A Fungal pH-Responsive Signaling Pathway Regulating *Aspergillus* Adaptation and Invasion into the Cornea

Xia Hua, Xiaoyong Yuan, and Kirk R. Wilhelmus

PURPOSE. To investigate the role of PalB and PacC, two components of a pH-responsive signal-transduction pathway of *Aspergillus nidulans*, during the pathogenesis of fungal infection of the cornea.

METHODS. Fungal strains included an *A. nidulans* wild-type isolate (A83), loss-of-function *A. nidulans* mutants of the *palB* (B7) or *pacC* (C6309) genes, and reconstituted genotypic strains (B7R and C6309R). Doubling times and radial growth rates were examined under neutral and acidic conditions. Corneal virulence was assessed ex vivo by topical inoculation of scarified porcine or human corneas with *A. nidulans* strains maintained in buffered medium until histologic examination after days 1, 3, and 5.

RESULTS. In vitro growth kinetics were similar for *A. nidulans* strains in liquid medium at pH 6.0 ($P = 0.24$) and 7.3 ($P = 0.75$). The *pacC* mutant C6309 grew more slowly ($P < 0.001$) on solid medium, whereas *palB* and *pacC* rescuants had growth kinetics comparable to those of the wild-type. Wild-type *A. nidulans* germinated on porcine corneas and produced hyphae that progressively invaded the stroma, reaching an average maximum penetration of $56\% \pm 9\%$ at 5 days after exposure. In contrast, hyphal invasion was significantly less by mutant strains B7 ($P = 0.005$) and C6309 ($P = 0.003$). Fungal penetration by C6309 was also significantly less than the wild-type ($P = 0.0005$) on explanted human corneas. Both fungal rescuants showed stromal invasion similar to the wild-type.

CONCLUSIONS. Corneal invasion by filamentous hyphae is attenuated by *palB* and *pacC* mutant strains of *A. nidulans*. The PacC pathway is involved in regulating fungal filamentation during ex vivo *Aspergillus* infection of the cornea. (*Invest Ophthalmol Vis Sci.* 2010;51:1517-1523) DOI:10.1167/iovs.09-4348

Fungi of the genus *Aspergillus* are epidemiologically important. These ascomycetes were among the first recognized etiologies of corneal infection and remain common isolates from oculomycoses.^{1,2} Reflecting diverse ecologic niches and risk factors, the relative prevalence of *Aspergillus* among patients with fungal keratitis varies widely from region to region.³⁻¹¹ *Aspergillus fumigatus* and *Aspergillus flavus* are established ophthalmic pathogens, and species such as *Aspergillus nidulans* are emerging causes of fungal keratitis.^{9,12-14}

The antifungal treatment of *Aspergillus* keratitis has been studied in animal models,¹⁵⁻¹⁷ but few investigations have examined the cellular and molecular pathogenesis of corneal aspergillosis.¹⁸ Recent knowledge gained from research into systemic and mucosal mycoses indicate that fungal infections involve adaptive interactions between host and pathogen.¹⁹⁻²¹ To infect tissue, fungi adjust to local conditions such as ambient pH, temperature, and nutrient supply. *Aspergillus* and other fungi that are pathogenic for the eye have the capabilities of colonizing and invading the injured ocular surface through mechanisms that enable growth and survival.

Metabolic systems shared by several fungi include a pathway that detects and responds to extracellular pH. Known as the Pal/PacC pathway in *Aspergillus* sp. and the RIM101 pathway in *Candida* sp.,²² this process consists of cell-surface receptors and an intracellular cascade. Gene products involved in pH perception, signal transduction, and molecular processing include the fungal sensors PalI/Rim9p and PalH/Rim21p; the intracellular proteins PalF/Rim8p, PalC, and PalA/Rim20p; a calpainlike protease PalB/Rim13p; and the transcription factor PacC/Rim101p. PalB-mediated proteolytic cleavage of the PacC zinc-finger-containing transcription factor results in a functional subunit that regulates gene expression of phosphatases, proteases, and other enzymes.²³⁻²⁵

The Pal/PacC pathway affects fungal pathogenicity. Strains of *Fusarium oxysporum*, *Colletotrichum acutatum*, and *Sclerotinia sclerotiorum* that contain disruptive mutations in homologues of the *pacC* gene have significantly altered virulence for plants and, in the case of *F. oxysporum*, for mice.²⁶⁻²⁸ The Pal/PacC pathway also plays an operative part in the production of experimental aspergillosis.²⁹ Furthermore, studies of *C. albicans* infections have shown the orthologous RIM101 pathway to be pivotal in candidal infection of mucous membranes and the cornea.³⁰⁻³² This conserved pathway influences virulence by several fungi of clinical importance.

In this study, we evaluated the role of the Pal/PacC pathway in experimental *Aspergillus* corneal infection. We used *A. nidulans* to dissect the molecular intermediates affecting fungal growth and virulence, because mutant construction and complementation is relatively easier than with other *Aspergillus* species.^{25,33,34} However, since related fungi such as *Aspergillus niger* and *A. fumigatus* encode PacC and other intermediates of the Pal/PacC pathway, studies with *A. nidulans* can exemplify the virulence mechanisms of *Aspergillus* species.^{35,36}

We used ex vivo models of corneal wounding and infection that allowed an economical, reproducible method of producing fungal infection of porcine and human corneal tissues.^{37,38} Prototrophic fungal strains with *palB* or *pacC* loss-of-function mutations and their isogenic reconstituted cognates were topically applied to the epithelial surface of injured corneas. Our findings indicate that PalB-mediated signaling and PacC-regulated genetic expression affect fungal invasion and pathogenicity during the onset of *Aspergillus* infection of cornea.

From the Sid W. Richardson Ocular Microbiology Laboratory, Department of Ophthalmology, Cullen Eye Institute, Baylor College of Medicine, Houston, Texas.

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Corresponding author: Kirk R. Wilhelmus, Cullen Eye Institute, 6565 Fannin Street, Houston, TX 77030; kirkw@bcm.edu.

TABLE 1. *A. nidulans* Strains

Strain	Phenotype	Genotype	Reference
A83	Wild-type	<i>yA2;(veA+)</i>	Barratt et al. ⁴⁰
B7	PalB-negative	<i>palB7</i>	Denison et al. ⁴²
B7R	PalB-positive rescuant	<i>palB^R</i>	Bignell et al. ²⁹
C6309	PacC-negative	<i>pacC-6309; pacC^C63</i>	Fernández-Martínez et al. ⁴³
C6309R	PacC-positive rescuant	<i>pacC^R</i>	Bignell et al. ²⁹

MATERIALS AND METHODS

Fungal Strains

Five *A. nidulans* strains were used in this study (Table 1), with all strains carrying markers in standard use.³⁹ The wild-type strain A83, *yA2;(ve+)* was obtained from the Fungal Genetics Stock Center (FGSC), University of Missouri (Kansas City, MO).^{40,41} A83 is a virulent prototrophic strain generated by the backcross of the Glasgow wild-type strain A4;*(ve+)* with FGSC strain A76 (*adG14 proA1 pabaA1 yA2;wA3*).⁴⁰ Mutant strains were provided by the Department of Infectious Disease, Imperial College London.^{29,42,43} The *palB*^{-/-} strain was constructed by transformation of an *A. nidulans* strain of genotype *yA2 pabaA1 pyrG89* with linearized plasmid pUC19.18Δ *Bam*HI which contained the *Sph*I/*Kpn*I fragment including the *palB* disrupted at the *Bam*HI restriction site with a fragment including the *pyr4* gene of *Neurospora crassa*.⁴² The resultant transformant yielded B7, a mutant strain with a loss-of-function mutation in the *palB* gene, making B7 unable to catalyze the pH-dependent initial proteolytic cleavage of PacC that normally occurs in response to alkaline pH.^{24,42} The *palB* rescuant B7R was reconstituted from B7 by replacement of the mutated version with the corresponding genomic region released from plasmid pAnBS by *Not*I digestion before *A. nidulans* protoplast transformation.²⁹ The *pacC*^{-/-} mutant strain C6309 (*pabaA1 yA2 wA3 palA1*) was fashioned by constitutive mutations of *pacC*^C63^{43,44}. C6309R had restoration of the *pacC* locus by using plasmid p4RC to provide wild-type functionality.^{29,43} All strains were stored in 25% glycerol at -80°C and were recultivated in yeast-peptone-dextrose (YPD) culture medium at 25°C.

In Vitro Growth

Triplicate samples of 5000 culturable units (CU) of each strain were inoculated into 25 mL M199 liquid medium (Invitrogen, Grand Island, NY) buffered with Tris-HCl to pH 6.0 or 7.3, then incubated at 25°C with continuous shaking. Doubling times of fungal strains were estimated by measuring optical density (OD) at a wavelength of 600 nm in a spectrophotometer (Ultraspec 2000; Pharmacia Biotech, Princeton, NJ), with a conversion factor of one OD₆₀₀ unit equivalent to 5.3 × 10⁵ CU/mL.⁴⁵

A 10-day-old colony culture of *A. nidulans* strains grown on a Sabouraud agar plate was used to obtain spore suspensions that were diluted in sterile distilled water to an OD of 0.01. Growth rates were determined by measuring colony radial growth on Sabouraud dextrose agar that was buffered with Tris-HCl to pH 5.6 or 7.3.⁴⁶ A 25-μL conidial suspension of each strain was spotted onto the center of the Sabouraud agar plate and inoculated at 25°C. Without opening the Petri dish, the longest and orthogonal diameters of colonies were measured and averaged each day.⁴⁷ Five replicates were used for each strain at each condition. The radial growth rate (millimeters/hour) was obtained from the linear regression slope of the growth curves during the period when colonies grew at a constant rate.⁴⁸ Results are presented as the mean with standard deviation. One-way analysis of variance (ANOVA) was used for statistical analysis.

Ex Vivo Cornea Models

Thirty fresh porcine eyes obtained from VisionTech, Inc. (Mesquite, TX) were immersed in a saline solution of penicillin, streptomycin, and

amphotericin B (Antibiotic-Antimycotic; Invitrogen, Grant Island, NY). An 18-mm diameter trephine (VisionPak, Lexington, KY) was used to excise corneal buttons that were then fixed onto an artificial chamber (Refractive Technologies, Cleveland, OH). The corneas were superficially scarified with a 22-gauge needle in a cross-hatched pattern, similar to a protocol previously developed for an experimental fungal keratitis model.⁴⁹ The corneal surface was then topically inoculated with a 10-μL solution containing 1 × 10⁴ conidia of *A. nidulans* that had been cultured in YPD medium for 4 days. Inoculated corneas were placed into a six-well culture dish (Corning, Corning, NY), so that corneal rims were immersed in modified supplemented hormonal epithelial medium (SHEM), consisting of equal volumes of Dulbecco's modified Eagle's medium and Ham's F12 medium. Buffered SHEM at pH 7.3 contained 5 ng/mL epidermal growth factor, 5 μg/mL insulin, 5 μg/mL transferrin, 5 μg/mL sodium selenite, 0.5 μg/mL hydrocortisone, 30 ng/mL cholera toxin A, 0.5% dimethylsulfoxide, 50 μg/mL gentamicin, and 5% fetal bovine serum. The tissues were incubated at 34°C in 5% carbon dioxide with 95% humidity, and SHEM was changed daily. After 3 and 5 days, porcine corneas were removed for embedding in OCT compound (Sakura Finetec, Torrance, CA). The tissues were frozen at -80°C for histopathologic processing.

Nine human corneas were obtained from the Lions Eye Bank of Texas, Houston, after informed consent for research use was obtained from the decedents' next-of-kin. The corneas were managed in compliance with the guidelines of the Declaration of Helsinki for research involving human tissue. Donor corneas were initially stored at 4°C in preservative (Optisol-GS; Bausch & Lomb, Irvine, CA), then transferred to modified SHEM that was buffered with 2 M Tris-HCl to pH 7.3. After superficial scarification of the corneas with a 22-gauge needle, 10 μL of 1 × 10⁵ CU *A. nidulans* in YPD was topically applied to the corneal surface. Inoculated corneas were put into six-well culture dishes, immersing the corneal rim in modified SHEM. The tissues were incubated at 34°C in 5% CO₂ with 95% humidity, with the SHEM changed daily. After 24 hours, the corneas were embedded in OCT compound and frozen at -80°C.

Hyphal Penetration

Ten-micrometer frozen sections were cut, and three sections were examined for each porcine or human cornea at 16-μm intervals, extending 160 μm from the central cornea. Corneal sections were stained with periodic acid-Schiff (PAS) or Gomori's methenamine-silver (GMS) reagents (Sigma-Aldrich, St. Louis, MO). Images were captured of an entire limbus-to-limbus section from each cornea with a digital camera (model DS-Fil; Nikon, Tokyo, Japan) attached to a microscope (model Y-FL; Nikon). We measured hyphal penetration under 10× magnification at three equidistant points along the corneal length. The total corneal thickness was then measured at 4× magnification for the same positions with an image-analysis system (NIS-Element, ver. 3.0; Nikon). The maximum percentage of hyphal penetration was estimated from measurements taken in three regions of each corneal section. To ensure that representative regions were selected, the results from the largest hyphal-depth percentages among three points for each histologic section were recorded for each corneal slide, and values were averaged from all three slides. The data were expressed as a percentage of corneal thickness, and results were compared by one-way ANOVA and the *t*-test.

TABLE 2. In Vitro Growth of *A. nidulans*

Strain	Neutral Media		Acidified Media	
	Doubling Time (h)*	Radial Growth Rate (mm h ⁻¹)	Doubling Time (h)*	Radial Growth Rate (mm h ⁻¹)
A83	4.04 ± 0.98	0.12 ± 0.02	3.22 ± 0.41	0.29 ± 0.03
B7	3.86 ± 0.54	0.02 ± 0.005*	3.53 ± 0.56	0.30 ± 0.03
B7R	4.81 ± 1.11	0.11 ± 0.01	3.47 ± 0.40	0.32 ± 0.04
C6309	4.76 ± 1.01	0*	3.80 ± 0.29	0.06 ± 0.05*
C6309R	4.51 ± 1.30	0.12 ± 0.02	3.99 ± 0.32	0.28 ± 0.03

Doubling times were measured in liquid media at pH 7.3 and 6.0. Radial growth rates were tested on solid medium at pH 7.3 and 5.6. Results are expressed as the mean ± SD.

* $P < 0.001$, compared with the wild-type strain.

RESULTS

In Vitro Fungal Growth

The doubling times for all *A. nidulans* strains (Table 2) were similar in M199 medium at pH 6.0 ($P = 0.24$) and 7.3 ($P = 0.75$). Mock-inoculated control cultures remained negative for growth throughout 24 hours of observation. On pH 5.6 Sabouraud agar, the *pacC* mutant strain C6309 grew slower ($P < 0.001$) than did the wild-type strain, whereas other strains had comparable ($P = 0.10$) in vitro radial growth rates (Fig. 1). On pH 7.3 Sabouraud agar, all strains grew more slowly than on pH 5.6. Strain C6309 did not grow sufficiently to estimate its radial growth rate on neutral medium. Compared with the wild-type, strain B7 grew more slowly ($P < 0.001$) at neutral pH but was not significantly different ($P = 0.10$) at pH 5.6. Growth rates of rescuant strains B7R and C6309R were not significantly different ($P = 0.10$ and $P = 0.48$) than those of the wild-type strain on solid medium.

Ex Vivo Corneal Virulence

At 3 days after inoculation of *A. nidulans* onto porcine corneas incubated in SHEMA, the fungal hyphae of strains A83, B7, B7R, and C6309R were present in the anterior corneal stroma, but strain C6309 produced no fungi within the stromal tissue

FIGURE 1. Radial growth rates of *A. nidulans* strains A83, B7, B7R, C6309, and C6309R at 25°C on Sabouraud agar plates. Strain C6309 showed reduced radial growth compared with the wild-type ($P < 0.001$).

(Table 3). By day 5, all *A. nidulans* strains had progressed into the corneal stroma (Table 3). Strains A83, B7R, and C6309R invaded the deeper stroma, but strains B7 and C6309 were limited to the anterior stromal layers (Fig. 2).

Comparison of corneal penetration at days 3 and 5 after inoculation showed no significant differences between wild-type strain A83 and rescuant strains B7R ($P = 0.19$) or C6309R ($P = 0.30$). The *palB* mutant strain was less invasive at day 5 than was the wild-type strain A83 ($P = 0.005$). Hyphal invasion of strain C6309 was significantly less than that of wild-type strain A83 at days 3 ($P = 0.0001$) and 5 ($P = 0.003$).

A. nidulans strain A83 rapidly invaded the human corneas within 24 hours after inoculation (Table 4). Septate hyphae grew in linear filaments that branched at acute angles. Strain B7 did not have significantly less penetration ($P = 0.22$) than the wild-type. Strain C6309 showed no hyphal invasion (Fig. 3).

DISCUSSION

Aspergillus species are widespread molds and relatively common causes of fungal keratitis.⁵⁰ Conidial adherence, spore germination, and hyphal extension enable tissue invasion.⁵¹ Upon attachment to the injured ocular surface, *Aspergillus* produces filamentous forms.⁵² Tips of septate hyphae sprout in linear and branching patterns that insinuate through stromal lamellae.^{53,54} Fungal proteases may contribute to destruction of the extracellular matrix,⁵⁵ but the pathophysiological processes that bring about *Aspergillus* keratitis remain unclear.

Virulence factors are genes or proteins that affect fungal pathogenicity.¹⁹ The eight chromosomes of *A. nidulans* contain nearly 10,000 genes, and genetically controlled pathogenicity may be multifactorial.⁵⁶ Fungal constituents implicated in the virulence of *Aspergillus* sp. comprise cell wall components, fungal enzymes such as aspartyl proteases and metalloproteases, and metabolic pathways.^{57,58}

We evaluated the pathogenicity of *A. nidulans* in an ex vivo model of corneal infection to determine the role of a pH-responsive signaling pathway. *A. nidulans* was used as a representative filamentous ascomycete for linking fungal physiology with mammalian virulence.⁵⁹ The genomics of this species have been well-studied, and genetic construction of selective mutations is feasible. Other *Aspergillus* species share similar signaling and metabolic pathways.⁶⁰ Fungal mutants were generated by reverse genetics to study PalB and PacC,

TABLE 3. Penetration of *A. nidulans* Strains onto Explanted Porcine Corneas

Strain	Day 3		Day 5	
	Maximum Penetration (Mean ± SD)	P^*	Maximum Penetration (Mean ± SD)	P^*
A83	26.10 ± 2.55	—	56.36 ± 9.43	—
B7	17.89 ± 5.31	0.073	24.11 ± 3.06	0.005
B7R	30.48 ± 18.84	0.71	61.21 ± 14.31	0.65
C6309	0	0.0001	15.50 ± 5.19	0.003
C6309R	37.18 ± 7.29	0.068	55.85 ± 22.34	0.97

$n = 3$ for all strains.

* Compared to wild-type strain A83.

FIGURE 2. Invasion of *A. nidulans* strains in porcine corneal tissue at pH 7.3 on day 3 after scarification and topical inoculation. (A) Wild-type strain A83 proliferated on the corneal surface and formed hyphae that branched into the anterior stroma. (B) Noninfected control cornea. (C) Strain B7, lacking PalB protease, produced scant hyphae in the superficial cornea. (D) Rescuer strain B7R was similar to wild-type, with extensive fungal invasion throughout the anterior cornea. (E) Strain C6309, lacking the PacC transcription factor, produced no hyphae, and corneal epithelium remained intact. (F) Rescuer strain C6309R produced hyphae that invaded the anterior third of the cornea. PAS staining. Scale bar, 50 μ m. Magnification, $\times 200$.

two key intermediates in the Pal/PacC pathway. Our model was based on the observation that the fungal load of experimental infection is proportional to the relative virulence of the infecting strain.⁶¹

PacC is a fungal zinc-finger-containing transcription factor that is normally held in an inert form. At a neutral or slightly alkaline milieu, proteins at the fungal plasma membrane sense ambient pH and transmit the signal intracellularly. PalB, a calcium-dependent cysteine protease, then cleaves PacC into a smaller molecule with a DNA-binding domain that, on further processing, enters the fungal cell nucleus to transcriptionally activate promoters of genes controlling the production of secreted and cytoplasmic enzymes.⁶²

TABLE 4. Penetration of *A. nidulans* Strains onto Explanted Human Corneas

Strain	Maximal Penetration (Mean % $\pm$ SD)	P*
A83	21.73 $\pm$ 3.69	—
B7	18.13 $\pm$ 2.24	0.22
C6309	0	0.0005

n = 3 for each strain.

* Compared with wild-type strain A83.

Fungal gene-disruption experiments suggest that the Pal/PacC pH-responsive pathway functions during filamentous fungal growth in host tissue.²⁹ A fungal strain with a *palB*-null phenotype has reduced pathogenicity. Use of this mutant and its corresponding, virulent rescuer corroborates that the Pal/PacC pathway is active during *Aspergillus* infection. Similarly, a *pacC*-null strain was practically incapable of producing tissue invasion,²⁹ although use of this pleiotropic strain may partly reflect lower proliferation potential since in vitro growth can correlate with in vivo virulence.⁶³

This study indicates that molecular alterations in an intracellular signaling cascade that affect PacC-regulated gene expression attenuates the development of experimental corneal aspergillosis by *A. nidulans*. We hypothesize that the Pal/PacC pathway regulates the transcription of genes associated with fungal invasion by *A. nidulans* (Fig. 4). The near-neutral pH of the normal ocular surface and cornea,^{64,65} along with the slight pH shift that occurs during microbial keratitis,⁶⁶ provides an appropriate environment for PacC activation in the setting of fungal exposure and injury.

That PacC has a role in corneal invasion by *A. nidulans* is consistent with observations in studies of an analogous regulatory pathway in other fungi. Homologues of *pacC* and other genes coding for the Pal/PacC pathway are known for different *Aspergillus* spp. and other filamentous fungi.²²

Furthermore, Rim101p, the PacC orthologue in *C. albicans*, regulates candidal filamentation and infection of the cornea.³² Thus, intermediates of the PacC regulatory system are conserved in several fungi, and this mechanism may be operative among ocular fungal pathogens.⁶⁷

Further interpretation of our experimental findings is limited by the model system. We noted that mutant strains retained some ability to form invasive filaments, so additional mechanisms contributing to corneal infection may be involved in the corneal infection model.⁶⁸ Also, the relationship of our ex vivo studies to in vivo infection is needed to establish the role of the Pal/PacC pathway in the pathogenesis of fungal keratitis.

FIGURE 3. Histopathology of scarified human corneas infected by *A. nidulans* strains 1 day after inoculation. (A) Wild-type strain A83 proliferated on the corneal surface and branched throughout the anterior cornea, appearing to preferentially invade through sites of superficial scarification. (B) The *palB* mutant strain B7 penetrated to an extent similar to that of the wild-type, but the fungal biomass was slightly less. (C) The *pacC* mutant strain C6309 survived on the corneal surface but failed to produce invasive filaments. GMS staining. Magnification $\times 200$; scale bars, 50 μm .

FIGURE 4. Model of *Aspergillus* PacC activation. Receptors at the fungal cell membrane sense ambient pH conditions and signal an intracellular pathway to proteolytically cleave PacC into an active transcription factor that upregulates selected fungal genes. (Modified from Peñas MM, Hervas-Aguilar A, Munera-Huertas T, et al. Further characterization of the signaling proteolysis step in the *Aspergillus nidulans* pH signal transduction pathway. *Eukaryot Cell*. 2007;6:960–970, doi:10.1128/EC.00047-07. Amended with permission from the American Society for Microbiology.)

Fungi adapt to their local ecosystem, and conditions at the ocular surface may elicit changes in fungal metabolism and gene expression that influence pathogenicity. Studies of functional genomics and the regulation of fungal invasion are helping to reveal how aspergillosis occurs and progresses.^{69,70} *Aspergillus* keratitis is a serious infection that can progress despite treatment.⁷¹ Ongoing research into specific fungal virulence factors should indicate new targets for interventions in the treatment and prevention of keratomycosis.

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