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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 Δ*pkaR* 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 *BCY1*, 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).

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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 Δ*pkaR* 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, except where noted (8, 30). To measure growth rates, 10^4 conidia were spotted onto the center of an AMM plate, and the diameter of the colony was measured every 24 h for 7 days (28). All experiments were performed in triplicate. Growth rates were defined as the difference in colony diameters over time. Growth rates were compared using a paired *t* test (SigmaStat, Point Richmond, CA). To measure the germination rate, 10^5 conidia ml^{-1} were incubated in YG (0.5% yeast extract, 2% glucose) at 37°C. In each of three independent assays, 100 conidia were scored for germination at timed intervals. Germination was defined as the production of a germ tube (12).

Construction of the *pkaR* isogenic set. The *pkaR* coding sequence (accession no. AF401202) with 5′and 3′ flanking DNA was amplified from H237 genomic DNA using a 5′ primer (5′-CCTCCCTCCTACTACTACCCC-3′) and a 3′ primer (5′-GGTTGCGTTCGAGTCTCC-3′) and cloned into pCR 2.1-TOPO (Invitrogen, Carlsbad, CA). The PCR product was moved as a SpeI-XhoI fragment into plasmid pSL1180 (GE Healthcare Bioscience, Little Chalfont, United Kingdom) that had been cut with SpeI-SalI, yielding plasmid pSL1180-*pkaR* (4). The hygromycin resistance marker was removed from plasmid pMAD91 by digestion with XhoI and XbaI, and the overhangs were filled in (46). The coding region of *pkaR* was removed from pSL1180-*pkaR* by digestion with SmaI and SacI, the SacI site was blunted, and the hygromycin cassette was cloned into the modified SmaI-SacI-cut DNA. This plasmid was digested with EcoRI to release the deletion construct; approximately 10 μg of the deletion construct was used to transform protoplasts of *A. fumigatus*, using standard techniques (31). Hygromycin-resistant colonies were selected, screened for homologous recombination by PCR, and confirmed by Southern blotting.

A complement ation construct was built by removing the phleomycin resistance cassette as an XhoI fragment from pBCPhleo and blunting the ends (39). pSL1180-*pkaR* was digested with SpeI and filled in, and the phleomycin marker was ligated into the plasmid to make plasmid pSL1180-*pkaR*(phleo). To reconstitute the *pkaR* gene at its native locus, Δ*pkaR* protoplasts were transformed with undigested pSL1180-*pkaR*(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 membrane (Hybond-N; GE Healthcare Bioscience). Membranes were hybridized to a ^{32}P-labeled random-primed *pkaR* DNA probe and washed under stringent conditions according to the manufacturer's recommendations.

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 Δ*pkaR* 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 detection of cAMP-dependent protein kinase from Promega, Madison, WI). Purified cAMP-dependent PKA catalytic subunit from bovine heart (Promega) was used as a standard. The incubation time was 30 min at room temperature. Quantitation was performed with a phosphorimager (Storm; GE Healthcare Bioscience). Activity 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, Δ*pkaR* 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, 10^6 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 germlings at 30°C on AMM plates (24 h for WT and *pkaR* C′ strains and 30 h for the Δ*pkaR* 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. H_2O_2 (30% stock) was purchased from Fisher (Fairlawn, NJ), and menadione (menadione sodium bisulfite), diamide (*N,N,N′,N′*-tetramethyazodicarboxamide), and paraquat (methyl viologen) were purchased from Sigma-Aldrich (St. Louis, MO).

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}; Cytoxan; Baxter Healthcare Corporation, Deerfield, IL) on day −3 and triamcinolone acetonide (40 mg kg^{-1}; Kenalog-10; BMS Company, Princeton, NJ) injected subcutaneously in the nape of the neck on day −1 (12). Tetracycline hydrochloride (Sigma) was provided in the water at a concentration of 500 μg ml^{-1} to prevent bacterial infection. Groups of 16 mice per strain were anesthetized with 3.5% isofluorane and inoculated intranasally with (1 to 5) × 10^5 conidia in 20 μl of sterile saline on day 0. A group of 8 mice was inoculated with saline as a control. Mortality was monitored for the next 14 days, and mice that appeared moribund were sacrificed by CO_2 euthanasia. The right lung and right kidney and the brain of each mouse were plated onto inhibitory mold agar (BD, Franklin Lakes, NJ) 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 Kruskall-Wallis with pairwise analysis performed post hoc by using Dunn's procedure (SigmaStat).

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). Monoconidial isolates of hygromycin-resistant colonies were screened for homologous recombination by PCR (data not shown) and confirmed by Southern blotting (Fig. 1D). Single-copy, homologous integration was confirmed by reprobing the blots with a fragment of the hygromycin resistance gene (data not shown). A homologous integrant, the Δ*pkaR* strain, was identified by the loss of the 4.2-kb WT PstI fragment and the appearance of the expected 2.7-kb PstI fragment (Fig. 1D). To complement the mutation, a plasmid carrying a wild-type copy of *pkaR*, flanked by a phleomycin resistance gene (Fig. 1C), was used to transform Δ*pkaR* protoplasts. Monoconidial isolates of phleomycin-resistant transformants were selected for Southern blotting analysis. A strain with a homologous integration of the complementation plasmid, which showed the 2.7-kb band from the deletion event plus a 6.1-kb band representing the WT gene, selection marker, and plasmid sequence (Fig. 1D), was chosen for further analysis. The complemented strain was designated *pkaR* C′.

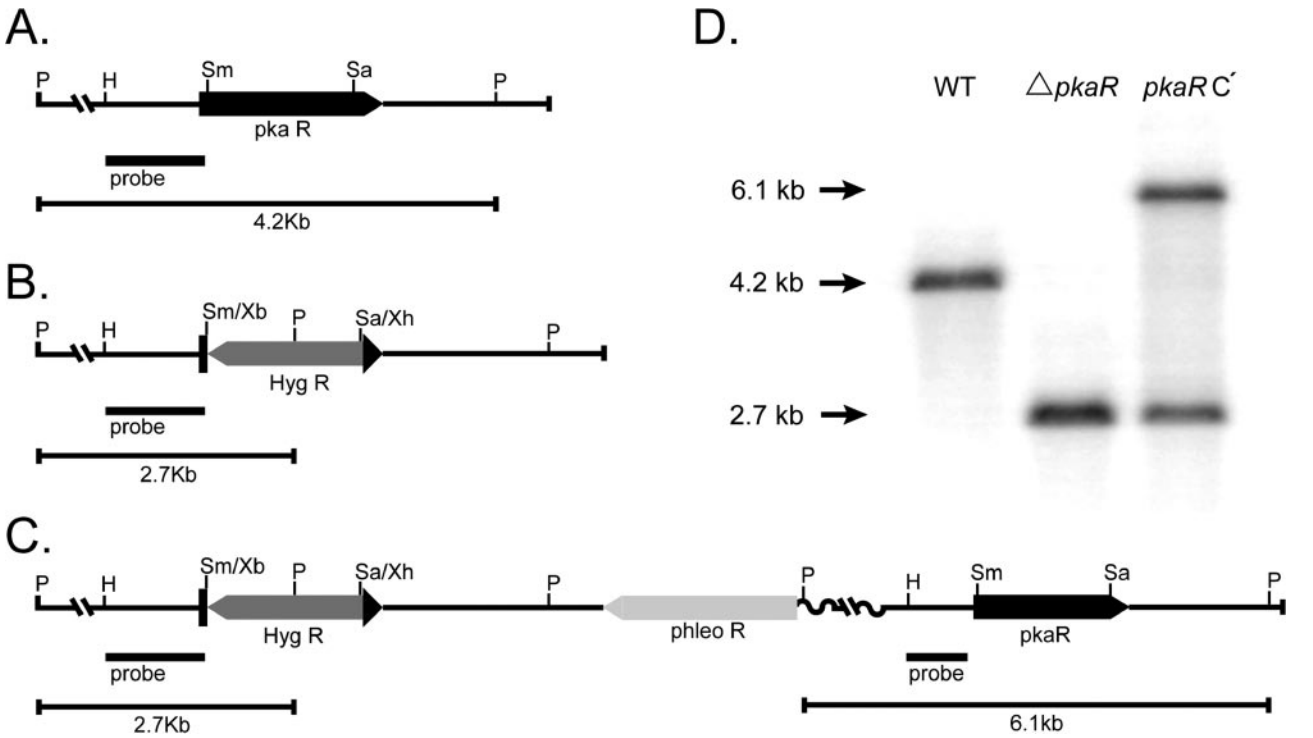


FIG. 1. Deletion and complementation of the *pkaR* gene. A. The wild-type locus for *pkaR*. B. The majority of the open reading frame (black arrow) for *pkaR* in *A. fumigatus* was deleted and replaced with the hygromycin resistance gene (Hyg R) by homologous recombination to produce Δ*pkaR*. C. A wild-type gene, flanked by the phleomycin resistance gene (phleo R), was integrated into the right flanking DNA in the deletion strain to complement the mutation, producing *pkaR* C′. D. Genomic DNA from each member of the isogenic set was cut with PstI and probed with a piece of the left arm (black box marked probe) to detect the wild-type (4.2 kb), deletion (2.7 kb), and recombinant (6.1 kb) bands. P, PstI; H, HindIII; Sm, SmaI; Sa, SalI; Xb, XbaI; Xh, XhoI.

PKA activity is not regulated by cAMP in the Δ*pkaR* 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 Δ*pkaR* mutant.

TABLE 1. PKA activity in the Δ*pkaR* mutant is not regulated by cAMP[a]

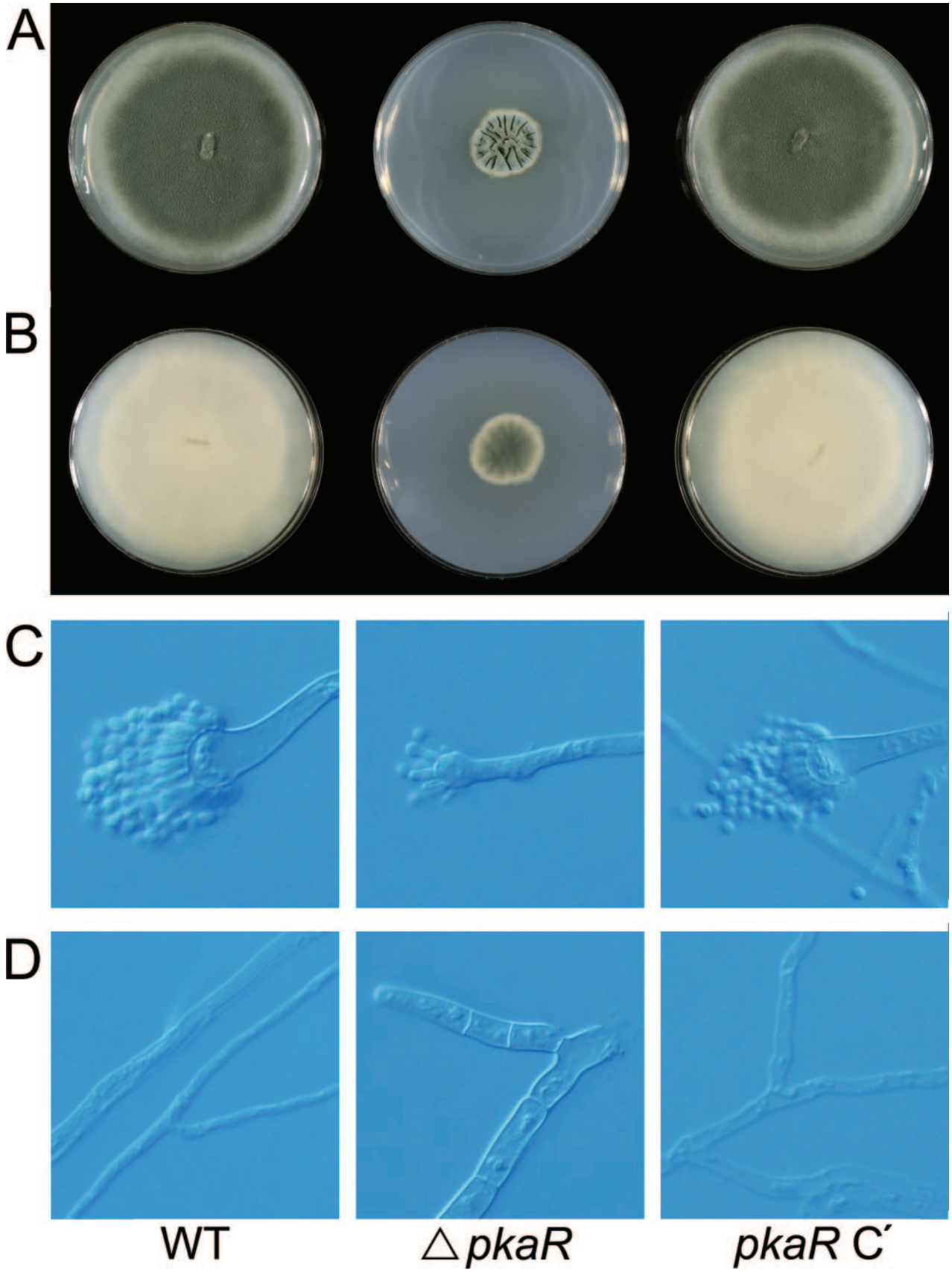
Genotype	PKA activity (U mg^{-1} of protein)	
	Basal	+cAMP
Wild type (H237)	0.24 ± 0.11	0.65 ± 0.18[b]
Δ*pkaR*	0.41 ± 0.22	0.40 ± 0.18
pkaR C′	0.27 ± 0.15	0.51 ± 0.15[b]

[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.

The Δ*pkaR* 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 Δ*pkaR* 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 Δ*pkaR* mutant appeared thicker and more pigmented than did that of the WT (Fig. 2D), the more striking difference was seen on the reverse of the colonies; the reverse side of the Δ*pkaR* 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 Δ*pkaR* 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


A
B
C
D
WT
△pkaR
pkaR C′

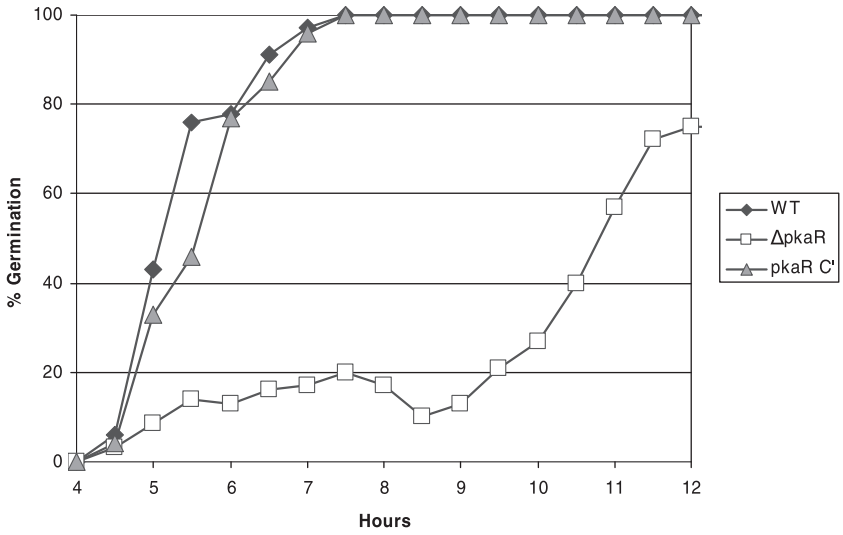


FIG. 3. Germination in AMM. Conidia were incubated in AMM at 37°C, and the presence of germ tubes was scored as percent germination at indicated time intervals.

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 *pkaR* 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 Δ*pkaR* 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 *pkaR* C′ colonies almost tripled in size, whereas the mutant colony only doubled. By 72 h, only the Δ*pkaR* 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 Δ*pkaR* 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 H_2O_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 Δ*pkaR* 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, H_2O_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.

Δ*pkaR* 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 to 5) $\times$ 10^5 conidia from the WT, Δ*pkaR*, and *pkaR* 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 Δ*pkaR* group actually received almost 3.5 times more conidia than the mice in the WT or *pkaR* C′ groups, yet the mice inoculated with the Δ*pkaR* 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

FIG. 2. Morphology of the Δ*pkaR* 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 Δ*pkaR* 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 Δ*pkaR* mutant are wider, thicker, and darker than those of the WT. C and D were photographed with differential interference contrast at magnification ×40.

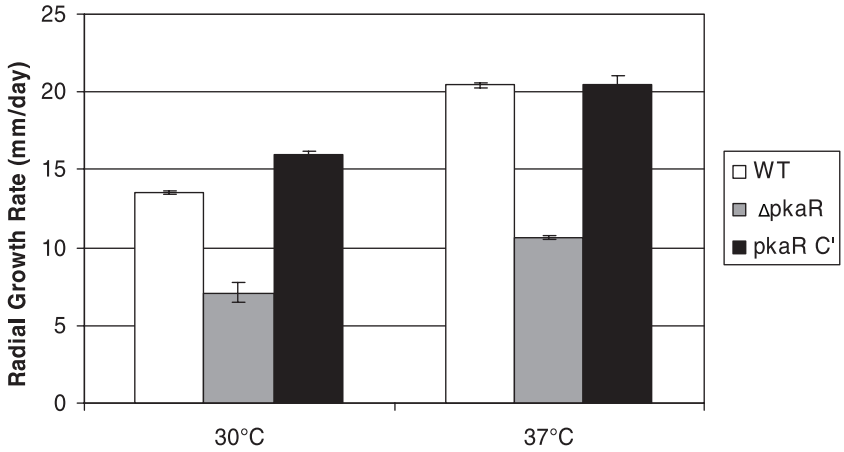


FIG. 4. Radial growth rates on AMM. Ten-microliter drops containing 10^4 conidia were inoculated into the middle of 50-mm plates and incubated at 30°C and 37°C. Colony diameters were measured at 24-h intervals, and the rate of radial growth was calculated for the interval between 24 and 48 h.

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

Treatment group	% Survival for genotype		
	WT	Δ*pkaR*	*pkaR* C′
Conidia			
H_2O_2			
0.005%	97	46	84
0.05%	84[b]	0	69[b]
Diamide			
3 mg ml^{-1}	82[c]	14	46[d]
10 mg ml^{-1}	15[d]	0	19[d]
Menadione			
25 mg ml^{-1}	92[d]	50	87
100 mg ml^{-1}	67[b]	1	64[b]
Paraquat			
22.5 mg ml^{-1}	60	37	71
45 mg ml^{-1}	59[d]	27	66[d]
Hyphae			
H_2O_2			
0.005%	57	64	55
0.01%	1[d]	10	0[d]
Diamide			
1 mg ml^{-1}	99	96	93
3 mg ml^{-1}	41[c]	3	18
Menadione			
50 mg ml^{-1}	98	100	100
150 mg ml^{-1}	14	22	26
Paraquat			
1 mg ml^{-1}	97	90	95
3 mg ml^{-1}	73[b]	12	72[b]

[a] 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.
[b] $P < 0.001$ compared with results for Δ*pkaR* strain.
[c] $P < 0.01$ compared with results for Δ*pkaR* strain.
[d] $P < 0.05$ compared with results for Δ*pkaR* strain.

pkaR C′ 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 it 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

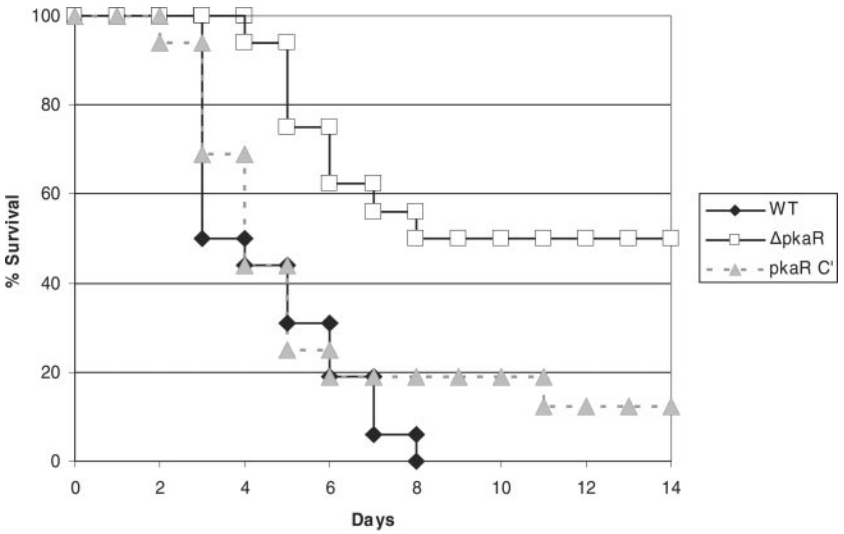


FIG. 5. Survival of mice inoculated with members of PkaR isogenic set. Immunosuppressed mice (groups of 16) were inoculated intranasally with (1 to 5) $\times 10^4$ conidia, and survival was followed for 14 days. Control mice (group of 8; not shown) that were given saline intranasally all survived for 14 days.

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 *A. fumigatus* conidia in alveolar macrophages and to be produced 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 *S. cerevisiae* have constitutive defense systems that are fairly specific 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_2O_2 can lead to production of hydroxyl radicals via the Fenton 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, resulting in the formation of H_2O_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

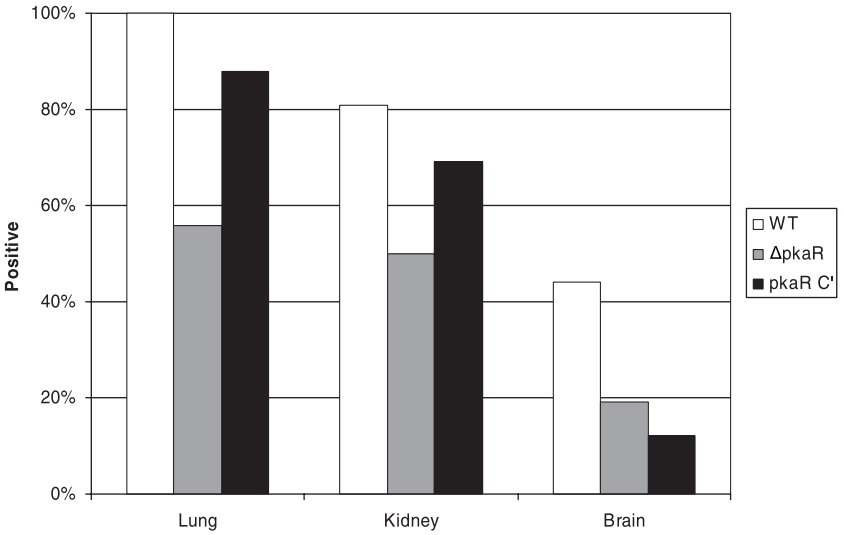


FIG. 6. Fungal clearance and dissemination. Organs were cultured from mice that died and from survivors that were sacrificed after 14 days. Nothing grew from the organs of the control mice (saline inoculated). All lung isolates were phenotyped by testing for hygromycin and phleomycin resistance.

chance to detect phenotypic changes in the *pkaR* mutant than would the use of a single agent (43).

In order to determine whether the Δ*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 Δ*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 *A. niger* mutant has approximately the same level of activity as in the wild type, whereas the activity in the *Colletotrichum* mutant is significantly increased over the basal level (40, 41).

Deletion mutants of *pkaR* were phenotypically different from the WT in both macroscopic and microscopic features. The slower radial growth seen with the *A. fumigatus* Δ*pkaR* mutant appears to be a common feature of regulatory subunit mutants of filamentous fungi, since it has also been reported for *Neurospora crassa*, *Colletotrichum lagenarium*, and *A. niger* (1, 40, 41). Germination rates are similar to the phenotypes described for *A. niger* mutants. Conidiation defects, ranging from failure to conidiate (*A. niger*) to delayed (*A. fumigatus*) or decreased (*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 *A. niger* Δ*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 *Neurospora crassa* mutants that are defective in the regulatory subunit of PKA (1, 40). The hyphal diameter of the *A. fumigatus* Δ*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 *A. fumigatus* Δ*pkaR* colony appears dark, presumably due to pigmentation of the hyphae. Expression of *pksP* is decreased in *A. fumigatus* isolates deleted for the major catalytic subunit of PKA, and our data would suggest that *pksP* may be inappropriately expressed in the Δ*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 *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_2O_2 stress resistance, whereas those conditions that lead to increased PKA pathway activity, such as deletion of *BCY1*, the regulatory subunit, result in decreased resistance to H_2O_2 stress (15, 43, 44). The decreased resistance to oxidative stress in the *bcy1* strain may be because the mutant fails to activate Yap1p-dependent gene transcription following exposure to H_2O_2 (15).

In the plant- and human-pathogenic yeasts, *Ustilago maydis*, *Candida albicans*, and *Cryptococcus neoformans*, PKA signaling plays an important role in morphogenesis, growth, and virulence. Disruption of *adr1*, one of the PKA catalytic subunits in *U. maydis*, results in a constitutively filamentous phenotype and loss of virulence (10). The regulatory subunit, encoded by *ubc1*, is required for filamentous growth, and deletion mutants are multiply budded. Although the *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 *C. albicans* are involved in the control of morphogenesis in the organism (3). For *TPK2*, this control is mediated through its activation of the transcription factor Efg1p. Because of the importance of the yeast-to-hyphae switch in the virulence of *C. albicans*, mutants lacking *TPK2* are attenuated in a mouse model of candidiasis (19, 22). Contrary to what is known about regulatory subunit mutants in other organisms, homozygous *bcy1* mutants of *C. albicans* are not viable, but the homozygous mutation can be created in a Δ*tpk2* 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 *C. neoformans* differs depending on which variety is being studied. In the more-common variety *grubii* (serotype A), Pka1 controls mating and production of the virulence factors melanin and capsule; Δ*pka1* strains are avirulent. Variety *grubii* mutants lacking the regulatory subunit, *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 *neoformans*), Pka2 plays the predominant role. In this variety, Δ*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 *neoformans*. Perhaps the most surprising finding is that both Δ*pka2* and Δ*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, *N. crassa* has been used as a model to study many developmental processes. The loss-of-growth-polarity phenotype described for the *mcb* regulatory subunit mutant is suppressed by a second mutation in PKAC-1, the major catalytic subunit in *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 *pkac-1* mutant but not for the *mcb* mutant (1). Mutation of the regulatory subunit of PKA in *A. nidulans* has not been reported. However, when *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 *A. niger* and *A. fumigatus* (38). Deletion of *pkaB* in *A. nidulans* does not lead to a growth defect, and deletion of the *pkaB* homologue in *A. niger* or *A. fumigatus* has not been reported. Neither overexpression nor deletion of *pkaB* in *A. nidulans* yielded a thermotolerant phenotype, as described for *N. crassa*. However, both deletion of *pkaA* and overexpression of *pkaB* does lead to increased susceptibility of the hyphae to H_2O_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_2O_2 may be due to an increase in melanin 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 Δ*pkaR* 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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REFERENCES

1. **Banno, S., N. Ochiai, R. Noguchi, M. Kimura, I. Yamaguchi, S. Kanzaki, T. Murayama, and M. Fujimura.** 2005. A catalytic subunit of cyclic AMP-dependent protein kinase, PKAC-1, regulates asexual differentiation in *Neurospora crassa*. Genes Genet. Syst. **80:**25–34.
2. **Bencina, M., H. Panneman, G. Ruijter, M. Legisa, and J. Visser.** 1997. Characterization and overexpression of the *Aspergillus niger* gene encoding the cAMP-dependent protein kinase catalytic subunit. Microbiology **143:** 1211–1220.
3. **Bockmuhl, D. P., S. Krishnamurthy, M. Gerads, A. Sonneborn, and J. F. Ernst.** 2001. Distinct and redundant roles of the two protein kinase A isoforms Tpk1p and Tpk2p in morphogenesis and growth of *Candida albicans*. Mol. Microbiol. **42:**1243–1257.
4. **Brosius, J.** 1989. Superpolylinkers in cloning and expression vectors. DNA **8:**759–777.
5. **Cassola, A., M. Parrot, S. Silberstein, B. B. Magee, S. Passeron, L. Giasson, and M. L. Cantore.** 2004. *Candida albicans* lacking the gene encoding the regulatory subunit of protein kinase A displays a defect in hyphal formation and an altered localization of the catalytic subunit. Eukaryot. Cell **3:**190–199.
6. **Charizanis, C., H. Juhnke, B. Krems, and K.-D. Entian.** 1999. The oxidative stress response mediated via Pos9/Skn7 is negatively regulated by the Ras/PKA pathway in *Saccharomyces cerevisiae*. Mol. Gen. Genet. **261:**740–752.
7. **Cordonnier, C., P. Ribaud, R. Herbrecht, N. Milpied, D. Valteau-Couanet, C. Morgan, A. Wade, and Société Française de Greffe de Moelle et de Thérapie Cellulaire.** 2006. Prognostic factors for death due to invasive aspergillosis after hematopoietic stem cell transplantation: a 1-year retrospective study of consecutive patients at French transplant centers. Clin. Infect. Dis. **42:**955–963.
8. **Cove, D. J.** 1966. The induction and repression of nitrate reductase in the fungus Aspergillus nidulans. Biochim. Biophys. Acta **113:**51–56.
9. **D'Souza, C., J. Alspaugh, C. Yue, T. Harashima, G. Cox, J. Perfect, and J. Heitman.** 2001. Cyclic AMP-dependent protein kinase controls virulence of the fungal pathogen *Cryptococcus neoformans*. Mol. Cell. Biol. **21:**3179–3191.
10. **Durrenberger, F., K. Wong, and J. W. Kronstad.** 1998. Identification of a cAMP-dependent protein kinase catalytic subunit required for virulence and morphogenesis in *Ustilago maydis*. Proc. Natl. Acad. Sci. USA **95:**5684–5689.
11. **Estruch, F.** 2000. Stress-controlled transcription factors, stress-induced genes and stress tolerance in budding yeast. FEMS Microbiol. Rev. **24:**469–486.
12. **Fortwendel, J. R., W. Zhao, R. Bhabhra, S. Parks, D. S. Perlin, D. S. Askew, and J. C. Rhodes.** 2005. A fungus-specific Ras homolog contributes to the hyphal growth and virulence of *Aspergillus fumigatus*. Eukaryot. Cell **4:**1982–1989.
13. **Fradin, C., M. Kretschmar, T. Nichterlein, C. Gaillardin, C. d'Enfert, and B. Hube.** 2003. Stage-specific gene expression of *Candida albicans* in human blood. Mol. Microbiol. **47:**1523–1543.
14. **Gold, S. E., S. M. Brogdon, M. E. Mayorga, and J. W. Kronstad.** 1997. The *Ustilago maydis* regulatory subunit of a cAMP-dependent protein kinase is required for gall formation in maize. Plant Cell **9:**1585–1594.
15. **Hasan, R., C. Leroy, A.-D. Isnard, J. Labarre, E. Boy-Marcotte, and M. B. Toledano.** 2002. The control of the yeast H_2O_2 response by the Msn2/4 transcription factors. Mol. Microbiol. **45:**233–241.
16. **Hicks, J. K., C. A. D'Souza, G. M. Cox, and J. Heitman.** 2004. Cyclic AMP-dependent protein kinase catalytic subunits have divergent roles in virulence factor production in two varieties of the fungal pathogen *Cryptococcus neoformans*. Eukaryot. Cell **3:**14–26.
17. **Hospenthal, D. R., K. J. Kwon-Chung, and J. E. Bennett.** 1998. Concentrations of airborne Aspergillus compared to the incidence of invasive aspergillosis: lack of correlation. Med. Mycol. **36:**165–168.
18. **Jahn, B., K. Langfelder, U. Schneider, C. Schindel, and A. Brakhage.** 2002. PKSP-dependent reduction of phagolysosome fusion and intracellular kill of *Aspergillus fumigatus* conidia by human monocyte-derived macrophages. Cell. Microbiol. **4:**793–803.
19. **Lane, S., C. Birse, S. Zhou, R. Matson, and H. Liu.** 2001. DNA array studies demonstrate convergent regulation of virulence factors by Cph1, Cph2, and Efg1 in *Candida albicans*. J. Biol. Chem. **276:**48988–48996.
20. **Liebmann, B., S. Gattung, B. Jahn, and A. Brakhage.** 2003. cAMP signaling in *Aspergillus fumigatus* is involved in the regulation of the virulence gene *pksP* and in defense against macrophages. Mol. Genet. Genomics **269:**420–435.
21. **Liebmann, B., M. Muller, A. Braun, and A. Brakhage.** 2004. The cyclic AMP-dependent protein kinase A network regulates development and virulence in *Aspergillus fumigatus*. Infect. Immun. **72:**5193–5203.
22. **Liu, H.** 2001. Transcriptional control of dimorphism in *Candida albicans*. Curr. Opin. Microbiol. **4:**728–735.
23. **McNeil, M. M., S. L. Nash, R. A. Hajjeh, M. A. Phelan, L. A. Conn, B. D. Plikaytis, and D. W. Warnock.** 2001. Trends in mortality due to invasive mycotic diseases in the United States, 1980–1997. Clin. Infect. Dis. **33:**641–647.
24. **Millner, P. D., P. B. Marsh, R. B. Snowden, and J. F. Parr.** 1977. Occurrence

of *Aspergillus fumigatus* during composting of sewage sludge. Appl. Environ. Microbiol. **34:**765–772.
25. **Mitchell, T. K., and R. A. Dean.** 1995. The cAMP-dependent protein kinase catalytic subunit is required for appressorium formation and pathogenesis by the rice blast pathogen *Magnaporthe grisea*. Plant Cell **7:**1869–1878.
26. **Moye-Rowley, W. S.** 2003. Regulation of the transcriptional response to oxidative stress in fungi: similarities and differences. Eukaryot. Cell **2:**381–389.
27. **Ni, M., S. Rierson, J.-A. Seo, and J.-H. Yu.** 2005. The *pkaB* gene encoding the secondary protein kinase A catalytic subunit has a synthetic lethal interaction with *pkaA* and plays overlapping and opposite roles in *Aspergillus nidulans*. Eukaryot. Cell **4:**1465–1476.
28. **Oliver, B. G., J. C. Panepinto, D. S. Askew, and J. C. Rhodes.** 2002. cAMP alteration of growth rate of *Aspergillus fumigatus* and *Aspergillus niger* is carbon-source dependent. Microbiology **148:**2627–2633.
29. **Oliver, B. G., J. C. Panepinto, J. R. Fortwendel, D. L. Smith, D. S. Askew, and J. C. Rhodes.** 2001. Cloning and expression of *pkaC* and *pkaR*, the genes encoding cAMP-dependent protein kinase of *Aspergillus fumigatus*. Mycopathologia **154:**85–91.
30. **Panepinto, J. C., B. G. Oliver, T. W. Amlung, D. S. Askew, and J. C. Rhodes.** 2002. Expression of the *Aspergillus fumigatus rheb* homologue, *rhbA*, is induced by nitrogen starvation. Fungal Genet. Biol. **36:**207–214.
31. **Panepinto, J. C., B. G. Oliver, J. R. Fortwendel, D. L. H. Smith, D. S. Askew, and J. C. Rhodes.** 2003. Deletion of the *Aspergillus fumigatus* gene encoding the Ras-related protein RhbA reduces virulence in a mouse model of invasive pulmonary aspergillosis. Infect. Immun. **71:**2819–2826.
32. **Paris, S., D. Wysong, J. P. Debeaupuis, K. Shibuya, B. Philippe, R. D. Diamond, and J. P. Latge.** 2003. Catalases of *Aspergillus fumigatus*. Infect. Immun. **71:**3551–3562.
33. **Paul, A., S. Hackbarth, R. D. Vogt, B. Roder, B. K. Burnison, and C. E. W. Steinberg.** 2004. Photogeneration of singlet oxygen by humic substances: comparison of humic substances of aquatic and terrestrial origin. Photochem. Photobiol. Sci. **3:**273–280.
34. **Perfect, J. R., G. M. Cox, J. Y. Lee, C. A. Kauffman, L. deRepentigny, S. W. Chapman, V. A. Morrison, P. Pappas, J. W. Hiemenz, D. A. Stevens, and the Mycoses Study Group.** 2001. The impact of culture isolation of *Aspergillus* species: a hospital-based survey of aspergillosis. Clin. Infect. Dis. **33:**1824–1833.
35. **Philippe, B., O. Ibrahim-Granet, M. C. Prevost, M. A. Gougerot-Pocodalo, M. S. Perez, A. VanderMeeren, and J. P. Latge.** 2003. Killing of *Aspergillus fumigatus* by alveolar macrophages is mediated by reactive oxidant intermediates. Infect. Immun. **71:**3034–3042.
36. **Rhodes, J. C., B. G. Oliver, D. S. Askew, and T. W. Amlung.** 2001. Identification of genes of *Aspergillus fumigatus* up-regulated during growth on endothelial cells. Med. Mycol. **39:**253–260.
37. **Ryckboer, J., J. Mergaert, J. Coosemans, K. Deprins, and J. Swings.** 2003. Microbiological aspects of biowaste during composting in a monitored compost bin. J. Appl. Microbiol. **94:**127–137.
38. **Shimizu, K., and N. P. Keller.** 2001. Genetic involvement of a cAMP-dependent protein kinase in a G protein signaling pathway regulating morphological and chemical transitions in *Aspergillus nidulans*. Genetics **157:**591–600.
39. **Silar, P.** 1995. Two new easy to use vectors for transformations. Fungal Genet. News Lett. **42:**73.
40. **Staudohar, M., M. Bencina, P. vandeVondervoort, H. Panneman, M. Legisa, J. Visser, and G. Ruijter.** 2002. Cyclic AMP-dependent protein kinase is involved in morphogenesis in *Aspergillus niger*. Microbiology **148:**2635–2645.
41. **Takano, Y., K. Komeda, K. Kojima, and T. Okuno.** 2001. Proper regulation of cyclic AMP-dependent protein kinase is required for growth, conidiation, and appressorium function in the anthracnose fungus *Colletotrichum lagenarium*. Mol. Plant-Microbe Interact. **14:**1149–1157.
42. **Tekaia, F., and J.-P. Latge.** 2005. *Aspergillus fumigatus*: saprophyte or pathogen? Curr. Opin. Microbiol. **8:**385–392.
43. **Temple, M. D., G. G. Perrone, and I. W. Dawes.** 2005. Complex cellular responses to reactive oxygen species. Trends Cell Biol. **15:**319–326.
44. **Toda, T., S. Cameron, P. Saas, M. Zoller, J. D. Scott, B. McMullen, M. Hurwitz, E. G. Krebs, and M. Wigler.** 1987. Cloning and characterization of *BCY1*, a locus encoding a regulatory subunit of the cyclic AMP-dependent protein kinase in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **7:**1371–1377.
45. **van Heerden, I., C. Cronjé, S. H. Swart, and J. M. Kotzé.** 2002. Microbial, chemical and physical aspects of citrus waste composting. Bioresour. Technol. **81:**1–76.
46. **Woods, J. P., E. L. Heinecke, and W. E. Goldman.** 1998. Electrotransformation and expression of bacterial genes encoding hygromycin phosphotransferase and beta-galactosidase in the pathogenic fungus *Histoplasma capsulatum*. Infect. Immun. **66:**1697–1707.
47. **Zurita-Martinez, S. A., and M. E. Cardenas.** 2005. Tor and cyclic AMP-protein kinase A: two parallel pathways regulating expression of genes required for cell growth. Eukaryot. Cell **4:**63–71.

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