

# Salicylic Acid Reduces the Production of Several Potential Virulence Factors of *Pseudomonas aeruginosa* Associated with Microbial Keratitis

Mahesh B. K. Bandara,<sup>1,2</sup> Hua Zhu,<sup>1,3</sup> Padmaja R. Sankaridurg,<sup>1,2,3</sup> and Mark D. P. Willcox<sup>1,2,3</sup>

**PURPOSE.** *Pseudomonas aeruginosa* is a common cause of contact-lens-related microbial keratitis. This bacterium is becoming increasingly resistant to antibiotics, and even if the infection can be treated with antibiotics, damage to the cornea resulting from the combined effect of bacteria and host factors can lead to loss of vision. The purpose of this study was to test the effect of salicylic acid on the production of potential virulence factors during the growth of *P. aeruginosa*.

**METHODS.** Bacterial cells were grown in a subinhibitory concentration of salicylic acid, and supernatants were collected and analyzed for presence of proteases by using zymography and hydrolysis of chromogenic substrates. The supernatants were also analyzed for the amount of acetylated homoserine lactones by using bacterial reporter strains. *Pseudomonas* cells from salicylic acid cultures were analyzed for their twitching and swimming motility as well as their ability to invade or cause the death of corneal epithelial cells.

**RESULTS.** Growth in a subinhibitory concentration of salicylic acid resulted in a significant reduction in the number of bacterial cells and a reduction in the rate of the number of bacteria increasing during logarithmic growth, but the time to reach the stationary phase of growth was unchanged. These changes in growth pattern affected the amount of acylated homoserine lactones produced by *P. aeruginosa* 6294. Also affected by growth in salicylic acid was the ability of strain 6294 to show twitching or swimming motility. Salicylic acid also reduced the invasion of strain 6294 into corneal epithelial cells and the epithelial cell death caused by strain 6206. Furthermore, production of proteases by *P. aeruginosa* was significantly reduced by growth in salicylic acid.

**CONCLUSIONS.** The results of this study clearly demonstrate that salicylic acid has a significant impact on several potential virulence factors of *P. aeruginosa* that may be involved in the production of microbial keratitis. These effects were probably mediated by reduction in the cell density and concomitant reduction in the quorum-sensing signaling molecules, the acy-

lated homoserine lactones, produced by *P. aeruginosa*. (*Invest Ophthalmol Vis Sci.* 2006;47:4453-4460) DOI:10.1167/iov.06-0288

The bacterium *Pseudomonas aeruginosa* is the most common cause of microbial keratitis (MK) during contact lens wear and has been implicated in other adverse responses to contact lens wear.<sup>1-4</sup> Most of these adverse responses occur subsequent to the bacteria adhering to the contact lens, releasing toxins or interacting with epithelial cells to produce the signs and symptoms of clinical events. *P. aeruginosa* keratitis is an aggressive infection characterized by extensive ulceration and destruction of the cornea, with breakdown of collagen and perforation, if therapy is not initiated quickly. With the increase in resistance of *P. aeruginosa* to antibiotics being reported in both the United States<sup>1,5</sup> and India,<sup>6,7</sup> there is a clear need for new agents to help control the MK produced by this microorganism.

The mechanisms underlying the pathogenesis of *P. aeruginosa* infections have been researched, and a multitude of virulence factors and mechanisms allow *P. aeruginosa* to adhere and survive and replicate in corneal tissue.<sup>8</sup> Lipopolysaccharide and pili may mediate initial adhesion to contact lenses or the cornea.<sup>9-12</sup> Type IV pili-mediated twitching motility and flagella-associated motility are important pathogenic traits of this bacterium and allow bacteria to move in liquids or along surfaces.<sup>13-15</sup> In addition, invasion and cytotoxicity of certain *P. aeruginosa* strains toward corneal epithelial cells may facilitate the virulence of *Pseudomonas* keratitis.<sup>16</sup> Bacterial flagella also appear to play a key role in the invasion of cells by this microorganism.<sup>17</sup>

*P. aeruginosa* produces many extracellular virulence factors that may contribute to its pathogenesis.<sup>8</sup> The proteases elastase, alkaline protease, and protease IV are associated with virulence, extensive tissue damage, invasiveness, dissemination, and colonization, and are able to promote the destruction of the cornea.<sup>18-23</sup> Protease IV destroys a variety of host proteins.<sup>18</sup> Elastase and alkaline protease produced by the bacterium may mediate penetration through the corneal epithelium.<sup>20,24-28</sup>

Production of some extracellular virulence factors in *P. aeruginosa* is controlled by acylated homoserine lactones (AHLs) which is part of the quorum-sensing (QS) of this bacterium. Two QS systems, Las and Rhl, have been designated in *P. aeruginosa*. All strains of *P. aeruginosa* isolated from corneal infections appear to produce AHLs whereas strains isolated from other sources, such as noninfective corneal inflammatory events and contact lens cases, may be defective in *lasI*, *lasR*, or *rhlI* genes involved in QS.<sup>23,29,30</sup>

Salicylic acid is a nonsteroidal anti-inflammatory drug (NSAID) that inhibits growth and biofilm formation by *Staphylococcus epidermidis* both as a sole agent<sup>31</sup> and in combination with antibiotics,<sup>32</sup> and has shown antimicrobial effects in experimental rabbit models of *Staphylococcus aureus* endocarditis.<sup>33,34</sup> Our laboratory has reported that salicylic acid

From the <sup>1</sup>Vision Cooperative Research Centre, Sydney, Australia; <sup>2</sup>The School of Optometry and Vision Science, University of New South Wales, Sydney, Australia; and the <sup>3</sup>Institute for Eye Research, Sydney, Australia.

Supported in part by the Federal Australian Government through the CRC scheme and through a grant from the American Optometric Foundation.

Submitted for publication March 17, 2006; revised May 30, 2006; accepted July 27, 2006.

Disclosure: **B.M.K. Bandara**, None; **H. Zhu**, None; **P.R. Sankaridurg**, None; **M.D.P. Willcox**, None

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Corresponding author: Mark D. P. Willcox, Vision Cooperative Research Centre, Level 5, North Wing, Rupert Myers Building, The University of New South Wales Sydney NSW 2052, Australia; m.willcox@visioncrc.org.

reduces the attachment of *P. aeruginosa* and *S. epidermidis* to human corneal epithelial cells in vitro.<sup>35</sup> Furthermore, several authors have referred to the antibiofilm properties of salicylic acid.<sup>31,36-38</sup> Salicylic acid has been shown to downregulate some of the virulence factors in *P. aeruginosa* and to attenuate the bacterium's virulence on *Arabidopsis thaliana* and *Caenorhabditis elegans*.<sup>39</sup> Among the virulence factors that were downregulated by salicylic acid were total protease and elastase activities.

The present study was undertaken to investigate the effect of salicylic acid on bacterial growth and production of quorum-sensing molecules and on the production of cell-associated and extracellular virulence factors of *P. aeruginosa* such as type IV pilus and flagella motility, signal molecules, and proteases. Further, the effect of salicylic acid on acute cytotoxicity and invasion of *P. aeruginosa* in a human corneal epithelial cell line was also evaluated using strain 6294, which is an invasive type, and strain 6206, which is a cytotoxic type.<sup>16</sup>

## MATERIALS AND METHODS

### Bacteria Strains and Culture Conditions

Strains of *P. aeruginosa* 6294 (invasive phenotype) and 6206 (cytotoxic phenotype) were grown for 18 hours at 37°C in tryptone soy broth (TSB; Oxoid, Sydney, Australia), with or without salicylic acid (2-hydroxybenzoic acid; Sigma-Aldrich, St. Louis, MO). Reporter strain *Chromobacterium violaceum* (CV026) and *Agrobacterium tumefaciens* A136 were grown overnight in TSB or supplemental medium A, respectively, as described previously.<sup>25</sup>

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of salicylic acid were determined using 96-well microtiter plates. Bacteria were prepared in TSB to a final concentration of  $1 \times 10^7$  cfu/mL. Salicylic acid was diluted in TSB at concentrations ranging from 60 to 260 mM. The pH of TSB or TSB containing salicylic acid was measured by using a pH meter before inoculation of bacterial strains. There was no pH difference between TSB and TSB plus salicylic acid (pH 7.2). Fifty microliters of bacterial suspension was mixed with another 50  $\mu$ L of TSB containing salicylic acid in each well of the 96-well plate. Triplicate wells were included for each concentration of salicylic acid tested. TSB only, without SA, was used as the positive control. Bacterial growth was monitored after 24 hours of incubation by reading the absorbance at 660 nm. The MIC end point was determined as the concentration in which there was 80% or greater reduction in growth compared with the control. For determination of the MBC, the viable bacterial counts in each well were enumerated on nutrient agar plates after a 24-hour incubation. The MBC end point was determined as the concentration at which there was no growth on the nutrient agar plate. The final concentration of salicylic acid used in subsequent aspects of the study was a subinhibitory concentration of 30 mM. Salicylic acid was added to TSB before incubation of the strains in this growth medium.

The growth of *P. aeruginosa* 6294 in the presence of a subinhibitory concentration of salicylic acid was examined. Overnight cultures of strains were resuspended to the final concentration of  $1 \times 10^7$  cfu/mL in TSB, with or without 30 mM salicylic acid, and incubated at 37°C. The optical density of bacterial growth was monitored and recorded automatically every 60 minutes for 24 hours. After 24 hours of growth, the number of colony-forming units was measured in each condition, after dilutions were plated on nutrient agar and incubated for 24 hours at 37°C. Tests for determining MIC and MBC and effect of growth in 30 mM salicylic acid were performed on two separate occasions, and identical results were obtained on each occasion.

### Twitching and Flagella Motility

After growth in the presence or absence of 30 mM salicylic acid, the cells of *P. aeruginosa* 6294 were washed twice with sterile PBS and resuspended in PBS at  $1 \times 10^8$  cfu/mL (OD of 0.1 at 660 nm). The

twitching motility assay was performed as described previously.<sup>40</sup> Briefly, cells were stabbed into a nutrient agar plate with a sterile toothpick and incubated overnight at 37°C. Plates were then removed from the incubator and incubated at room temperature for two more days. Colony edges and the zone of motility were measured with a phase-contrast microscope. Flagella motility was measured according to the method of O'Toole and Kolter.<sup>40</sup> Briefly, a colony of strain 6294 was inoculated into M63 agar (0.3% agar) supplemented with glucose (0.2%) and casamino acids (0.5%), incubated for 24 hours, and the distance of colony migration measured (in millimeters). Both twitching and swimming motility were tested on two separate occasions with two repeats per occasion.

### HCE Cells and Culture Conditions

Transformed human corneal epithelial (HCE) cells were maintained as described by Araki-Sasaki et al.<sup>41</sup> Briefly, HCE cells were cultured in modified hormone epithelium medium consisting of an equal volume of Eagle's minimum essential medium (Invitrogen-Gibco, Grand Island, NY) and Ham's F12 medium (Trace Bioscience, Sydney, Australia) supplemented with 5% fetal bovine serum (Invitrogen-Gibco), 50  $\mu$ g/mL gentamicin, 100  $\mu$ g/mL streptomycin, 100 U/mL penicillin, 2.5  $\mu$ g/mL amphotericin B, 100  $\mu$ g/mL cholera toxin, 5  $\mu$ g/mL insulin, 10 ng/mL epidermal growth factor, and 0.5% dimethyl sulfoxide (DMSO; all obtained from Sigma-Aldrich). Cells were grown at 37°C in an atmosphere of 5% CO<sub>2</sub> until confluent.

### Bacterial Invasion of HCE Cells

Bacterial invasion of HCE monolayers was quantified by a gentamicin survival assay as described previously.<sup>42</sup> Briefly, an invasive strain of *P. aeruginosa*, 6294,<sup>16</sup> ( $1 \times 10^6$  cfu/mL) was grown in the presence or absence of 30 mM salicylic acid, added to HCE cell monolayers and incubated at 37°C in an atmosphere of 5% CO<sub>2</sub> for 2 hours. Subsequently, the HCE cells were washed three times with prewarmed PBS and incubated in 0.5 mL of MEM containing 200  $\mu$ g/mL gentamicin (Sigma-Aldrich) at 37°C for 2 hours and lysed by adding 0.5 mL of 0.25% Triton X-100 (Sigma-Aldrich) at 37°C for 15 minutes. The released bacteria were counted by viable counts on nutrient agar plates. Tests were performed on three separate occasions and the results averaged.

### Bacterial Cytotoxicity to HCE Cells

The acute cytotoxic activity of the *P. aeruginosa* strain 6206<sup>16</sup> was evaluated by measuring the amount of lactate dehydrogenase (LDH) released into the media from dead HCE cells, as described previously,<sup>50</sup> using *P. aeruginosa* 6206 ( $1 \times 10^6$  cfu/mL) grown in the presence or absence of salicylic acid. The level of LDH released from the dead HCE cells was measured with a commercial assay kit (CytoTox 96; Promega, Madison, WI) according to the manufacturer's instructions.

### Exoprotease Activity

*P. aeruginosa* (strain 6294) was inoculated into 10 mL of TSB and allowed to grow at 37°C overnight. *P. aeruginosa* cells were harvested by centrifugation (3000g, 10 minutes, 20°C), washed twice in PBS, and resuspended in TSB at a concentration of  $1 \times 10^8$  cfu/mL (OD of 0.1 at 660 nm). The prepared bacteria were added 1:10 to fresh 400 mL of TSB, with or without 30 mM salicylic acid. The cultures were incubated at ambient temperature or 37°C for 20 hours and normalized to an optical density of 1.0 at 660 nm by adding TSB. Supernatants from normalized bacterial cultures were then collected by centrifugation (3000g, 10 minutes) and filtered through a 0.22- $\mu$ m filter to remove any remaining bacteria and stored at 20°C. Culture supernatants from growths at ambient temperature or 37°C were analyzed by zymography, and only culture supernatants from growths at ambient temperature were used for quantitative analysis of elastase and protease IV, by using chromogenic substrates.

The protease activity of the normalized bacterial culture supernatants was examined by zymography on SDS-PAGE gel containing 7.5% (wt/vol) SDS-polyacrylamide and gelatin 0.1% (wt/vol) as a substrate.<sup>43</sup> After electrophoresis and staining with 0.5% (wt/vol) Coomassie blue R-250, the clear bands in the stained gelatin background were visualized and recorded using the calibrated densitometer (GS-800; Bio-Rad, Hercules, CA). As a control for the apparent molecular mass of elastase, porcine pancreas elastase (200 µg/mL; ICN Biomedicals Inc., Irvine, CA) was used.

LasB (elastase) of the normalized bacterial culture supernatants was also determined by Congo red assay, as described previously.<sup>44,45</sup> The appearance of a red color in the supernatant, due to the cleavage of ECR, was measured with a spectrometer (Spectrafluor Plus; Tecan Austria GmbH, Grödig, Austria) at 495 nm. The protease IV activity of the normalized bacterial culture supernatants was quantified by a chromogenic substrate (Chromozym PL; Roche Diagnostics, Indianapolis, IN) as described by O'Callaghan et al.<sup>21</sup> The optical density of the reaction mixture was measured after 30 minutes at 405 nm with a microtiter plate reader (Spectrafluor Plus; Tecan) and the differences in optical density between test and control samples were calculated. Tests were performed on three separate occasions and the results averaged.

### AHL Detection by Thin-Layer Chromatography and Bioassay

The level of AHLs in normalized bacterial culture supernatants was quantified by measuring the degrees of activation in the  $\beta$ -galactosidase reporter strain *A. tumefaciens* (A136) and *Chromobacterium violaceum* (CV026), as described previously.<sup>30,46</sup> The experiments were performed at least twice.

### Statistical Analysis

Data are expressed as the mean  $\pm$  SEM, and all experiments were repeated three times in duplicate. Two-way analysis of variance (ANOVA) was used to determine the significance.  $P < 0.05$  was considered a significant difference.

## RESULTS

### Determination of MIC and MBC and the Effect of Salicylic Acid on the Growth of *P. Aeruginosa*

The MIC for salicylic acid for strain 6294 (invasive strain) was 120 mM and for strain 6206 (cytotoxic strain) was 100 mM. The MBC for strain 6294 was 240 mM and for 6206 was 180 mM. Therefore, a concentration of 30 mM was used to determine the effect of salicylic acid on growth and other factors. Figure 1 demonstrates the effect on growth of 30 mM salicylic acid by strain 6294 (similar findings were found for strain 6206 [data not shown]). This concentration of salicylic acid slightly reduced the overall number of bacterial cells (as measured by absorbance at 660 nm) that grew over the 24-hour period. After 24 hours of growth, the number of colony-forming units per milliliter of cells grown in the absence of salicylic acid was  $4.98 \times 10^9$  cfu/mL  $\pm$   $2.02 \times 10^8$  for strain 6294 or  $4.43 \times 10^9$  cfu/mL  $\pm$   $3.55 \times 10^8$  for strain 6206, and  $3.62 \times 10^9$  cfu/mL  $\pm$   $2.93 \times 10^8$  for strain 6294 or  $4.42 \times 10^9$  cfu/mL  $\pm$   $1.53 \times 10^8$  for strain 6206 grown in the presence of salicylic acid.

### Flagella and Twitching Motility

Salicylic acid reduced the twitching motility of *P. aeruginosa* 6294. The colonies of *P. aeruginosa* in the absence of salicylic acid were flat with a rough appearance displaying irregular colony edges (Figs. 2A, 2B) and a hazy zone surrounding the colony. The cells were in a very thin layer. After 2 days of incubation at ambient temperature, the colony expansion due to twitching motility occurred very rapidly, and the diameter of the spreading hazy zone was  $27 \pm 5$  mm. Bacteria that were grown with the salicylic acid were incapable of producing twitching zones and had round, smooth, regular colony edges (Fig. 2C). Flagella-mediated swimming of *P. aeruginosa* 6294 in the presence of salicylic acid was also reduced (zone size, 2 mm) in comparison with the strain grown without salicylic acid (zone size, 15 mm).

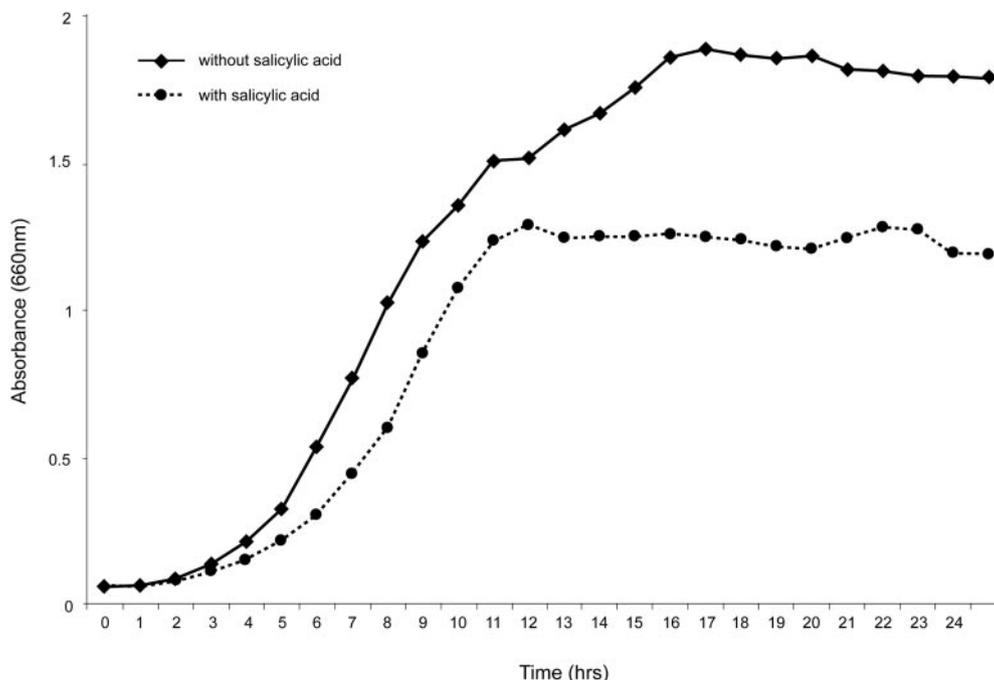
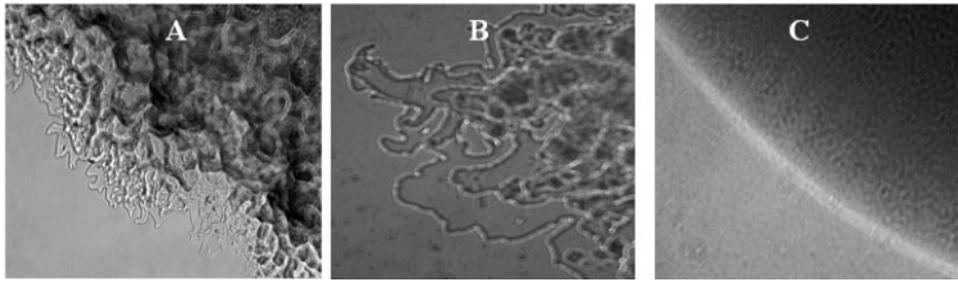


FIGURE 1. Growth of *P. aeruginosa* strain 6294 in the presence of a subinhibitory concentration (30 mM) of salicylic acid.



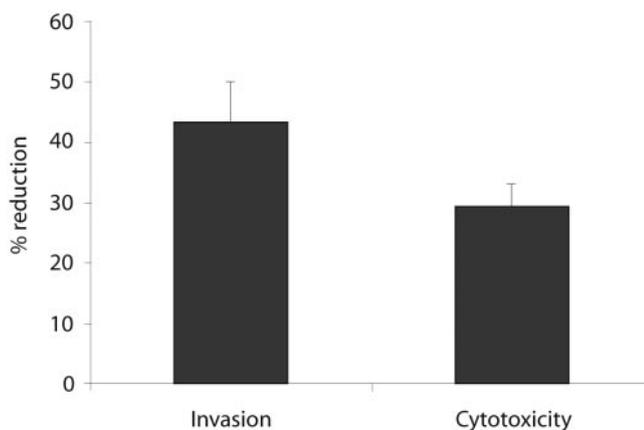
**FIGURE 2.** Light microscopic analysis of colony edges of *P. aeruginosa* in twitching motility plates in the presence or absence of salicylic acid. *P. aeruginosa* produced a flat, widely spread, irregularly shaped colony in the absence of salicylic acid (A, B). The colonies from the bacteria grown with 30 mM salicylic acid (C) were rounded, had a smooth domed shape and lacked a hazy zone surrounding the colony. Magnification: (A, C)  $\times 100$ ; (B)  $\times 400$ .

### Bacterial Invasion and Cytotoxicity to HCE Cells

Compared with the cultures without salicylic acid, the ability of *P. aeruginosa* strain 6294 to invade HCE cells was reduced by 43% when the cells were grown with 30 mM salicylic acid (Fig. 3),  $3.2 \times 10^5$  cfu/mL of control cells invaded the epithelial cells, whereas  $1.83 \times 10^5$  cfu/mL of salicylic acid-treated cells invaded the epithelial cells. The cytotoxicity of *P. aeruginosa* 6206 was reduced by 27% when the cells were grown in the presence of salicylic acid (Fig. 3) compared with those grown without salicylic acid.

### Exoprotease Production in the Presence of Salicylic Acid

Protease production by *P. aeruginosa* in response to salicylic acid exposure was analyzed by gelatin zymography. Three major bands of extracellular proteases at approximately 200 (near the top of the separating gel), 120, and 56 kDa were detected in the control samples (Fig. 4). On the basis of molecular weights and the apparent molecular mass of the control elastase, these three bands were classified as protease IV, elastase, and alkaline protease, respectively.<sup>21,47</sup> The cultures exposed to salicylic acid displayed a lower density of these bands compared with the control. There appeared to be an extra protease band in the salicylic acid culture supernatant (Fig. 4). This "extra" extracellular protease had an approximate molecular mass of between 70 and 100 kDa. Results of the elastin Congo red and chromozym PL assays showed that the elastase and protease IV activities in the culture supernatant of *P. aeruginosa* in response to 30 mM salicylic acid were also



**FIGURE 3.** Mean reduction in invasion and acute cytotoxicity of *P. aeruginosa* in HCE cells grown in the presence of salicylic acid. Results are expressed as the mean percentage reduction of three independent experiments compared with HCE cells incubated with bacteria grown without salicylic acid. In the absence of salicylic acid,  $3.2 \times 10^5$  cfu/mL of strain 6294 invaded the epithelial cells. In the absence of salicylic acid, strain 6206 gave 95% cytotoxicity compared with the positive control of Triton X-100 lysed cells.

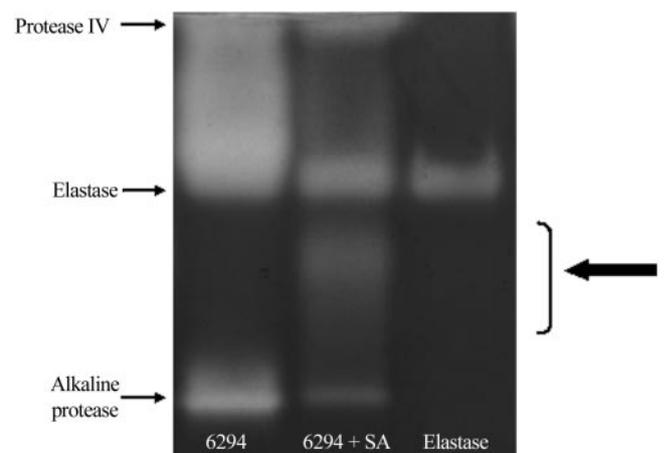
significantly decreased ( $P < 0.05$ ; by 46% and 37%, respectively; Table 1).

### Thin Layer Chromatography and Bioassay of AHLs

AHLs produced by *P. aeruginosa* were detected after reversed phase thin-layer chromatography (TLC) and overlay with monitor bacterium *C. violaceum* (CV 026). In comparison to the control, lower levels of AHLs from *P. aeruginosa* were detected in the culture by using salicylic acid-grown *P. aeruginosa* (Fig. 5). The bioassay of AHLs showed that the amounts of  $\beta$ -galactosidase induced by AHLs in the supernatant of the control were significantly higher ( $P < 0.05$ ) than the culture supernatants that were exposed to salicylic acid. The results of the mean of three individual experiments of AHLs bioassays are shown in Table 1. The untreated bacteria produced  $219 \pm 17$  units of  $\beta$ -galactosidase, whereas *P. aeruginosa* grown in the presence of 30 mM salicylic acid produced only  $22 \pm 4$  units.

### DISCUSSION

In this study, the effects of salicylic acid on the production of extracellular and cell associated virulence factors of *P. aeruginosa* were investigated in vitro. Results clearly demonstrated that salicylic acid had a significant impact on the number of cells that could grow in its presence; on the production of elastase, protease IV, and alkaline protease; on twitching and on flagella-associated motility and affected the production of



**FIGURE 4.** Gelatin zymography of *P. aeruginosa* strain 6294, grown with (lane 2) and without 30 mM salicylic acid (lane 1) and control elastase (lane 3; porcine pancreas elastase, 200  $\mu$ g/mL). **Bold arrow:** an unknown protease activity of *P. aeruginosa* in response to salicylic acid exposure. Supernatants from cells grown in the presence or absence of salicylic acid were normalized to an OD of 1.0 at 660 nm by adding TSB to account for the small difference in growth in the presence of salicylic acid.

**TABLE 1.** Comparison of the Production of Protease IV, Elastase, and AHLs by *P. aeruginosa* in the Presence of Salicylic Acid

Virulence Factor	Culture without Salicylic Acid	Culture with 30 mM Salicylic Acid	% Reduction
Protease IV (U × 1000)	32 ± 9	20 ± 7	37
Elastase (mU)	11 ± 1	6 ± 3	46
AHL production (β-galactosidase units)	219 ± 17	22 ± 4	89

Results are expressed as the mean percentage reduction ± SE of three independent experiments performed in triplicate.

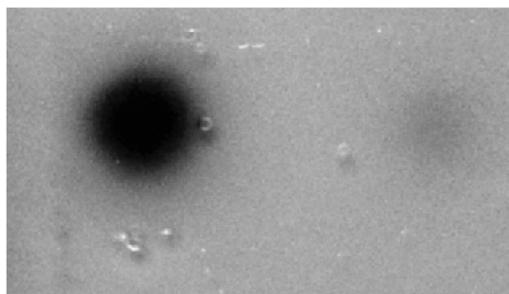
acylated homoserine lactones in *P. aeruginosa*. The results also demonstrated that salicylic acid inhibited invasion and acute cytotoxicity of *P. aeruginosa* in HCE cells.

Bacterial adhesion and colonization on the host surface is a complex process. A study by O'Toole and Kolter<sup>40</sup> found that type IV pili and flagella-mediated motility in *Pseudomonas* was required for biofilm formation on abiotic surfaces and the formation of bacterial microcolonies. Type IV pili in *P. aeruginosa* are important for cell-to-cell contacts, adhesion, colonization, and cytotoxicity toward epithelial cells in vitro and in vivo.<sup>17,40,48-50</sup> Flagella are essential for the spread of infection, allowing bacteria to swim in aqueous environments and playing possible roles in virulence, including mediation of inflammatory response, cellular invasion, and modulation of adhesion to mucins.<sup>17,51,52</sup> Previously, we had demonstrated that growth in salicylic acid significantly reduced the adhesion of *P. aeruginosa* strain 6294 to contact lenses by approximately 50% at a concentration of 30 mM.<sup>35</sup> Also, the adhesion of *P. aeruginosa* strain Paer1 to corneal epithelium was inhibited by approximately 30% at a concentration of salicylic acid of 20 mM.<sup>35</sup> The results of the present study demonstrated that type IV pili-mediated (twitching) motility and flagellum-mediated swimming of *P. aeruginosa* was reduced in the presence of salicylic acid.

Subsequent to adhering to surfaces, *P. aeruginosa* can either invade cells or cause cell death. Invasion and cytotoxicity are two important virulence mechanisms and these have been demonstrated both in vitro and in vivo.<sup>16,53</sup> The bacteria that have invaded can replicate within the cells and both the host cells and the intracellular bacteria can remain viable up to 24 hours.<sup>54</sup> Salicylic acid reduced the invasion of *P. aeruginosa* into corneal epithelial cells by approximately 43%. Sali-

cylic acid also significantly reduced the cytotoxic activity of strain 6206. The reduced cytotoxic activity and invasion of *P. aeruginosa* by salicylic acid may also reduce pathogenicity in the eye. It is possible that there would have been a significantly greater change in both invasion and cytotoxicity of cells if salicylic acid had been added to the experiment throughout (i.e., also added at the time the bacteria were allowed to interact with the epithelial cells). However, we chose not to add salicylic acid to the epithelial cells, as this may have complicated the analysis of the results, given that salicylic acid is a potent inhibitor of eukaryotic cell functions—that is, it acts as a nonsteroidal anti-inflammatory substance. It is of interest to note that the invasion of strain 6294 was reduced in the presence of salicylic acid, even though protease activity was also reduced. The proteases LasA and LasB appear to cleave the invasion-inhibiting toxins ExoS and ExoT of *P. aeruginosa*.<sup>55</sup> Thus, a reduction in LasB (elastase, the 120 kDa protein shown on zymography) may have been hypothesized to increase invasion. Either the apparent upregulation of the novel protease activity at 70 to 100 kDa is even better at cleaving ExoS and ExoT, or salicylic acid might reduce the production of these toxins as well as the proteases.

The next step in corneal virulence is likely to be destruction of host tissue by the bacterium, and this may be mediated by toxins or proteases. Salicylic acid was capable of reducing the production of extracellular proteases in *P. aeruginosa*. The amounts of protease IV and elastase in the culture supernatants of *P. aeruginosa* exposed to salicylic acid were reduced compared with the control. Using plate assays for total protease (plates containing skim milk) or for elastase (plates containing gelatin), Prithiviraj et al.<sup>39</sup> have also demonstrated a significant decrease in these activities in the presence of subinhibitory concentrations of salicylic acid for *P. aeruginosa* PA14. Several studies have demonstrated that *Pseudomonas* serine protease IV is a significant virulence factor during corneal infections.<sup>18,19,46</sup> Further, Caballero et al.<sup>56</sup> suggest that the ability of *P. aeruginosa* to destroy elastin is a major virulence determinant during acute infection, and increased protease activities were associated with tissue damage. Therefore, a significant decrease in protease IV and elastase production with salicylic acid suggests that salicylic acid may reduce corneal virulence and the inflammatory response of the eye during corneal infection. The appearance of an unknown enzyme activity just above the alkaline protease seen in the gel may be an uncharacterized protease expressed in response to salicylic acid exposure. The characteristics of this protease band remain to be defined, but we have demonstrated very low levels of a protease of apparent similar molecular mass (98 kDa) in strain 6294 in other experiments.<sup>30</sup> There has been a report of a protease of ~80 kDa called PASP produced by *P. aeruginosa* PA103 and produced from the gene *PA0423*. This protease can be seen in a mutant that is deficient in protease IV activity.<sup>57</sup> In other experiments, if biofilms of *P. aeruginosa* are exposed to ciprofloxacin, there is a 38% to 65% decrease in total proteolytic activity.<sup>58</sup> In that study<sup>58</sup> *P. aeruginosa* strain PA1230



Control in  
absence of  
salicylic acid

In presence  
of 30 mM  
salicylic acid

**FIGURE 5.** TLC of acylated homoserine lactones of *P. aeruginosa* strain 6294 grown with (lane 2) and without 30 mM salicylic acid (lane 1). For the TLC analysis the samples were chromatographed on silica gel 60 C<sub>18</sub> reversed phase thin-layer plates, and the spots were visualized with biosensor *C. violaceum*.

was shown to have a protease profile on zymography very similar to the profile of 6294 in the present study. Of note, although there was no report of additional protease bands appearing during treatment, more total protease was reported to be produced in response to incubation of biofilms in two times the MIC.<sup>58</sup> Most of the protease experiments were conducted at a temperature of 37°C in vitro, whereas the temperature of the cornea is approximately 33°C.<sup>59</sup> This difference is unlikely to affect the results; indeed protease IV, for example, of *P. aeruginosa* is active in the corneas of mice.<sup>19</sup>

The differentiation of the *P. aeruginosa* mode of growth to a biofilm form<sup>60</sup> and the expression of many virulence factors, such as elastase (*lasB*),<sup>61</sup> LasA protease (*lasA*),<sup>62</sup> protease IV<sup>63</sup> and PA0423 (PASP),<sup>64</sup> and the twitching motility<sup>65</sup> in *P. aeruginosa* are regulated by quorum sensing (QS). Furthermore, QS is used to regulate adhesion and colonization (including biofilm formation) of *P. aeruginosa*.<sup>66,67</sup> Examination of the TLC profiles of *P. aeruginosa* in this study clearly demonstrated that salicylic acid was capable of reducing the expression of AHL signal molecules even in the presence of only a small change in cell numbers. These effects were probably mediated by reduction in the cell density and concomitant reduction in the quorum-sensing signaling molecules, acylated homoserine lactones, produced by *P. aeruginosa*. In addition, salicylic acid can downregulate the QS gene *rhlR*,<sup>39</sup> which in itself is involved in the regulation of the production of LasB (elastase), LasA, protease IV (PrpL), and alkaline protease.<sup>64</sup> Perhaps the reduced production of the QS signal molecules in the presence of salicylic acid is the global switch that downregulates all (or many) of the pathogenicity traits shown in the present investigation.

The results of this study demonstrate that salicylic acid has a significant impact on the virulence factors produced by *P. aeruginosa*. This indicates that this compound is a strong candidate for potential ocular application designed to prevent bacterial contamination of contact lenses or for the treatment of corneal infections. Further experiments should be conducted to examine the possible toxicity of salicylic acid used as a topical agent on the eye. We have previously published that concentrations of salicylic acid of up to 60 mM cause no inhibition of the growth of corneal epithelial cells in tissue culture.<sup>35</sup> There is very little other published information concerning the potential toxic effect of salicylic acid on the cornea. Topical application of acetylsalicylic acid at a concentration of 0.3% applied twice daily for 30 days on the eyes of rats was not associated with reported cytotoxicity.<sup>68</sup> Acetylsalicylic acid is rapidly hydrolyzed in corneas by endogenous esterases,<sup>69</sup> indicating that salicylic acid is most probably nontoxic to corneas. Salicylic acid also has potent anti-inflammatory effects, as it is an inhibitor of the arachidonic acid cascade. It is well known that initially the inflammatory cascade that occurs during *Pseudomonas* keratitis is essential to remove the infecting bacteria.<sup>70</sup> However, it is the continued presence of inflammation, particularly PMNs in the cornea, that contributes to a large extent to the corneal destruction associated with keratitis.<sup>70</sup> Perhaps the addition of salicylic acid on presentation of a patient with *Pseudomonas* keratitis would benefit from the combined anti-virulence factor and anti-inflammatory functions of salicylic acid. If 1 mg/mL sodium salicylate is applied to the corneas of guinea pigs 4 hours after initiation of *P. aeruginosa* infection and subsequently every hour for 6 hours,<sup>71</sup> there is no apparent reduction in the number of PMNs; however, the number of bacteria were not recorded. Further studies should be performed to determine whether salicylic acid can mitigate the course of *P. aeruginosa* corneal infection.

## References

- Alexandrakis G, Alfonso E, Miller D. Shifting trends in bacterial keratitis in South Florida and emerging resistance to fluoroquinolones. *Ophthalmology*. 2000;107:1497-1502.
- Boonpasart S, Kasetsuwan N, Puangricharern V, Pariyakanok L, Jitpoonkukul T. Infectious keratitis at King Chulalongkorn Memorial Hospital: a 12-year retrospective study of 391 cases. *J Med Assoc Thailand*. 2002;85:S217-S230.
- Lam D, Houang E, Fan D, et al. Incidence and risk factors for microbial keratitis in Hong Kong: comparison with Europe and North America. *Eye*. 2002;16:608-618.
- Sun X, Deng S, Li R, et al. Distribution and shifting trends of bacterial keratitis in north China (1989-98). *Br J Ophthalmol*. 2004;88:165-166.
- Chaudhry NA, Flynn HW, Murray TG, et al. Emerging ciprofloxacin-resistant *Pseudomonas aeruginosa*. *J Ophthalmol*. 1999;128:509-510.
- Garg P, Sharma S, Rao GN. Ciprofloxacin-resistant *Pseudomonas* keratitis. *Ophthalmology* 1999;106:1319-1323.
- Kunimoto DY, Sharma S, Garg P, Rao GN. In vitro susceptibility of bacterial keratitis pathogens to ciprofloxacin: emerging resistance. *Ophthalmology*. 1999;106:80-85.
- O'Brien TP. Management of bacterial keratitis: beyond exorcism towards consideration of organism and host factors. *Eye*. 2003;17:957-974.
- Fletcher EL, Fleiszig SM, Brennan NA. Lipopolysaccharide in adherence of *Pseudomonas aeruginosa* to the cornea and contact lenses. *Invest Ophthalmol Vis Sci* 1993;34:1930-1936.
- Gupta SK, Berk RS, Masinick S, Hazlett LD. Pili and lipopolysaccharide of *Pseudomonas aeruginosa* bind to the glycolipid asialo GM1. *Infect Immun*. 1994;62:4572-4579.
- Gupta SK, Masinick S, Garrett M, Hazlett LD. *Pseudomonas aeruginosa* lipopolysaccharide binds galectin-3 and other human corneal epithelial proteins. *Infect Immun*. 1997;65:2747-2753.
- Zaidi TS, Fleiszig SM, Preston MJ, Goldberg JB, Pier GB. Lipopolysaccharide outer core is a ligand for corneal cell binding and ingestion of *Pseudomonas aeruginosa*. *Invest Ophthalmol Vis Sci*. 1996;37:976-986.
- Hahn H P. The type-4 pilus is the major virulence-associated adhesin of *Pseudomonas aeruginosa*. *Gene*. 1997;192:99-108.
- Semmler AB, Whitchurch CB, Mattick JS. A re-examination of twitching motility in *Pseudomonas aeruginosa*. *Microbiology*. 1999;145:2863-2873.
- Zolfaghari I, Evans DJ, Fleiszig SM. Twitching motility contributes to the role of pili in corneal infection caused by *Pseudomonas aeruginosa*. *Infect Immun* 2003;71:5389-5393.
- Fleiszig S M, Zaidi TS, Preston MJ, Grout M, Evans DJ, Pier GB. Relationship between cytotoxicity and corneal epithelial cell invasion by clinical isolates of *Pseudomonas aeruginosa*. *Infect Immun*. 1996;64:2288-2294.
- Fleiszig SM, Arora SK, Van R, Ramphal R. FlhA, a component of the flagellum assembly apparatus of *Pseudomonas aeruginosa*, plays a role in internalization by corneal epithelial cells. *Infect Immun*. 2001;69:4931-4937.
- Engel LS, Hill JM, Caballero AR, Green LC, O'Callaghan RJ. Protease IV, a unique extracellular protease and virulence factor from *Pseudomonas aeruginosa*. *J Biol Chem*. 1998;273:16792-16797.
- Engel LS, Hill JM, Moreau JM, Green LC, Hobden JA, O'Callaghan RJ. *Pseudomonas aeruginosa* protease IV produces corneal damage and contributes to bacterial virulence. *Invest Ophthalmol Vis Sci*. 1998;39:662-665.
- Hobden JA. *Pseudomonas aeruginosa* proteases and corneal virulence DNA. *Cell Biol*. 2002;21:391-396.
- O'Callaghan RJ, Engel LS, Hobden JA, Callegan MC, Green LC, Hill JM. *Pseudomonas* keratitis: the role of an uncharacterized exoprotein, protease IV, in corneal virulence. *Invest Ophthalmol Vis Sci*. 1996;37:534-543.
- Pillar CM, Hobden JA. *Pseudomonas aeruginosa* exotoxin A and keratitis in mice. *Invest Ophthalmol Vis Sci*. 2002;43:1437-1444.

23. Zhu H, Thuruthyl SJ, Willcox MDP. Production of N-acyl homoserine lactones by gram-negative bacteria isolated from contact lens wearers. *Clin Exp Ophthalmol*. 2001;29:150-152.
24. Bejarano PA, Langeveld JP, Hudson BG, Noelken ME. Degradation of basement membranes by *Pseudomonas aeruginosa* elastase. *Infect Immun*. 1989;57:3783-3787.
25. Burns FR, Gray RD, Paterson CA. Inhibition of alkali-induced corneal ulceration and perforation by a thiol peptide. *Invest Ophthalmol Vis Sci*. 1990;31:107-114.
26. Heck LW, Morihara K, Abrahamson DR. Degradation of soluble laminin and depletion of tissue-associated basement membrane laminin by *Pseudomonas aeruginosa* elastase and alkaline protease. *Infect Immun*. 1986;54:149-153.
27. Howe TR, Iglewski BH. Isolation and characterization of alkaline protease-deficient mutants of *Pseudomonas aeruginosa* in vitro and in a mouse eye model. *Infect Immun*. 1984;43:1058-1063.
28. Twining SS, Davis SD, Hyndiuk RA. Relationship between proteases and descemetocoele formation in experimental *Pseudomonas* keratitis. *Curr Eye Res*. 1986;5:503-510.
29. Zhu H, Bandara R, Conibear TC, et al. *Pseudomonas aeruginosa* with *lasI* quorum-sensing deficiency during corneal infection. *Invest Ophthalmol Vis Sci*. 2004;45:1897-1903.
30. Zhu H, Thuruthyl SJ, Willcox MD. Determination of quorum-sensing signal molecules and virulence factors of *Pseudomonas aeruginosa* isolates from contact lens-induced microbial keratitis. *J Med Microbiol*. 2002;51:1063-1070.
31. Muller E, Al-Attar J, Wolff A, Farber B. Mechanism of salicylate-mediated inhibition of biofilm in *Staphylococcus epidermidis*. *J Infect Dis*. 1998;177:501-503.
32. Polonio RE, Mermel LA, Paquette GE, Sperry JF. Eradication of biofilm-forming *Staphylococcus epidermidis* (RP62A) by a combination of sodium salicylate and vancomycin. *Antimicrob Agent Chemother*. 2001;45:3262-3266.
33. Kupferwasser L, Yeaman M, Shapiro S, et al. Acetylsalicylic acid reduces vegetation bacterial density, hematogenous bacterial dissemination and frequency of embolic events in experimental *Staphylococcus aureus* endocarditis through antiplatelet and antibacterial effects. *Circulation*. 1999;99:2791-2797.
34. Nicolau DP, Freeman CD, Nightingale CH, et al. Reduction of bacterial titers by low-dose aspirin in experimental aortic valve endocarditis. *Infect Immun*. 1993;61:1593-1595.
35. Bandara BM, Sankaridurg PR, Willcox MDP. Non-steroidal anti-inflammatory agents decrease bacterial colonisation of contact lenses and prevent adhesion to human corneal epithelial cells. *Curr Eye Res* 2004;29:245-251.
36. Farber BF, Hsieh H, Donnenfeld ED, Perry HD, Epstein A, Wolff A. A novel antibiofilm technology for contact lens solutions. *Ophthalmology*. 1995;102:831-836.
37. Perilli R, Marziano ML, Formisano G, Caiazza S, Scorcio G, Baldassarri L. Alteration of organized structure of biofilm formed by *Staphylococcus epidermidis* on soft contact lenses. *J Biomed Mat Res*. 2000;49:53-57.
38. Tomlinson A, Simmons PA, Seal DV, McFadyen AK. Salicylate inhibition of *Acanthamoeba* attachment to contact lenses. *Ophthalmology* 2000;107:112-117.
39. Prithiviraj B, Bais HP, Weir T, et al. Down regulation of virulence factors of *Pseudomonas aeruginosa* by salicylic acid attenuates its virulence on *Arabidopsis thaliana* and *Caenorhabditis elegans*. *Infect Immun*. 2005;73:5319-5328.
40. O'Toole GA, Kolter R. Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. *Mol Microbiol*. 1998;28:449-461.
41. Araki-Sasaki K, Ohashi Y, Sasabe T, et al. An SV40-immortalized human corneal epithelial cell line and its characterization. *Invest Ophthalmol Vis Sci*. 1995;36:614-621.
42. Fleiszig SMJ, Zaidi TS, Fletcher EL, Preston MJ, Pier GB. *Pseudomonas aeruginosa* invades corneal epithelial cells during experimental infection. *Infect Immun*. 1994;62:3485-3493.
43. Heussen C, Dowdle EB. Electrophoretic analysis of plasminogen activators in polyacrylamide gels containing sodium dodecyl sulfate and copolymerized substrates. *Anal Biochem*. 1980;102:196-202.
44. Bjorn MJ, Sokol PA, Iglewski BH. Influence of iron on yields of extracellular products in *Pseudomonas aeruginosa* cultures. *J Bacteriol*. 1979;138:193-200.
45. Schad PA, Bever RA, Nicas TI, Leduc F, Hanne F, Iglewski BH. Cloning and characterization of elastase genes from *Pseudomonas aeruginosa*. *J Bacteriol* 1987;169:2691-2696.
46. Miller JH. *Experiments in Molecular Genetics*. Woodbury, NY: Cold Spring Harbor Laboratory Press; 1972:11797-12924.
47. Traidej M, Caballero AR, Marquart ME, Thibodeaux BA, O'Callaghan RJ. Molecular analysis of *Pseudomonas aeruginosa* protease IV expressed in *Pseudomonas putida*. *Invest Ophthalmol Vis Sci*. 2003;44:190-196.
48. Kang PJ, Hauser AR, Apodaca G, et al. Identification of *Pseudomonas aeruginosa* genes required for epithelial cell injury. *Mol Microbiol*. 1997;24:1249-1262.
49. O'Toole GA, Roberto K. Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. *Mol Microbiol*. 1998;30:295-304.
50. Tang H, Kays M, Prince A. Role of *Pseudomonas aeruginosa* pili in acute pulmonary infection. *Infect Immun*. 1995;63:1278-1285.
51. Arora SK, Ritchings BW, Almira EC, Lory S, Ramphal R. The *Pseudomonas aeruginosa* flagellar cap protein, FlhD, is responsible for mucin adhesion. *Infect Immun*. 1998;66:1000-1007.
52. Feldman M, Bryan R, Rajan S et al. Role of flagella in pathogenesis of *Pseudomonas aeruginosa* pulmonary infection. *Infect Immun*. 1998;66:43-51.
53. Cole N, Willcox MDP, Fleiszig SMJ, et al. Different strains of *Pseudomonas aeruginosa* isolated from ocular infections or inflammation display distinct corneal pathologies in an animal model. *Curr Eye Res*. 1998;7:730-735.
54. Fleiszig SMJ, Zaidi TS, Pier GB. *Pseudomonas aeruginosa* invasion of and multiplication within corneal epithelial cells in vitro. *Infect Immun*. 1995;63:4072-4077.
55. Cowell BA, Twining SS, Hobden JA, Kwong MS, Fleiszig SMJ. Mutation of *lasA* and *lasB* reduces *Pseudomonas aeruginosa* invasion of epithelial cells. *Microbiol*. 2003;149:2291-2299.
56. Caballero A, Thibodeaux B, Marquart M, Traidej M, O'Callaghan RJ. *Pseudomonas* keratitis: protease IV gene conservation, distribution, and production relative to virulence and other *Pseudomonas* proteases. *Invest Ophthalmol Vis Sci*. 2004;45:522-530.
57. Marquart ME, Caballero AR, Chomnawang M, Thibodeaux BA, Twining SS, O'Callaghan RJ. Identification of a novel secreted protease from *Pseudomonas aeruginosa* that causes corneal erosions. *Invest Ophthalmol Vis Sci*. 2005;46:3761-3768.
58. Oldak E, Trafny EA. Secretion of proteases by *Pseudomonas aeruginosa* biofilms exposed to ciprofloxacin. *Antimicrob Agent Chemother*. 2005;49:3281-3288.
59. Shiloh R, Munitz H, Portuguese S, et al. Corneal temperature in schizophrenia patients. *Int J Neuropsychopharmacol*. 2005;8:537-547.
60. Juhas M, Eberl L, Tummler B. Quorum sensing: the power of cooperation in the world of *Pseudomonas*. *Environ Microbiol*. 2005;7:459-471.
61. Brint JM, Ohman DE. Synthesis of multiple exoproducts in *Pseudomonas aeruginosa* is under the control of RhlR-RhlI, another set of regulators in strain PAO1 with homology to the autoinducer-responsive LuxR-LuxI family. *J Bacteriol*. 1995;177:7155-7163.
62. Toder DS, Gambello M, Iglewski BH. *Pseudomonas aeruginosa* LasA: a second elastase under the transcriptional control of *lasR*. *Mol Microbiol*. 1991;5:2003-2010.
63. Arevalo-Ferro C, Hentzer M, Reil G, et al. Identification of quorum-sensing regulated proteins in the opportunistic pathogen *Pseudomonas aeruginosa* by proteomics. *Environ Microbiol*. 2003;5:1350-1369.
64. Nouwens AS, Beatson SA, Whitchurch CB, et al. Proteome analysis of extracellular proteins regulated by the *las* and *rhl* quorum sensing systems in *Pseudomonas aeruginosa* PAO1. *Microbiol*. 2003;149:1311-1322.

65. Glessner A, Smith RS, Iglewski BH, Robinson JB. Roles of *Pseudomonas aeruginosa* las and rhl quorum-sensing systems in control of twitching motility. *J Bacteriol* 1999;181:1623-1629.
66. Fuqua C, Winans SC, Greenberg EP. Census and consensus in bacterial ecosystems: the LuxR-LuxI family of quorum-sensing transcriptional regulators. *Ann Rev Microbiol*. 1996;50:727-751.
67. Rumbaugh KP, Griswold JA, Hamood AN. The role of quorum sensing in the in vivo virulence of *Pseudomonas aeruginosa*. *Microbes Infect*. 2000;2:1721-1731.
68. Gupta SK, Joshi S, Tandon R, Mathur P. Topical aspirin provides protection against galactosemic cataract. *Indian J Ophthalmol*. 1997;45:221-225.
69. Valeri P, Romanelli L, Martinelli B, Guglielmotti A, Catanese B. Time-course of aspirin and salicylate in ocular tissues of rabbits. *Lens Eye Tox Res*. 1989;6:465-475.
70. Hazlett LD. Corneal response to *Pseudomonas aeruginosa* infection. *Prog Retin Eye Res*. 2004;23:1-30.
71. Chusid MJ, Shea ML. Quantitation of corneal inflammation by chemiluminescence. *Arch Ophthalmol*. 1986;104:1540-1544.