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The Molecular Pathogenicity of *Fusarium* Keratitis:

A Fungal Transcriptional Regulator Promotes Hyphal Penetration of the Cornea

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Abstract

Purpose—The pathogenic mechanisms of fungal infection during human keratomycosis were investigated in an *ex vivo* corneal model that used strains of *Fusarium oxysporum* differing in the production of a fungal transcription factor.

Methods—A *pacC*$^{-}$ loss-of-function mutant and a *pacC*^{c} dominant-activating mutant were constructed from a wild-type isolate of *F. oxysporum*, and the three strains were characterized by *in vitro* growth kinetics. Twenty-seven human donor corneas maintained in tissue culture were superficially scarified and topically inoculated with the wild-type, the *pacC*$^{-}$ loss-of-function mutant, or the *pacC*^{c} dominant-activating strain. Relative hyphal invasion into the stroma was compared histopathologically in corneal sections.

Results—*F. oxysporum* strains demonstrated comparable exponential growth rates *in vitro*. Wild-type *F. oxysporum* invaded into corneal tissue within one day and penetrated through the anterior stroma during the next 4 days. The *pacC*$^{-}$ loss-of-function mutant invaded explanted corneas significantly less than the wild-type on day 1 ($P<0.0001$) and on day 3 ($P=0.0003$). The *pacC*^{c} dominant-activating strain adhered and penetrated explanted corneas similar to the wild-type strain.

Conclusion—The PacC pathway regulating the transcription of fungal genes allows fungal adaptation to the ocular surface and enables invasion of the injured cornea by *F. oxysporum*.

Keywords

filamentous fungi; fungal keratitis; keratomycosis; ocular mycology

Fusarium species are widespread environmental moulds, important community-acquired pathogens, and prevalent causes of fungal keratitis.[1-3] Trauma, contact lens wear, and immunosuppression can overcome the eye's natural defenses and predispose to *Fusarium* keratitis.[4, 5] Infection ensues when *Fusarium* spp. produce filamentous forms that invade compromised tissues. The pathogenesis of keratomycosis involves a dynamic relationship between host susceptibility and fungal virulence factors.[6]

Hyphal invasion relies on fungal metabolic pathways that have an effect on pathogenicity.[7] Several genetically directed processes influence fungal infection.[8] One pathway involves a pH-responsive signaling cascade leading to activation of the PacC/Rim101p transcription

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factor.[9] Orthologues of this regulatory intermediate have been found in several hyphomycetes including *Acremonium chrysogenum*,[10] *Aspergillus nidulans*,[11] *Aspergillus niger*,[12] *Colletotrichum acutatum*,[13] *Fusarium oxysporum*,[14] *Fusarium verticillioides*,[15] *Penicillium chrysogenum*,[16] *Sclerotinia sclerotiorum*,[17] and *Trichoderma harzianum*.[18]

Once activated, this transcription factor regulates genes involved in infection.[14, 19] In studies on experimental *Candida albicans* keratitis, proteolytic activation of Rim101p, a homologue of the PacC transcription factor, promotes hyphal formation and fungal invasion.[20,21] A comparable mechanism appears to be pivotal in the pathogenesis of infections caused by *Fusarium* and *Aspergillus*.[22, 23]

We hypothesized that PacC is important for filamentous fungal infection of the cornea. We used *F. oxysporum* genetic mutants in an *ex vivo* model of fungal adaptation and corneal infection to study whether *pacC* mutation would attenuate fungal invasion.[24] We further determined the relative invasiveness of a strain with a dominant-activating *pacC* allele.

Materials and Methods

Fungal Strains

F. oxysporum f. sp. *lycopersici* strain 4287 (race 2) is a wild-type phytoisolate from the Instituto Nacional de Investigación y Tecnología Agraria y Almentaria, Madrid. The *pacC* gene is present as a single copy in the *F. oxysporum* genome and encodes a 610-amino acid protein (GenBank accession no. AY125958). A $pacC^{-}$ loss-of-function mutant (strain $pacC^{+/-}12$) was developed from this wild-type strain by targeted gene replacement as reported previously, and complementation of the mutated strain with the wild-type *pacC* gene was able to restore pathogenicity.[14] A $pacC^{c}$ dominant-activating merodiploid (strain $pacC^{c}9$) was also constructed that carried both the wild-type *pacC* gene and an ectopic $pacC^{c}$ allele containing a mutation that removed the carboxy terminus of a truncated transcript.[14]

Fungal strains were stored as microconidial suspensions in glycerol at -80°C and then grown in potato-dextrose broth (PDB, Difco, Detroit, MI) at 27°C. Aliquots of each strain were harvested during exponential growth and suspended in sterile phosphate-buffered saline (PBS). Triplicate samples of 5,000 culturable units (CU) of each strain were inoculated into 25 mL M199 liquid medium (Invitrogen, Grand Island, NY) and buffered with Tris-HCl to pH 6.0, pH 7.3, and pH 8.0 to produce acidic, physiologic, and slightly alkaline conditions. A pH of 7.3 was chosen to approximate the pH of the normal human cornea; a pH of 8.0 was used to ensure adequate environmental activation of the PacC pathway;[8] and an acidic pH was selected to determine whether this environment would result in altered fungal pathogenicity since the PacC pathway is typically selectively activated at normal-to-alkaline conditions.

Flasks were incubated at 27°C with continuous shaking. Growth was estimated using optical density (OD) at a wavelength of 600 nm in an Ultraspec 2000 spectrophotometer (Pharmacia Biotech, Princeton, NJ), applying a conversion factor of one OD_{600} unit equivalent to 5.3×10^5 CU/mL that was determined from *in vitro* growth of *Fusarium solani*.[4] These inocula based on OD values consisted of suspensions of hyphal spherules from log-phase growth in shaken cultures in PDB.

Ex Vivo Cornea Model

Human corneas were obtained from the Lions Eye Bank of Texas, Houston, after informed consent for research use was obtained from decedents' next-of-kin. Donor corneas were initially stored at 4°C in Optisol-GS (Bausch & Lomb, Irvine, CA) then transferred to

modified supplemented hormonal epithelial medium (SHEM), consisting of equal volumes of Dulbecco's modified Eagle's medium and Ham's F12 medium supplemented with epidermal growth factor, insulin, transferrin, sodium selenite, hydrocortisone, cholera toxin A, dimethylsulfoxide, 50 μg/mL gentamicin, and 5% fetal bovine serum that was buffered with 2 M Tris-HCL to pH 7.3 or pH 8.0. An artificial anterior chamber (Refractive Technologies, Cleveland, OH) stabilized corneal buttons during superficial scarification performed by a 22-gauge needle, similar to a protocol previously described for an experimental fungal keratitis model.[25] Ten μL of 1×10^5 CU *F. oxysporum* in PDB were topically applied to the corneal surface.[4] Inoculated corneas were transferred into 6-well culture dishes (Corning, Corning, NY), immersing each corneoscleral rim in modified SHEM. Tissues were incubated at 34°C in 5% CO_2 with 95% humidity, changing SHEM daily. After 24, 72, and 120 hours, corneas were removed, embedded in OCT compound (Sakura Finetec, Torrance, CA), placed in liquid nitrogen, and stored overnight in a -80°C freezer.

Histopathology

Ten μm-thick sections were stained with periodic acid-Schiff (PAS) reagent (Sigma-Aldrich, St. Louis, MO). Three sections were examined for each cornea, and images were captured at 10-μm intervals from the corneal mid-point with a DS-Fil digital camera (Nikon, Tokyo, Japan) attached to a Nikon Y-FL microscope. The depth of hyphal penetration of the corneal thickness was obtained at 5 equidistant points along the corneal length, using the NIS-Element 3.0 image analysis system (Nikon). The maximal percentage of hyphal penetration was estimated at regions demonstrating the greatest depth of corneal involvement, and the three largest hyphal-depth percentages were averaged from 5 measurements of each histological section. Three corneas were pooled to calculate the mean ± standard deviation, and collated results were compared by the Student *t*-test.

Results

In Vitro Comparison

F. oxysporum strains demonstrated similar lag, log-growth, and plateau phases *in vitro* (Fig. 1). The doubling times among fungal strains were similar at pH 7.3 and at pH 8.0 (Table 1). At pH 6.0, the wild-type strain doubled at a mean of 4.4 ± 0.2 hrs, and generation times of the *pacC*$^-$ loss-of-function strain and of the dominant-activating strain *pacC*^{c}9 averaged 3.3 ± 0.3 hrs and 4.6 ± 0.3 hrs, respectively.

Ex vivo Corneal Virulence

One day after inoculation of *F. oxysporum* onto human corneas incubated in SHEM buffered to pH 7.3, wild-type fungi adhered to the disrupted epithelial surface and invaded Bowman's layer into the anterior stroma through scarification marks, producing branched, septate hyphae that extended across and between stromal lamellae (Fig. 2). The *pacC*$^-$ loss-of-function mutant, on the other hand, formed a mycelial mat on the corneal surface but produced no fungal hyphae within corneal tissue by one day of incubation. The *pacC*^{c} dominant-activating strain formed invasive hyphae similar to the wild-type (Table 2).

By the third day following inoculation, wild-type *F. oxysporum* continued to penetrate into the corneal stroma, to an average maximal depth twice that at day 1 (Table 2). However, the *pacC*$^-$ loss-of-function mutant produced scanty hyphae, few of which invaded into the anterior stroma. In contrast, the dominant-activating pacC^c strain showed progressive invasion which exceeded that of the wild-type isolate (Fig. 2). While no significant differences in hyphal penetration were found on the fifth day following fungal inoculation,

interpretation was limited by mycelial overgrowth of some sections by the dominant-activating strain.

In SHEM buffered to pH 8.0, wild-type and dominant-activating strains showed similar initial invasiveness, averaging maximal penetration of 4.4% ± 1.5% and 6.7% ± 1.5%, respectively, after one day postinoculation. At day 1 postinoculation maximal penetration percentages of wild-type and dominant-activating strains (Fig. 3) were significantly less at pH 8.0 compared to those at pH 7.3 (P=0.005 and P=0.005). The loss-of-function mutant *pacC*$^{+/-}$12 did not produce fungal hyphae in corneal tissue at day 1. *In vitro* fungal kinetics are not necessarily associated with tissue pathogenicity during infection, and the *ex vivo* data did not correlate with projections from the *in vitro* growth curves.

Discussion

Fusarium is a leading cause of fungal keratitis that can complicate corneal injury and contact lens wear.[3, 5, 26] *Fusarium solani, F. oxysporum, F. verticillioides*, and related species are phytopathogens dispersed throughout the environment.[27, 28] These filamentous fungi have several cellular and molecular attributes that allow colonization and infection of plants and animals.[29] This investigation examined a fusarial virulence pathway associated with fungal attachment and penetration into host tissues.

We modified an *ex vivo* system to study the pathogenic mechanisms of hyphal invasion into human corneas.[24] Explanted donor corneas, lacking ocular surface defenses and systemic immunity, were used to analyze the relative pathogenicity of fungi for the corneal stroma. Similar to other alternative models,[30] tissue culture of eye-bank eyes provides a method to selectively examine the initial events during microbial adherence and invasion of ocular tissue.

We used *F. oxysporum*, a plant pathogen and occasional human corneal isolate that is moderately less virulent than *F. solani*.[31-33] Selected genetic mutants of pathogenic fungi aid in identifying specific virulence factors required for mycotic infection.[34, 35] Therefore, applying molecular disruption techniques, a single gene of *F. oxysporum* was altered that encodes a sequence-specific protein potentially involved in disease pathogenicity.[36]

We found that deletion of the *pacC* gene retarded invasiveness of *F. oxysporum*, indicating that the transcription factor PacC may be involved in filamentous fungal survival and growth at the ocular surface. Slightly more fungal penetration of the cornea was observed with a dominant-activating PacC strain, but this was not statistically significant. Our findings are consistent with studies demonstrating that PacC is needed to establish invasive fusariosis or aspergillosis.[22, 23] PacC-regulated genes appear to be involved in fungal adaptation to the corneal microenvironment and in filamentous growth into stromal tissue.

PacC is part of an intracellular signaling system in several filamentous fungi that responds to ambient pH.[37] Synthesized as an inactive polypeptide, PacC is activated at neutral to alkaline pH through enzymatic proteolysis.[38] Zinc-finger DNA-binding domains then regulate the expression of several fungal genes, including phosphatases and proteases.[9] The PacC-regulated phenotype could thereby affect adaptive filamentous growth at the ocular surface and facilitate opportunistic fungal invasion into the traumatized cornea.

The cornea is susceptible to diverse fungi, and experimental models provide ways to study the virulence of yeasts and filamentous moulds. The emergence of fungal genomics offers additional opportunities for examining the multifactorial processes of fungal growth and pathogenicity in oculomycoses.[39] Molecular mechanisms of tissue invasion by *Fusarium*

spp., such as the PacC pathway, present potential targets for improved control of fungal eye disease.

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FIGURE 1.
Estimation of *in vitro* growth kinetics of *F. oxysporum* strains. Triplicate samples of each strain were inoculated into liquid M199 media at pH 7.3. Mean fungal concentrations (culturable units/ml) are plotted with standard deviations.

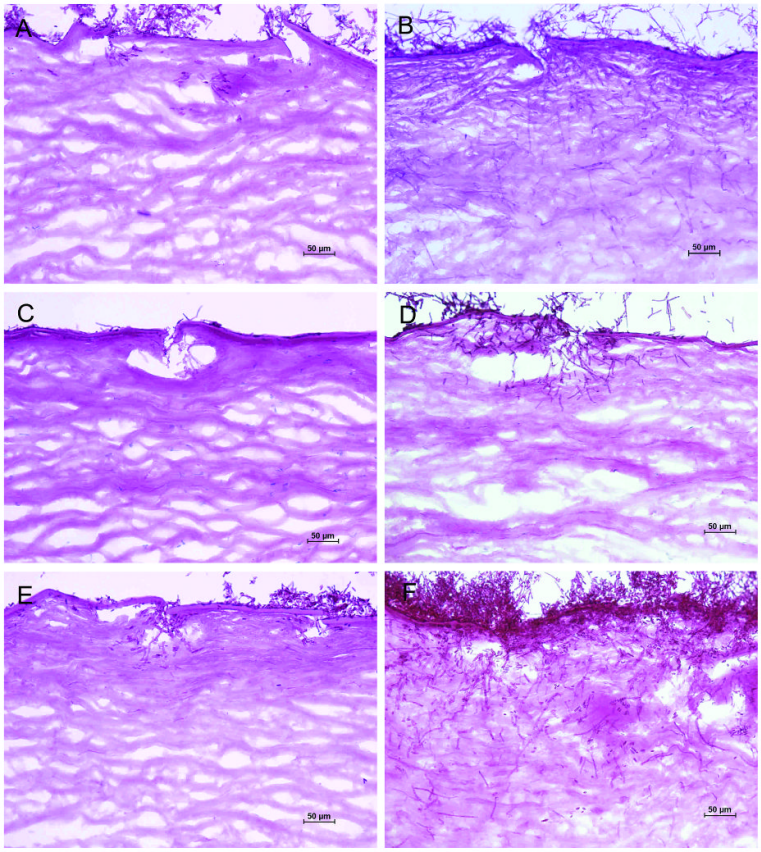

FIGURE 2.

F. oxysporum invasion into human corneas incubated at pH 7.3 (periodic acid-Schiff; original magnification, ×200). A, Wild-type fungi invaded the superficial corneal surface within one day. B, The wild-type strain progressed into the deeper corneal stroma by the third day following inoculation. C, The loss-of-function mutant *pacC*$^{+/-}$12 produced no fungal hyphae within corneal tissue on day 1. D, On the third day after inoculation the *F. oxysporum pacC*$^{+/-}$12 mutant produced hyphae that invaded into the anterior stroma. E, The dominant-activating strain *pacC*^{c}*9* formed hyphae in the superficial stroma on day 1. F, The p*acC*^{c}9 strain proliferated on the corneal surface and continued to extend into the corneal stroma on day 3.

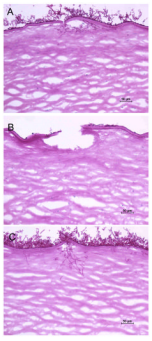

FIGURE 3.

F. oxysporum infection at pH 8.0 on first day following inoculation (periodic acid-Schiff; original magnification, ×200). A, The wild-type strain invaded into the anterior cornea. B, The oss-of-function mutant *pacC*$^{+/-}$12 did not produce invasive fungal hyphae. C, The dominant-activating strain *pacC*^{c}*9* invaded the superficial cornea similar to the wild-type.

TABLE 1

Estimation of Generation Time (hrs) of *F. oxysporum* Strains at Different pH Conditions

Strain	Genotype	pH 7.3	*P*	pH 8.0	*P*
Wild-type	4287	4.1 ± 0.2	-	4.7 ± 0.2	-
pacC$^{+/-}$12	*pacC*$^{-}$ loss-of-function	3.3 ± 0.5	0.09	5.0 ± 0.5	0.33
pacC^{c}9	*pacC*^{c} dominant-activating	4.7 ± 0.4	0.11	5.0 ± 0.3	0.16

A fungal spherule suspension was used as the inoculum. Values expressed as mean ± standard deviation. *P* value compares growth of each mutant strain to wild-type.

TABLE 2

Maximal Penetration of *F. oxysporum* Strains into Explanted Human Corneas under Physiological Conditions

	Wild-Type		*pacC*$^{+/-}$12			*pacC*^{c}9		
Day	**n**	**Penetration (%)**	**n**	**Penetration (%)**	***P***	**n**	**Penetration (%)**	***P***
1	3	9.1 ± 0.9	3	0	<0.0001	3	8.9 ± 1.9	0.87
3	3	18.3 ± 1.0	3	7.5 ± 3.8	0.0003	3	27.8 ± 6.2	0.10
5	3	65.0 ± 9.1	3	65.0 ± 8.9	1.00	3	74.9 ± 2.5	0.14

Values expressed as mean ± standard deviation for pools of 3 corneas per group. *P* value compares corneal invasion of each mutant strain to wild-type.