delayed germination, to

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**TABLE 1. PKA activity in the ΔpkaR mutant is not regulated by cAMP**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>PKA activity (U mg⁻¹ of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basal</td>
</tr>
<tr>
<td>Wild type (H237)</td>
<td>0.24 ± 0.11</td>
</tr>
<tr>
<td>ΔpkaR</td>
<td>0.41 ± 0.22</td>
</tr>
<tr>
<td>pkaR C′</td>
<td>0.27 ± 0.15</td>
</tr>
</tbody>
</table>

$^a$ PKA activity was measured on cell-free hyphal extracts before (basal) and after (+ cAMP) the addition of cAMP to a final concentration of 1 μM.

$^b$ The +cAMP value significantly differs from the basal level, with a P value of <0.01.
decreased viability of the conidia, to a decreased rate of apical extension, or to a combination of factors.

The germination rates of the three strains were measured over a 14-h period at 37°C, and the results from a representative experiment are shown in Fig. 3. The WT and pkacR \textit{C}' strains began to show germ tubes shortly after 4 h; the proportion of conidia showing germ tubes increased rapidly over the next 2 h, and 100% of the conidia had germinated by 7.5 h. The mutant was slow to germinate; approximately 20% of the conidia had germinated by 9 h. Between 9 and 12 h, an additional 50% of the conidia germinated, with slightly decreased kinetics compared with the WT. However, germination of the \textit{\Delta pkacR} conidia never exceeded 75 to 80%. Indeed, when conidia from plates older than 5 days were used, total germination in the mutant fell to \(<50\%\), whereas the germination of the WT and complemented strains was unchanged. Morphologically, the germ tubes of the mutant were shorter and broader than were those of the WT.

The radial growth of the isogenic set was measured at 37°C on AMM plates. Following 24 h of incubation, the colony sizes were very similar, reflecting the size of the drops of the conidial inocula. In the next 24 h, however, the WT and \textit{\Delta pkacR} \textit{C}' colonies almost tripled in size, whereas the mutant colony only doubled. By 72 h, only the \textit{\Delta pkacR} strain had not yet filled the petri dish. Growth rates at 30° and 37°C, calculated between 24 and 48 h, were consistent with the colony diameters; the growth rates of the mutant were approximately half that of the WT at both temperatures (Fig. 4). Therefore, it is likely that a combination of all three factors contributed to the smaller colony size of the mutant.

**Susceptibility to oxidative damage is increased in \textit{\Delta pkacR} conidia.** The PKA pathway has been reported to control response to oxidative damage in numerous organisms. Therefore, we sought to examine the susceptibility of a mutant with unregulated PKA activity to several oxidative agents with different modes of action. The activities of \(\text{H}_2\text{O}_2\), diamide, menadione, and paraquat were tested against both conidia and hyphae of the members of the isogenic set. As shown in Table 2, the conidia of the \textit{\Delta pkacR} strain were more susceptible to killing by all four of the oxidative agents. When the conidia were allowed to germinate before exposure to the oxidant, only paraquat and diamide were markedly more active against the mutant. Interestingly, \(\text{H}_2\text{O}_2\) was slightly less active against the germlings. Although the decrease in susceptibility seen when testing the mutant hyphae with hydrogen peroxide was small, it was consistent, even at concentrations not reported here.

**\textit{\Delta pkacR} strain had reduced virulence in a murine model of IA.** In order to determine the effect of unregulated PKA on the pathogenesis of IA, \((1 \times 10^5)\) conidia from the WT, \textit{\Delta pkacR}, and \textit{pkacR} \textit{C}' strains were inoculated intranasally into immunosuppressed mice (Fig. 5). Because of the lower viability of the mutant, the total number of conidia delivered was adjusted so that equivalent numbers of viable conidia were inoculated. The mice in the \textit{\Delta pkacR} group actually received almost 3.5 times more conidia than the mice in the WT or \textit{pkacR} \textit{C}' groups, yet the mice inoculated with the \textit{\Delta pkacR} mutant survived significantly longer \((P < 0.05)\). No deaths were recorded when mice were inoculated with saline. When inocula were reduced to approximately \(10^4\), none of the mice receiving the mutant died, whereas approximately 50% of the WT and

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**FIG. 2.** Morphology of the \textit{\Delta pkacR} mutant compared with the WT and the complemented strains. A. The forward side of the colonies is shown after growth on AMM at 30°C for 5 days. B. The reverse side of the same colonies, illustrating the highly pigmented reverse side of the \textit{\Delta pkacR} mutant. C. Microscopic morphology of the conidial heads of the isolates grown under the same conditions for 48 h, showing the poorly developed conidial heads in the mutant. D. The early hyphae of the \textit{\Delta pkacR} mutant are wider, thicker, and darker than those of the WT. C and D were photographed with differential interference contrast at magnification \(\times 40\).

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**FIG. 3.** Germination in AMM. Conidia were incubated in AMM at 37°C, and the presence of germ tubes was scored as percent germination at indicated time intervals.
groups did (data not shown). In the experiment shown in Fig. 5, the right lung, right kidney, and the brain of all animals were cultured at the time of death or following the end of the experiment when the survivors were sacrificed (Fig. 6). Virtually none of the mice inoculated with the WT or pkaR C' strain were able to clear the organism from their lungs, and most of them had evidence of dissemination to the kidney, even though the immunosuppression was transient in this model. Only about half of the lungs and the kidneys of the mice that received the mutant were still positive. Although there was some dissemination to the brain in all groups, the numbers were low overall. None of the control animals, which were inoculated with saline, had positive cultures in any organ. Finally, all the lung isolates from all animals were phenotyped for drug resistance markers, and the results showed that there was no cross-contamination among groups.

**DISCUSSION**

Although Aspergillus fumigatus is an important opportunistic pathogen of humans, its primary role in the environment is the recycling of carbon and nitrogen from plant and animal matter through the compost cycle (42). In order to survive in this niche, A. fumigatus must compete with many other microbes in the compost environment, sensing various forms of stress and nutrient deprivation and mounting an effective response (24, 33, 37). The adaptive responses that make A. fumigatus successful in one ecological niche may also be utilized when the organism finds itself in a different environment, that of the mammalian lung. Temperature shifts, wide variations in pH, and oxidizing agents may be common stressors within both environments (13, 24, 33, 37). We have been interested in how A. fumigatus responds to various stresses and whether these responses may be involved in its pathogenicity, since the prevalence of A. fumigatus as a pathogen exceeds its prevalence in the air (17). When the fungus is cocultured with mammalian cells, steady-state message levels of the regulatory subunit for PKA increase (29, 36). This suggests that tight control of PKA activity may be required for the optimal response to the stress

**TABLE 2. Survival from oxidative damage by conidia and hyphae of the pkaR isogenic set**

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>% Survival for genotype</th>
<th>WT</th>
<th>∆pkaR</th>
<th>pkaR C'</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conidia</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H₂O₂</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.005%</td>
<td>97</td>
<td>46</td>
<td>84</td>
<td></td>
</tr>
<tr>
<td>0.05%</td>
<td>84&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0</td>
<td>69&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Diamide</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 mg ml&lt;sup&gt;−1&lt;/sup&gt;</td>
<td>82&lt;sup&gt;c&lt;/sup&gt;</td>
<td>14</td>
<td>46&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>10 mg ml&lt;sup&gt;−1&lt;/sup&gt;</td>
<td>15&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0</td>
<td>19&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Menadione</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25 mg ml&lt;sup&gt;−1&lt;/sup&gt;</td>
<td>92&lt;sup&gt;d&lt;/sup&gt;</td>
<td>50</td>
<td>87</td>
<td></td>
</tr>
<tr>
<td>100 mg ml&lt;sup&gt;−1&lt;/sup&gt;</td>
<td>67&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1</td>
<td>64&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Paraquat</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>22.5 mg ml&lt;sup&gt;−1&lt;/sup&gt;</td>
<td>60</td>
<td>37</td>
<td>71</td>
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</tr>
<tr>
<td>45 mg ml&lt;sup&gt;−1&lt;/sup&gt;</td>
<td>59&lt;sup&gt;d&lt;/sup&gt;</td>
<td>27</td>
<td>66&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Hyphae</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H₂O₂</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.005%</td>
<td>57</td>
<td>64</td>
<td>55</td>
<td></td>
</tr>
<tr>
<td>0.01%</td>
<td>1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>10</td>
<td>0&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Diamide</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 mg ml&lt;sup&gt;−1&lt;/sup&gt;</td>
<td>99</td>
<td>96</td>
<td>93</td>
<td></td>
</tr>
<tr>
<td>3 mg ml&lt;sup&gt;−1&lt;/sup&gt;</td>
<td>41&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>Menadione</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 mg ml&lt;sup&gt;−1&lt;/sup&gt;</td>
<td>98</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>150 mg ml&lt;sup&gt;−1&lt;/sup&gt;</td>
<td>14</td>
<td>22</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>Paraquat</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 mg ml&lt;sup&gt;−1&lt;/sup&gt;</td>
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<td>90</td>
<td>95</td>
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</tr>
<tr>
<td>3 mg ml&lt;sup&gt;−1&lt;/sup&gt;</td>
<td>73&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12</td>
<td>72&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Survival of water-treated controls was set at 100% for each strain. There were no significant differences when data from the pkaR C' strain were compared with those from the wild type.

<sup>b</sup> P < 0.001 compared with results for ∆pkaR strain.

<sup>c</sup> P < 0.01 compared with results for ∆pkaR strain.

<sup>d</sup> P < 0.05 compared with results for ∆pkaR strain.

**FIG. 4.** Radial growth rates on AMM. Ten-microliter drops containing 10<sup>4</sup> conidia were inoculated into the middle of 50-mm plates and incubated at 30°C and 37°C. Colony diameters were measured at 24-h intervals, and the rate of radial growth was calculated for the interval between 24 and 48 h.
engendered by coculture. Therefore, we sought to compare the
susceptibility to environmental stress, specifically oxidative
damage, of a mutant that lacked the regulatory subunit of PKA
with that of the wild type and a complemented mutant. Since
reactive oxygen species are known to be responsible for killing
\textit{A. fumigatus} conidia in alveolar macrophages and to be pro-
duced in composts, we reasoned that oxidative damage would
be a biologically relevant stress for this opportunistic pathogen
(33, 35).

We have used four different agents to cause the oxidative
damage, since no one oxidant is representative of oxidative
stress. For example, it has been shown that yeast cells of \textit{S.}
\textit{cerevisiae} have constitutive defense systems that are fairly spec-
ific for each individual stressor, but the induced systems that
are responsible for repair, the general environmental stress
response, are largely shared (43). The reactive oxygen species
hydrogen peroxide is widely used because it is highly reactive
with a broad spectrum of targets. In addition, the presence of
H$_2$O$_2$ can lead to production of hydroxyl radicals via the Fent-
ton reaction. Menadione and paraquat are redox cycling
agents, and they work by generating superoxide by reducing
molecular oxygen at the expense of NADPH in aerobically
growing cells. The superoxide can then be dismutated, result-
ing in the formation of H$_2$O$_2$. Diamide can cause oxidative
stress more indirectly by oxidizing glutathione, shifting the
redox balance of the cell, and reacting with sulfhydryl groups
on proteins (42). Therefore, we reasoned that using these
different oxidizing agents should give us a significantly better

![Survival of mice inoculated with members of PkaR isogenic set.](image)

**FIG. 5.** Survival of mice inoculated with members of PkaR isogenic set. Immunosuppressed mice (groups of 16) were inoculated intranasally
with (1 to 5) $\times 10^6$ conidia, and survival was followed for 14 days. Control mice (group of 8; not shown) that were given saline intranasally all
survived for 14 days.

![Fungal clearance and dissemination.](image)

**FIG. 6.** Fungal clearance and dissemination. Organs were cultured from mice that died and from survivors that were sacrificed after 14 days.
Nothing grew from the organs of the control mice (saline inoculated). All lung isolates were phenotyped by testing for hygromycin and phleomycin
resistance.
chance to detect phenotypic changes in the \textit{pkaR} mutant than would the use of a single agent (43).

In order to determine whether the $\Delta pkaR$ mutant would show the lack of regulation predicted, PKA activity was tested in extracts from all three members of the isogenic set. The activity detected in the WT extract increased significantly when exogenous cAMP was added, whereas there was no increase seen in the mutant, confirming the lack of cAMP dependence in the PKA activity of the $\Delta pkaR$ strain. One feature noted was that the overall activity seen in the mutant was not extraordinarily high. There does not seem to be a consensus in how filamentous fungi respond to deletion of the PKA regulatory subunit; the \textit{A. niger} mutant has approximately the same level of activity as in the wild type, whereas the activity in the \textit{Colletotrichum} mutant is significantly increased over the basal level (40, 41).

Deletion mutants of \textit{pkaR} were phenotypically different from the WT in both macroscopic and microscopic features. The slower radial growth seen with the \textit{A. fumigatus} $\Delta pkaR$ mutant appears to be a common feature of regulatory subunit mutants of filamentous fungi, since it has also been reported for \textit{Neurospora crassa}, \textit{Colletotrichum lagenarium}, and \textit{A. niger} (1, 40, 41). Germination rates are similar to the phenotypes described for \textit{A. niger} mutants. Conidiation defects, ranging from failure to conidiate (\textit{A. niger}) to delayed (\textit{A. fumigatus}) or decreased (\textit{C. lagenarium}) conidiation, also appear to be common to PKA regulatory subunit mutations in filamentous fungi, supporting a key role for PKA signaling in asexual development of these organisms (1, 40, 41). An interesting morphological feature of the \textit{A. niger} $\Delta pkaR$ mutant is the distinctive bulbous cells that develop during submerged growth. This is similar to the loss of growth polarity that has been reported for \textit{Neurospora crassa} mutants that are defective in the regulatory subunit of PKA (1, 40). The hyphal diameter of the \textit{A. fumigatus} $\Delta pkaR$ mutant was increased, but there was no evidence of loss of polarity following 12 h in submerged culture. Macroscopically, when grown on solid medium, the reverse of the \textit{A. fumigatus} $\Delta pkaR$ colony appears dark, presumably due to pigmentation of the hyphae. Expression of \textit{pksP} is decreased in \textit{A. fumigatus} isolates deleted for the major catalytic subunit of PKA, and our data would suggest that \textit{pksP} may be inappropriately expressed in the $\Delta pkaR$ mutant, leading to pigmentation in hyphae that would normally be hyaline (20).

The PKA pathway has been studied with a number of other yeasts and molds, including some that are pathogenic for animals and plants. For the model organism \textit{S. cerevisiae}, PKA mediates the general stress response as well as regulating the specific oxidative response through the transcription factors Yap1p and Pos9p (6). Genetic conditions that result in decreased PKA pathway activity, such as deletion of one or more catalytic subunits, lead to increased H$_2$O$_2$ stress resistance, whereas those conditions that lead to increased PKA pathway activity, such as deletion of \textit{BCY1}, the regulatory subunit, result in decreased resistance to H$_2$O$_2$ stress (15, 43, 44). The decreased resistance to oxidative stress in the \textit{bcy1} strain may be because the mutant fails to activate Yap1p-dependent gene transcription following exposure to H$_2$O$_2$ (15).

In the plant- and human-pathogenic yeasts, \textit{Ustilago maydis}, \textit{Candida albicans}, and \textit{Cryptococcus neoformans}, PKA signaling plays an important role in morphogenesis, growth, and virulence. Disruption of \textit{adr1}, one of the PKA catalytic subunits in \textit{U. maydis}, results in a constitutively filamentous phenotype and loss of virulence (10). The regulatory subunit, encoded by \textit{ubc1}, is required for filamentous growth, and deletion mutants are multiply budded. Although the \textit{ubc1} mutants can colonize maize, they have impaired virulence, and the plant does not form galls in response to the growth of the mutant (14). Both PKA catalytic subunits of \textit{C. albicans} are involved in the control of morphogenesis in the organism (3). For \textit{TPK2}, this control is mediated through its activation of the transcription factor Elg1p. Because of the importance of the yeast-to-hypha switch in the virulence of \textit{C. albicans}, mutants lacking \textit{TPK2} are attenuated in a mouse model of candidiasis (19, 22). Contrary to what is known about regulatory subunit mutants in other organisms, homozygous \textit{bcy1} mutants of \textit{C. albicans} are not viable, but the homozygous mutation can be created in a $\Delta pka2$ mutant background. Although the role of unregulated PKA activity in virulence has not been examined, these double mutants display PKA activity that is not responsive to the addition of cAMP, reduced germination, and decreased viability (5). PKA regulation of virulence in \textit{C. neoformans} differs depending on which variety is being studied. In the more-common variety \textit{grubii} (serotype A), Pka1 controls mating and production of the virulence factors melanin and capsule; \textit{\Delta pka1} strains are avirulent. Variety \textit{grubii} mutants lacking the regulatory subunit, \textit{pkr1}, overproduce melanin and capsule and are hypervirulent (9). Pka2 does not function in regulating any of these virulence traits in serotype A, but in serotype D (variety \textit{neoformans}), Pka2 plays the predominant role. In this variety, \textit{\Delta pka2} mutants fail to mate, do not undergo haploid fruiting, and fail to produce melanin and capsule. Pka1 does not play any discernible role in regulating these processes in variety \textit{neoformans}. Perhaps the most surprising finding is that both \textit{\Delta pka2} and \textit{\Delta pkr1} mutants of serotype D have wild-type virulence, even though Pka2 regulates well-known cryptococcal virulence factors (16). Clearly, although the PKA signaling pathways are generally well conserved, their end targets share both similarities and differences. The evolutionary distance between these closely related pathogenic yeasts has resulted in highly divergent modes of regulation of virulence.

In the filamentous fungi, \textit{N. crassa} has been used as a model to study many developmental processes. The loss-of-growth-polarity phenotype described for the \textit{mbc} regulatory subunit mutant is suppressed by a second mutation in \textit{PKAC-1}, the major catalytic subunit in \textit{N. crassa}. Although these mutants were not tested for stress per se, increased thermotolerance, which was defined as a decreased susceptibility to heat shock, was reported for the \textit{pkac-1} mutant but not for the \textit{mbc} mutant (1). Mutation of the regulatory subunit of PKA in \textit{A. nidulans} has not been reported. However, when \textit{pkaA}, the major catalytic subunit, is deleted, radial growth of the colony is decreased, which is the same phenotype reported for the comparable mutant for both \textit{A. niger} and \textit{A. fumigatus} (38). Deletion of \textit{pkaB} in \textit{A. nidulans} does not lead to a growth defect, and deletion of the \textit{pkaB} homologue in \textit{A. niger} or \textit{A. fumigatus} has not been reported. Neither overexpression nor deletion of \textit{pkaB} in \textit{A. nidulans} yielded a thermostolerant phenotype, as described for \textit{N. crassa}. However, both deletion of \textit{pkaA} and overexpression of \textit{pkaB} do lead to increased susceptibility of the hyphae to H$_2$O$_2$. These findings suggested that the two
catalytic subunits in *A. nidulans* play opposite roles in the regulation of the response to oxidative stress (27). For *A. fumigatus*, our data support the hypothesis that correct regulation of PKA is required for wild-type resistance to oxidative damage inflicted by a range of mechanisms. Conidia appear susceptible to oxidative damage regardless of the agent used, perhaps because the processes involved in breaking dormancy are energetically expensive and redox sensitive. This might be analogous to the model suggested for *S. cerevisiae*, in which increased sensitivity to a number of different oxidants is due to a failure in some constitutive process required for resistance to oxidative stress (43). Although the hyphal response to the agents was more varied, the mutant was more susceptible to oxidative damage caused by the redox cycling agent paraquat and diamide, which targets sulfhydryl groups. It is tempting to speculate that the increased resistance of the hyphae to H$_2$O$_2$ may be due to an increase in melainin content of the hyphal walls, as suggested by the dark reverse of the colony, but one would expect that the quenching of reactive oxygen species by melanin would be a more general observation (18).

The *pkaR* mutant of *A. fumigatus* has reduced radial growth and increased susceptibility to oxidative damage, and both of these phenotypes have been associated with reduced virulence in *A. fumigatus*. In an immunocompromised mouse model of invasive aspergillosis, the group that received the *ΔpkaR* strain did experience reduced mortality compared with the groups receiving the wild type or the complemented strain. Reduced virulence has been reported for different filamentous fungal mutants, as well as for the yeasts, in which the regulation of PKA is impaired. Mutants of *Colletotrichum* that lack the regulatory subunit of PKA grow more slowly than the wild type and fail to infect intact cucumber leaves, although they can infect wounded leaves (41). When the catalytic subunit is deleted from *Magnaporthe*, the mutants are unable to infect rice leaves, whether or not they are abraded (25). And in *A. fumigatus*, *pkaC1* deletion mutants are almost avirulent (21). Although it is difficult to compare results from different models and mutants produced in different backgrounds, the virulence phenotype of the regulatory subunit mutants appears less severe. This suggests that it is more deleterious to virulence to have reduced PKA activity than to have unregulated activity. However, since mutational analysis is incomplete for many of these organisms, including *A. fumigatus*, it may be premature to speculate on the overall contribution of the signaling system. In addition, the cross talk between signaling pathways may be shown to be the primary factor controlling growth and virulence, similar to the role played by PKA and TOR in regulating growth in yeast (47). Clearly, the precedent in *Cryptococcus* suggests that although core signaling pathways may be conserved, regulation of these pathways and ultimate targets may lead to markedly different results for very similar organisms (16). Our current efforts are directed toward delineating PKA targets in *A. fumigatus* and elucidating their roles in regulating pathogenesis of this important opportunistic pathogen.

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