Contribution of ExsA–Regulated Factors to Corneal Infection by Cytotoxic and Invasive Pseudomonas aeruginosa in a Murine Scarification Model

Ellen J. Lee,¹ Brigitte A. Cowell,¹ David J. Evans,¹,² and Suzanne M. J. Fleiszig¹

PURPOSE. The exoenzyme S regulatory protein ExsA regulates a type III secretion system in Pseudomonas aeruginosa. In vitro, cytotoxic strains use this system to secrete exotoxin (ExoU) and ExoT causing cytotoxicity and inhibiting their phagocytosis by epithelial cells. Invasive P. aeruginosa secrete ExoT and ExoS, but exsA mutation has little impact on their short-term interactions with epithelia. In the present study, the contribution of these ExsA-regulated proteins toward corneal infections in vivo was investigated.

METHODS. After anesthesia, the left cornea of C57BL/6 mice was scratched injured and then inoculated with cytotoxic (PA103) or invasive (PAK) P. aeruginosa or with isogenic mutants in exsA-related genes. Inocula of 10^3 to 10^6 bacteria/5 μL were used, and at least five animals were assigned to each experimental group. Corneal disease was quantified at regular intervals for 14 days in masked fashion with two different scoring systems.

RESULTS. For the cytotoxic strain, mutation of either exoU or exoT alone had little effect on virulence, whereas simultaneous mutation of both exoT and exoU or of exsA resulted in a significantly reduced capacity to cause corneal disease. Complementation of the double exoUexoT mutant with exoT alone restored bacterial colonization levels (>3-log increase) and disease severity to wild-type levels. Complementation with exoT alone increased colonization (~3-log increase) and increased virulence to almost the same levels as wild-type or exsA-complemented infections. Virulence of the invasive strain was not reduced by mutation of exsA or of genes encoding the ExsA-regulated secreted proteins.

CONCLUSIONS ExsA contributed to corneal virulence of only cytotoxic P. aeruginosa, with contributions made by both ExoU and ExoT to bacterial survival and disease severity. This differs from cytotoxic P. aeruginosa virulence in the lung, which is ExoU-dependent. (Invest Ophthalmol Vis Sci. 2003; 44:3892–3898) DOI:10.1167/iovs.02-1302

The bacterium Pseudomonas aeruginosa is an opportunistic Gram-negative pathogen that can cause rapidly progressing, potentially blinding corneal infections.¹ It is also responsible for serious diseases in other locations of the body, particularly in people with cystic fibrosis or burn wounds or in those who are immunocompromised.² P. aeruginosa secretes multiple exoproteins that may contribute toward bacterial virulence in corneal infection. Of these, exotoxin A and protease IV,³–⁶ but not phospholipase C or alkaline protease,⁷,⁸ have been found to be important virulence determinants in the eye. The contributions of elastase (LasB) and LasA protease in P. aeruginosa keratitis are uncertain, due in part to the complex regulation of these factors.⁹,¹⁰

P. aeruginosa strains can be broadly classified as either cytotoxic or invasive based on their effects on epithelial cells.¹⁰ Invasive and cytotoxic strains differ genotypically in some genes of the Exoenzyme S regulon that are coordinately regulated by the transcriptional activator ExsA.¹¹,¹² ExsA-regulated genes that distinguish cytotoxic and invasive strains encode effector molecules that are delivered into mammalian cells through a contact-dependent (type III) secretion mechanism similar to that found in many other Gram-negative bacteria.¹²,¹³

In cytotoxic P. aeruginosa, ExsA regulates acute cytotoxic activity toward various mammalian cell types including epithelial cells, macrophages, and polymorphonuclear neutrophils (PMNs).¹¹,¹⁴,¹⁵ The ExsA-regulated effector molecules that have been identified in cytotoxic strains are (1) exotoxin (ExoU) (encoded by exoU), a 70-kDa protein that is required for full cytotoxic activity in epithelial cells and macrophages,¹¹,¹⁶ (2) ExoT (encoded by exoT), a 55-kDa form of exoenzyme S with less ADP-ribosyltransferase activity,¹¹,¹⁷ but which causes epithelial cell rounding and inhibition of bacterial internalization¹⁸,¹⁹ through the activation of RhogTPases.²⁰,²¹ In vitro research has shown that exsA mutation of a cytotoxic strain changes the way in which it interacts with cultured corneal epithelial cells. ExsA mutants (which do not secrete ExoU or ExoT) lose their acute cytotoxic activity and acquire the ability to invade cells.¹¹,²²

In contrast, invasive P. aeruginosa do not induce acute cytotoxicity, and they invade and multiply within epithelial cells in vitro and in vivo.²³–²⁵ Their lack of acute cytotoxic activity is explained by the absence of the exoU gene.¹¹,¹⁶ However, their ability to invade mammalian cells while encoding ExoT (which inhibits bacterial invasion in a cytotoxic strain background) is not yet understood. In this regard, ExoS, an ExsA-regulated effector (49 kDa) encoded only by invasive strains also has a potential inhibitive effect on invasion, and it exerts this effect when introduced into Yersinia spp.²⁶ or cytotoxic P. aeruginosa.¹⁹ Like ExoT, ExoS can activate Rho-family GTPases to disrupt the actin cytoskeleton.²⁷,²⁸ Some invasive strains have also been found to encode a third ExsA-regulated effector molecule, ExoY, which has adenylate cyclase activity.²⁹ Despite the possession of three ExsA-regulated factors that quickly affect cell function in other settings,²⁷–²⁹ exsA mutation in an invasive strain has little impact on its

From the ¹Morton D. Sarver Laboratory for Cornea and Contact Lens Research, School of Optometry, University of California, Berkeley, California; and the ²Touro University College of Osteopathic Medicine, Vallejo, California.

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Corresponding author: Suzanne M. J. Fleiszig, School of Optometry, University of California, Berkeley, CA 94720-2020; fleiszig@berkeley.edu.

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ability to interact with corneal epithelial cells in vitro, at least over a short (3-hour) period.13

The relative contributions of ExsA-effector molecules in causing corneal disease in vivo are not known for either invasive or cytotoxic strains, although introduction of exoU into an invasive strain through a plasmid was found to enhance its virulence in a lung model of infection.20 In this study, we investigated the roles of ExoU and ExoT in the virulence of a cytotoxic strain and of ExoS and ExoT in the virulence of an invasive strain. Given that type III protein secretion involves translocation of effector molecules directly from the bacterial cytoplasm to the host cell cytoplasm through a specialized delivery apparatus, we used isogenic bacterial mutants, rather than purified exoproducts, to examine the contribution of these ExsA-regulated factors toward P. aeruginosa infectious keratitis.

Methods

Bacterial Strains and Preparation of Inocula

ExsA-regulated effectors of cytotoxic P. aeruginosa were investigated with strain PA103 (serogroup O1) and various isogenic transposon mutants that affect type III secretion of ExoU and/or ExoT. These included an exsA mutant (PA103exoC::Tc), that does not produce any ExsA-regulated proteins,5 an exoT mutant (PA103exoC::Tc), an exoU mutant (PA103exoU::Tc), and an exoU/exoT double mutant (PA103exoU/exoT::Tc). Some experiments involved the use of the exoU/exoT double mutant complemented with either exoU or exoT through the plasmid pUC18. These were compared with wild-type strain PA103 or with the exoU/exoT mutant containing only the empty pUC18 vector.

ExsA-regulated effectors of invasive P. aeruginosa which produce ExoS and ExoT, but not ExoU, were investigated with strain PAK (serogroup O1) and the following isogenic mutants: an exsA mutant (PAKexoC::Gem), an exoT mutant (PAKexoC::Gem), and an exoU/exoT double mutant (PAKexoU/exoT::Gem). The properties of all the strains and mutants used are summarized in Table 1. None of the mutants used is defective in growth or survival, even when incubated in vitro with corneal epithelial or other cell types.14,15

Bacteria were grown overnight (18 hours) at 37°C on Luria-Bertani (LB) agar plates. Strains complemented with pUC plasmids were grown on LB agar supplemented with carbenicillin (100 μg/mL). Bacteria were suspended in buffered minimum essential Eagle’s medium (MEM; cat. no. M-6462; Sigma-Aldrich, St. Louis, MO) to a concentration of 2 × 10^8 cfu/mL and diluted in MEM to appropriate concentrations before inoculation of the cornea. Bacterial concentrations were confirmed by viable counts.

Murine Corneal Infection In Vivo

A murine scarification model of corneal infection was used.7 Female C57BL/6 mice (5–7 weeks old) were anesthetized, and three scratches (each 1 mm length) were made on the left cornea of each animal with a sterile needle. Scratched eyes were then topically inoculated with a strain PA103 or PAK, or their isogenic mutants at inocula of 10^5, 10^4, 10^3, or 10^2 total colony-forming units in 5 μL MEM. With the exception of pilot experiments, at least five animals were assigned to each treatment group. Progression of infections was monitored to detect any differences between mutant and wild-type strains during development and resolution of disease. Experiments were repeated at least once when necessary.

The overall severity of corneal infections was scored in a masked fashion at 1, 2, 4, 7, and 14 days after challenge, according to the following previously described grading system: grade 0, no visible disease; grade 1, faint opacity partially covering the pupil; grade 2, dense opacity covering the pupil; grade 3, dense opacity covering the entire anterior segment; and grade 4, perforation of the cornea and/or phthisis bulbi (shrinkage of the eyeball). Infections with wild-type bacteria were generally noted to progress in severity until 2 to 4 days after bacterial challenge, after which the severity of infection, as determined by overall scores, tended to remain unchanged. An eye with an overall grade of 2 or greater was considered infected.

An additional five-point grading system (grade 0, no visible disease, to grade 4, severe disease; Table 2) was also used for the assessment of multiple disease characteristics, including area and density of the central opacity, density of the peripheral opacity, and quality of the epithelial surface.22

The parameter grades were totaled to produce a single total score ranging from 0 (clear, normal) to a maximum of 16. Descriptive comments were recorded, and eyes were photodocumented in color using a camera (SLR Olympus, Tokyo, Japan) attached to a dissecting microscope (Stemi 2000C; Carl Zeiss Meditec, Thornwood, NY).

For enumeration of colonizing bacteria, eyes were enucleated and then homogenized in 1 mL tryptic soy broth. To determine the number of viable bacteria, serial dilutions of homogenates were plated on LB agar plates. For plasmid-complemented strains, homogenates were also plated on LB agar plates supplemented with carbenicillin (400 μg/mL) to examine retention of the plasmid during 2 days in vivo.

All procedures were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the University of California, Berkeley Animal Care and Use Committee.

Statistical Analysis

Data are presented as an overall grade of infection for each mouse (using the system of Beisel et al.), or as a median score with lower
and upper quartiles (using the system of Beisel et al. or the extended grading system of Cowell et al.)). Nonparametric statistics were used to determine the statistical significance of differences between groups. Thus, the Mann-Whitney test was used to compare two groups, and the Kruskal-Wallis test was used for comparisons between three or more groups. The χ² test was used where indicated. P < 0.05 was considered significant. Bacterial mutants in exsA- and exsA-regulated genes were found to alter the probability of disease occurrence rather than to prevent disease. Thus, some variability occurred among animals in the same treatment group. Because important conclusions related to the role of ExsA in corneal disease caused by cytotoxic and invasive P. aeruginosa strains emerged from the data already obtained (356 animals), the use of additional animals to perform more detailed statistical analysis was not clearly justifiable.

RESULTS

Role of Type III-Secreted Effectors in Corneal Infection by Cytotoxic P. aeruginosa

With an inoculum of 10⁶ cfu total bacteria, both wild-type PA103 and its isogenic exoU mutant infected all inoculated eyes (Table 3). In contrast, the exoU/exoT double mutant and the exsA mutant each caused corneal infection in only one of five inoculated eyes. The significant difference in the infection rates between the exoU mutant and the exoU/exoT double mutant with 10⁶ cfu bacteria (P = 0.0367, Mann-Whitney test) suggests a major role for ExoT in disease development. However, subsequent experiments showed that mutation of exoT alone did not reduce virulence compared with PA103 (Table 3). The difference in infection rates between bacteria lacking both ExoU and ExoT (exoA mutant and exoU/exoT double mutant) and those producing one or both of these effectors (exoU mutant and wild-type PA103) was significant (P = 0.0025, Mann-Whitney test). These data suggested that ExoU and ExoT played redundant roles in corneal disease caused by the cytotoxic P. aeruginosa strain PA103.

To determine whether there were subtle differences in disease severity between disease caused by the wild-type and exoU or exoT mutants that were not detected by the single-point grading system, a multifactorial five-point grading system was also used. Still, no differences were detected with mutation of either exoU or exoT alone (Table 4).

Mutations affecting both exoU and exoT reduced the probability of infection rather than preventing disease occurrence. When infections occurred (grade 2 or greater), they often resolved during the 14-day observation period, unlike those caused by bacteria producing ExoU or ExoT, which never improved (Table 3; χ² = 10.52, P < 0.01). Furthermore, examination of photographs of eyes revealed differences in disease characteristics that were not numerically assessed as part of the scoring systems used. For example, by day 7, the cornea in disease caused by PA103, or the exoU, or the exoT mutant took on a yellow or brown hue due to deposition of pigment within the cornea, and the limbal vessels were extremely dilated. This occurred in all animals infected with bacteria capable of secreting either ExoU or ExoT. In contrast, all eyes infected with the double exoU/exoT or the exsA mutant exhibited white corneal infiltrates and only minor limbal dilation (Fig. 1). There was a significant association between the presence of an ExsA-regulated effector molecule (ExoU or ExoT) and pigment deposition/limbal vessel dilation (χ² = 35.95, P < 0.01).

When eyes were infected with bacteria containing the pUCP18 plasmid, the duration of the experiment was limited to 2 days to ensure plasmid retention. The plasmid was retained by the bacteria until the end of the 2-day observation period. This was shown by comparison of the number of colonies that grew when eye homogenates were plated on agar

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**Table 3. Overall Disease Scores with PA103 or Isogenic Mutants at 4 Days after Inoculation**

<table>
<thead>
<tr>
<th>Strain</th>
<th>10⁶ cfu</th>
<th>10⁷ cfu</th>
<th>10⁸ cfu</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment 1</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PA103</td>
<td>3, 3, 3, 3, §</td>
<td>3, 3, 3, 0, 0</td>
<td>3, 3, 3, 0, 0</td>
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<tr>
<td>exoU</td>
<td>3, 3, 3, 3, §</td>
<td>3, 3, 3, 3, §</td>
<td>3, 3, 3, 0, 0</td>
</tr>
<tr>
<td>exoU/exoT</td>
<td>3, 0, 0, 0, 0</td>
<td>3, 3, 3, 1, §</td>
<td>3, 3, 0, 0, 0</td>
</tr>
<tr>
<td>exsA</td>
<td>3, 0, 0, 0, 0</td>
<td>3, 0, 0, 0, 0</td>
<td>3*, 0, 0, 0, 0</td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PA103</td>
<td>3, 3, 3, 0, 0</td>
<td>3, 3, 3, 3, §</td>
<td>3, 3, 0, 0, 0</td>
</tr>
<tr>
<td>exoT</td>
<td>3, 3, 3, 1, 0</td>
<td>3, 3, 3, 3, §</td>
<td>3, 3, 2, 1, 0</td>
</tr>
</tbody>
</table>

* Eye showed one grade improvement by day 14.
† Eye showed two grades improvement by day 14.
‡ One mouse was discontinued during the initial 24 h of the experiment for general health reasons.
§Eye showed one grade improvement by day 7.
with and without carbenicillin. As expected, wild-type PA103 was more virulent than the exoUexoT double mutant that was complemented with the empty plasmid vector (P = 0.0017; Table 5). Complementation of the exoUexoT double mutant with either exoU or exoT using that plasmid restored disease severity so that it was no longer significantly different from wild type (for exoU, P = 0.8983; for exoT, P = 0.1102). The effects of exoU and exoT complementation were statistically significant (P = 0.0181), with significantly worse disease associated with exoU. Although these results with the overall scoring system showed no statistical difference between disease caused by wild-type and exoT complemented mutant, it was apparent from the photographs that the disease characteristics differed (Fig. 2). This difference was revealed when statistical analysis of total scores, which take into account individual disease parameters, was performed (P = 0.0073). More detailed analysis showed statistical differences in area of main opacity (P = 0.0088), surrounding opacity density (P = 0.0181), and surface regularity (P = 0.0027), but not in density of main opacity (P = 0.0845). Because these experiments were terminated after 2 days to minimize plasmid loss, differences in corneal pigment deposition and limbal vessel dilation occurring later in the disease process (7 days after inoculation) were not evaluated.

Viable counts of bacteria recovered 2 days after inoculation showed that double mutation of exoU and exoT reduced the ability of bacteria to survive in vivo by more than 3 log units (P = 0.0017; Fig. 3).

Complementation of the exoUexoT double mutant with either exoU or exoT significantly increased the number of viable bacteria recovered from infected eyes (P = 0.0017, exoU complementation; P = 0.0181, exoT complementation). The increase with exoT was striking (~1000-fold), but colonization still fell short of wild-type levels by approximately 50% (P = 0.0040). In contrast, wild-type colonization levels were completely restored with exoU complementation (P = 0.4062).

**Role of Type III–Secreted Effectors in Corneal Infection by Invasive *P. aeruginosa***

To determine the potential effects of ExsA-regulated factors on corneal disease induced by invasive *P. aeruginosa*, a pilot experiment was performed in which eyes were inoculated with 10⁵, 10⁴, 10³, or 10⁰ cfu of strain PAK or its isogenic exsA mutant, and overall disease scores were assigned 4 days after inoculation. The exsA mutation did not result in reduced corneal disease (Table 6).

A second experiment was performed in which eyes were scored by both scoring systems. This time, an inoculum of 10⁵ cfu was used because the pilot study showed that this inoculum was optimal for infecting corneas with wild-type PAK. Again, results revealed no significant difference between PAK and the exsA mutant at each time point with either scoring system (Table 7; P > 0.05 Mann-Whitney test).

ExsA regulates the type III secretion of ExoS and ExoT. The results (Table 8) unexpectedly showed that the total disease severity scores for exsA mutant and exoT mutant infections were significantly higher than those for the wild-type strain PAK or double exsAexoT mutant infections at all time points tested (2 days, P = 0.0393; 4 days, P = 0.0124; 7 days, P = 0.0240: simultaneous comparison of all groups with the Kruskal-Wallis test).

**DISCUSSION**

ExsA-regulated factors of cytotoxic and invasive *P. aeruginosa* had different effects on virulence in a cornea model. The significant reduction in virulence of the exsA mutant of a cytotoxic *P. aeruginosa* strain suggested a role for the ExsA-

<table>
<thead>
<tr>
<th>Strain</th>
<th>Overall Scores</th>
<th>Total*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA103</td>
<td>4, 4, 4, 3, 3, 3</td>
<td>13.5 (12.5:15.5)</td>
</tr>
<tr>
<td>exoUexoT + pUCP18</td>
<td>2, 2, 2, 2, 1, 1</td>
<td>5.5 (2:3)</td>
</tr>
<tr>
<td>exoUexoT + pUCPexoU</td>
<td>4, 4, 4, 4, 3, 3</td>
<td>13.5 (13.5:14)</td>
</tr>
<tr>
<td>exoUexoT + pUCPexoT</td>
<td>3, 3, 3, 3, 3, 1</td>
<td>9.5 (9:9.5)</td>
</tr>
</tbody>
</table>

* Median score (lower quartile:upper quartile).
regulated type III secretion system in virulence in corneal tissues. In contrast, mutation in \textit{exsA} had no effect on virulence of an invasive strain, whereas mutation of single effector molecules regulated by this system actually caused small, but significant, increases in \textit{P. aeruginosa}–induced disease. For cytotoxic \textit{P. aeruginosa}, mutation of \textit{exsA}, or of both \textit{exoU} and \textit{exoT} in PA103 reduced virulence by a similar amount. This suggested that ExoU and ExoT are the principal ExsA-regulated virulence factors in this corneal disease model. The fact that \textit{exsA} mutation did not decrease virulence below levels noted with the \textit{exoUexoT} double mutant suggested that other ExsA-regulated virulence mechanisms, such as the type III secretion apparatus itself, which has cytotoxic effects on mammalian cells in vitro, play a significant role. Mutation of either \textit{exoU} or \textit{exoT} alone did not reduce virulence, which suggests that either of these exoproteins alone was sufficient to confer virulence, whereas neither was absolutely required. Complementation experiments showed that ExoU was somewhat more damaging than ExoT.

Complementation experiments also showed that either ExoU or ExoT was required for bacterial survival in the eye in vivo, which probably relates to their role in the disease process. The inability of \textit{exoUexoT} double mutants to colonize corneas in vivo is not due to a generalized defect in growth or survival, because they grow efficiently in vitro, even in the presence of mammalian cells. In vitro, ExoT inhibits \textit{P. aeruginosa} internalization by corneal epithelial cells and disrupts actin cytoskeleton function through the activation of RhoGTPases. It has also been shown to inhibit wound healing of airway epithelial cells in vitro. ExoU is essential for acute cytotoxicity in vitro in various mammalian cell types including corneal cells, and it is absolutely essential for virulence of \textit{P. aeruginosa} in an animal model of acute pneumonia. That ExoT was able to substitute partially for ExoU in the cornea suggests that there could be differences in pathogenesis between the lung and the eye. It also suggests that some of the contribution of ExoU to \textit{P. aeruginosa} virulence in the cornea may reflect activity separate from its acute cytotoxic effects. The mechanism by which ExoU induces acute cytotoxicity in vitro or disease in vivo is not yet known. It has been proposed that ExoU, like other ExsA-regulated effectors, may have more than one mechanism of action. Thus, ExoU and ExoT may share activities that contribute to corneal disease. Otherwise, the role(s) of ExoU and ExoT in \textit{P. aeruginosa} corneal virulence differ entirely, despite significant redundancy.
A commonly used method of comparing virulence in animal models is calculation of the ID$_{50}$ (the inoculum at which disease develops in 50% of challenged animals). The 

\textit{exoUexoT} double mutant and \textit{exsA} mutant of PA103 occasionally caused disease in the cornea, albeit with lower probability than wild-type or single \textit{exoU} or \textit{exoT} mutants. Furthermore, inoculation with wild-type bacteria at the highest challenge dose used (10$^6$ cfu per 5-$\mu$L drop) did not always guarantee infection would result, whereas inoculation with the lowest challenge dose (10$^5$ cfu per 5-$\mu$L drop) occasionally caused disease. Thus, it was not possible to calculate an ID$_{50}$ for wild-type or for any of the mutants. A significant reduction, but not total elimination of capacity to cause disease by \textit{exsA} mutation, shows that ExsA is not absolutely necessary for corneal disease to develop. Rather, the ExsA system, through either ExoU or ExoT, increases the probability that bacterial inoculation will result in disease.

When infections occurred with the \textit{exsA} or \textit{exoUexoT} double mutant, there were visible differences in resultant disease that were not detected by either scoring system. Severe limbal blood vessel engorgement and accumulation of pigment that were not detected by either scoring system. Severe limbal disease in the cornea, albeit with lower probability than wild-type bacteria at the highest challenge dose used (10$^6$ cfu per 5-$\mu$L drop) did not always guarantee infection would result, whereas inoculation with the lowest challenge dose (10$^5$ cfu per 5-$\mu$L drop) occasionally caused disease. Thus, it was not possible to calculate an ID$_{50}$ for wild-type or for any of the mutants. A significant reduction, but not total elimination of capacity to cause disease by \textit{exsA} mutation, shows that ExsA is not absolutely necessary for corneal disease to develop. Rather, the ExsA system, through either ExoU or ExoT, increases the probability that bacterial inoculation will result in disease.

In conclusion, the data suggest distinct pathogenic mechanisms for cytotoxic and invasive \textit{P. aeruginosa} in the cornea. Each causes approximately 50% of \textit{P. aeruginosa} corneal infections.40 Thus, these results are significant for future research into the pathogenic mechanisms of \textit{P. aeruginosa} and for the design of therapeutic interventions to reduce the probability of bacteria-induced corneal disease.
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