Virulence Factor Profiles and Antimicrobial Susceptibilities of Ocular *Bacillus* Isolates

**ABSTRACT** *Bacillus* causes one of the most rapidly blinding intraocular infections: endophthalmitis. In this study, *Bacillus* spp. were isolated from ocular infection cases, taxonomically characterized by riboprint analysis, and screened for the presence of putative virulence factors. The ability of these isolates to kill retinal and corneal cells was examined, as were antibiotic susceptibility profiles. The majority of isolates belonged to the *B. cereus* taxonomic group of microorganisms and were identified as *B. cereus* (53%) or *B. thuringiensis* (26%). Toxins were identified in most *B. thuringiensis* and *B. cereus* isolates. Most *B. cereus* and *B. thuringiensis* killed corneal and retinal cells within 6 h. All isolates were susceptible to most antibiotics tested, with quinolones and vancomycin being the most potent. These findings represent the first report of *B. thuringiensis* as an important ocular pathogen, demonstrates the potential ocular toxicity of *B. cereus* and *B. thuringiensis* isolates, and identifies antibiotics whose efficacy against *Bacillus* were superior to those used clinically.

**KEYWORDS** *Bacillus cereus*; *Bacillus thuringiensis*; endophthalmitis; eye; keratitis; toxins

**INTRODUCTION**

With the exception of *Bacillus anthracis*, members of the *Bacillus cereus* taxonomic group and other *Bacillus* spp. are considered to be of minimal significance in the context of human infection. *B. cereus* is commonly considered an agent of mild food poisoning outbreaks but has also been reported to cause severe infections in both immunocompromised and immunocompetent individuals.\(^1\)\(^-\)\(^8\) *B. cereus* is a common cause of the post-traumatic and endogenous forms of severe endophthalmitis, a rare intraocular infection that can result in significant vision loss, if not loss of the eye itself, within a few days.\(^9\)\(^-\)\(^13\) Clinical reviews total the number of *Bacillus* endophthalmitis cases resulting in total vision loss at 70%, with nearly half of those resulting in enucleation or evisceration.\(^10\)\(^-\)\(^13\) During intraocular infection, *Bacillus* grows rapidly in the vitreous, migrates into all tissues of the eye, and produces a plethora of toxins that, in a coordinately regulated fashion, contribute to virulence.\(^14\),\(^15\) An intense intraocular inflammatory response also occurs, which may also contribute to pathogenesis through bystander damage to retinal tissues. *Bacillus* is also the leading bacterial cause of endogenous endophthalmitis, an often bilaterally blinding form of the disease resulting from metastatic spread from the bloodstream into the eye.\(^10\)\(^-\)\(^13\),\(^16\)
**Materials and Methods**

**Bacterial Isolates and Media**

Thirty-nine ocular *Bacillus* isolates were collected from the Dean A. McGee Eye Institute/Presbyterian Hospital (Oklahoma City, OK, USA), Alcon Laboratories (Ft. Worth, TX, USA), Bascom Palmer Eye Institute (Miami, FL, USA), and University of Pittsburgh Medical Center (Pittsburgh, PA, USA). Isolates were collected from cases of keratitis, blepharitis, conjunctivitis, and endophthalmitis and from infected contact lenses.

All isolates were propagated in brain heart infusion (BHI, Becton Dickinson, Franklin Lakes, NJ, USA) for genotypic and phenotypic analyses, on sporulation media for identification of parasporal crystals, and in cell culture media (described below) for analysis of ocular cell toxicity.

**Riboprint Analysis of Bacillus Isolates**

The RiboPrinter® Microbial Characterization System (DuPont Qualicon, Wilmington, DE, USA) was used taxonomically to identify the ocular isolates. (McLean, C. and D. W. Stroman, Abstr. 98th Amer. Soc. Microbiol., abstr. C126, 1996). The RiboPrinter performed the following steps: bacterial lysis, restriction enzyme digestion of chromosomal DNA, electrophoresis, transfer, hybridization (5.5-kb rRNA operon probe derived from other members of the *B. cereus* group is by detection of parasporal crystals in spore preparations of *B. thuringiensis*.

We hypothesize that members of the *B. cereus* group of organisms other than *B. cereus* sensu stricto are also responsible for ocular infections. Further, these organisms are capable of producing toxins that may facilitate the development of rapid and severe ocular infections. We therefore analyzed the genotypic and phenotypic profiles of *Bacillus* isolates from various types of ocular infections. In addition to *B. cereus*, *B. thuringiensis* was identified from a number of *Bacillus* ocular infection cases. Few differences in virulence factor profiles among *B. cereus* and *B. thuringiensis* ocular isolates were found. Most ocular isolates in the *B. cereus* taxonomic group produced toxins and were able to kill retinal and corneal cells *in vitro*, suggesting high potential of these strains for ocular toxicity. We have also identified two antibiotics whose superior efficacy may be exploited to improve therapeutic outcomes of *Bacillus* ocular infections.

*B. thuringiensis* is used worldwide as an organic insecticide because of its plasmid-encoded insecticidal crystal toxins. *B. thuringiensis* is rarely considered a human pathogen but has been shown to cause human disease. Cases of *B. thuringiensis* opportunistic wound infections, gastrointestinal disease outbreaks, oral infections, and ocular infections have been reported. Concern has been raised over the widespread use of *B. thuringiensis* as a pesticide because of its plasmid-encoded insecticidal cry toxins.

The classification of the members of the *B. cereus* group of organisms has been challenging due to their close genetic and phenotypic similarities. Numerous genomic fingerprinting and phenotyping methods have been employed to analyze the relationship between members of the *B. cereus* group (reviewed in Ref. 27). *B. anthracis*, *B. cereus*, and *B. thuringiensis* have been reported to belong to a single species (*B. cereus* sensu lato). Evaluations of taxonomic and phylogenetic relationships among members of the *B. cereus* group demonstrated that *B. cereus* and *B. thuringiensis* may be the closest genetically, while *B. anthracis* may represent a divergent phylogenetic group. Comparative genome hybridization, multilocus sequence analysis, and sequence analysis of ribosomal RNA have confirmed the close similarities of chromosomal genes among this group. Phenotypically (and with the obvious difference of possessing anthrax or insecticidal toxins aside), these organisms carry similar genes for several virulence factors, including hemolysins, proteases, and enterotoxins. *B. cereus* has also been shown to exhibit anthrax-like characteristics further complicating the exact identification of these organisms.

Because of the phenotypic similarities between *B. cereus* and *B. thuringiensis*, standard microbiological testing may result in a misidentification of these organisms. *B. thuringiensis* and *B. cereus* have similar colony morphologies, appear identical on Gram stain, and hemolyze blood agar. Manual biochemical identification systems can identify *Bacillus* spp. but are subject to varying accuracy when differentiating *B. cereus* from *B. thuringiensis*. PCR, rep-PCR, restriction fragment length polymorphism, and amplified fragment length polymorphism DNA fingerprinting have been used to differentiate *B. anthracis* from other *B. cereus* group organisms but cannot accurately differentiate *B. thuringiensis* from *B. cereus*. The only widely accepted method of differentiating *B. thuringiensis* from other...
Virulence and Antimicrobial Susceptibilities of Bacillus Ocular Isolates

Antimicrobial Susceptibility Testing

Minimal inhibitory concentration (MIC) analysis for six antibiotics were determined by microdilution methods recommended by the National Committee for Clinical Laboratory Standards as follows. For MIC determinations, 18-h cultures were diluted in Muller-Hinton Broth (MHB; Becton Dickinson) to obtain a final inoculum with an absorbance equivalent to that of a 0.5 McFarland turbidity standard. Microtiter plates were incubated overnight at 37°C and analyzed spectrophotometrically (OD650) to assess bacterial growth per well. The MIC was designated as the lowest antibiotic concentration that completely inhibited growth.

The following antibiotic powders were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified: amikacin sulfate salt, ceftazidime pentahydrate, clindamycin hydrochloride, vancomycin hydrochloride, and ciprofloxacin hydrochloride (US Biological, Swampscott, MA, USA). Stock solutions were prepared at a concentration of 1.0 mg/ml and diluted in MHB for MIC analyses.

### PCR Amplification of Putative Virulence Factors

The following genes for Bacillus toxins were analyzed by PCR: phosphatidylinositol-specific phospholipase C (PI-PLC; plcA), phosphatidylcholine-specific phospholipase C (PC-PLC; plcB), sphingomyelinase (SPH; sphA), and hemolysin BL (HBL; hblCA). Crude DNA extracts for PCR amplification were made by homogenizing 1.0-ml overnight cultures with 0.1-µm silica glass beads (5000 rpm; Biospec Products, Bartlesville, OK, USA). The primer sets used for amplification and predicted product sizes are listed in Table 1. PCR mixtures for all sets of primers contained 1X reaction buffer, 2.5 mM MgCl2, and 0.5 U Taq polymerase (TaKaRa LA Taq, Chemicon, Temecula, CA, USA); 0.2 mM dNTP mix (Idaho Technology Inc., Salt Lake City, UT, USA), 0.2 µM forward and reverse primers (Integrated DNA Technologies, Coralville, IA, USA), and 5 ng template DNA. PCR reactions were in final volumes of 10 µl for hot air thermocycling (Rapidcycler, Idaho Technology Inc.). General PCR cycling conditions were as follows: 95°C for 1 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at primer-dependent temperatures for 1 min, and elongation at 72°C for 1 min. Specific annealing temperatures for each primer were plcA, 58°C; plcB, 63°C; sphA and hblCA, 59°C. PCR products were analyzed by electrophoresis on an 0.8% agarose gel (in 0.5X Tris-borate-EDTA buffer).

### Phenotypic Evaluation of Putative Virulence Factors

The methods used for analysis of putative virulence factors of Bacillus culture filtrates and individual

---

**TABLE 1** PCR Primers Used to Analyze Bacillus Toxins in Ocular Isolates

<table>
<thead>
<tr>
<th>Targeted gene</th>
<th>Forward primer&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Reverse primer</th>
<th>Predicted size of amplified product (bp)</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>plcA</td>
<td>5′-GTGATAACATTAGCTGAG</td>
<td>5′-TTGAATTGCCAGCTACCT</td>
<td>1731</td>
<td>50</td>
</tr>
<tr>
<td>plcB</td>
<td>5′-GTTTATGAAAACCTTTAATTAG</td>
<td>5′-GTCGATACAAACAAAAACGC</td>
<td>1060</td>
<td>51</td>
</tr>
<tr>
<td>hblCA</td>
<td>5′-CAGCTAGAGGAAGTCCCAGC</td>
<td>5′-CAATATGCCCTAGAAGC CCCG</td>
<td>3300&lt;sup&gt;a&lt;/sup&gt;</td>
<td>41, 52</td>
</tr>
<tr>
<td>sphA</td>
<td>5′-TGGTTTGTATACGACGGAG</td>
<td>5′-AGAGTAATCATTATACGTATATTGTTG</td>
<td>1038</td>
<td>53, 54</td>
</tr>
</tbody>
</table>

<sup>a</sup>Amplicon product size based on Ref. 41.
colonies have been described previously. Briefly, PI-PLC activity was determined by quantifying acetylcholinesterase release. PC-PLC activity was determined by an egg yolk agar turbidity assay. SPH activity was determined by quantifying trinitrophenylaminolauroylsphingomyelin hydrolysis and by the CAMP test. Proteolytic activity was determined on hide azure powder and on BHI agar supplemented with 2.5% skim milk. Relative motility was determined by measuring the colony diameter on motility agar.

*B. thuringiensis* isolates were differentiated from other members of the *B. cereus* group by detection of parasporal crystals. *Bacillus* strains were grown on sporulation media agar for 7 days at 30°C. Cells were stained with carbolfuchsin and examined by phase contrast microscopy (Zeiss Axiovert 135, Thornwood, NY, USA).

Student’s *t*-test was used for statistical comparisons between strains. Values represent the mean ± standard deviation (SD) for *n* ≥ 2 separate assays of triplicate samples per strain, unless otherwise specified. A *p* value of ≤0.05 was considered significant.

**Ocular Cell Toxicity of Bacillus Ocular Isolates**

Human Muller cells (M10-M1; a kind gift from Dr. Astrid Limb, Moorfields Eye Institute, London, UK) were propagated to 80–100% confluency in Dulbecco’s modified Eagle medium (DMEM, Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (FCS) and 1% glutamine. Human corneal keratocytes (a kind gift from Dr. James Jester, University of Texas Southwest Medical Center, Dallas, TX, USA) were propagated to 80–100% confluency in DMEM/HAMS-F12 (1:1 vol/vol) medium supplemented with 10% FBS and 1% glutamine. *Bacillus* isolates were grown to a concentration of 10⁸ cfu/ml in cell culture medium. Media covering the monolayers was removed and replaced with media containing 10⁴ cfu/ml bacilli (an approximate MOI of 0.2), and cells were incubated at 37°C with 5% CO₂. Muller cells were infected with endophthalmitis isolates, while corneal keratocytes were infected with *Bacillus* spp. isolated from anterior segment infections. At 6 h postinfection, monolayers were stained with 0.04% trypan blue (wt/vol in PBS, pH 7.4) to quantify dead and live cells. Supernatants were harvested for analysis of lactate dehydrogenase (LDH) leakage resulting from cell membrane damage, according to the kit manufacturer’s instructions (CytotoxOne, Promega). Significant LDH release or cell death was considered to be ≥50% LDH release compared with that of uninfected or sham-infected monolayers, or ≥50% death of 2 × 10⁵ cells/well. Growth of bacilli on monolayers was confirmed by track dilution of supernatants onto BHI agar.

**RESULTS**

**Distribution of Bacillus Species in Ocular Isolates**

As depicted in Figure 1, the majority of *Bacillus* cultured from both posterior and anterior segment infections were identified as *B. cereus* or *B. thuringiensis*. Among all isolates, *B. cereus* was the most prevalent species (20 isolates, 52.6%), while *B. thuringiensis* was the next most prevalent (10 isolates, 26.3%). Other isolates included *B. subtilis*, *B. mycoides*, *B. pumilus*, *B. flexus*, and *Paenibacillus polymyxa*. Among anterior segment infection isolates (contact lens–associated, keratitis, blepharitis, conjunctivitis), *B. cereus* was the most prevalent species (10 isolates, 47.7%), while *B. thuringiensis* was the next most prevalent (8 isolates, 38%). Among endophthalmitis isolates, *B. cereus* was the most prevalent species (10 isolates, 47.7%), while *B. thuringiensis* and *B. mycoides* were the next most prevalent (2 isolates each, 11.8%).

**FIGURE 1** Distribution of *Bacillus* and *Paenibacillus* species among ocular isolates. The majority of *Bacillus* cultured from both posterior and anterior segment infections were identified as *B. cereus* or *B. thuringiensis*. 

M. C. Callegan et al.
TABLE 2  *In vitro* Activities of Five Antibiotics Against *Bacillus* Ocular Isolates

<table>
<thead>
<tr>
<th><em>Bacillus</em> spp. (No. of strains tested)</th>
<th>Antibiotic</th>
<th>MIC (µ/ml)</th>
<th>50%</th>
<th>90%</th>
<th>% of strains fully susceptible</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. cereus (20)</td>
<td>Amikacin</td>
<td>1–16</td>
<td>4</td>
<td>16</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>Ceftazidime</td>
<td>2–128</td>
<td>32</td>
<td>64</td>
<td>8.3</td>
</tr>
<tr>
<td></td>
<td>Clindamycin</td>
<td>1–16</td>
<td>8</td>
<td>16</td>
<td>84.1</td>
</tr>
<tr>
<td></td>
<td>Ciprofloxacin</td>
<td>0.03–1</td>
<td>0.25</td>
<td>0.5</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>Vancomycin</td>
<td>1–16</td>
<td>2</td>
<td>2</td>
<td>83.3</td>
</tr>
</tbody>
</table>

| B. thuringiensis (10)                  | Amikacin   | 2–16       | 4   | 16  | 100.0                         |
|                                        | Ceftazidime| 16–512     | 32  | 64  | 7.9                           |
|                                        | Clindamycin| 2–8        | 4   | 4   | 100.0                         |
|                                        | Ciprofloxacin| 0.12–0.25 | 0.12| 0.25| 100.0                         |
|                                        | Vancomycin | 1–4        | 2   | 4   | 100.0                         |

| Other *Bacillus* strains (8*)          | Amikacin   | 0.25–32    | 2   | 8   | 62.5                          |
|                                        | Ceftazidime| 0.5–16     | 2   | 8   | 62.5                          |
|                                        | Clindamycin| 2–16       | 4   | 4   | 87.5                          |
|                                        | Ciprofloxacin| 0.06–0.5 | 0.06| 0.25| 100.0                         |
|                                        | Vancomycin | 0.25–8     | 0.5 | 8   | 75.0                          |

*Other strains included B. pumilus (n = 1), B. flexus (n = 2), B. subtilis (n = 1), B. mycoides (n = 2), and P. polymyx (n = 2).*

Antimicrobial Susceptibilities of *Bacillus* Ocular Isolates

*B. cereus* and *B. thuringiensis* ocular isolates were generally sensitive to all antibiotics tested, except ceftazidime (Table 2). The only isolates sensitive to ceftazidime were *B. flexus*, *B. subtilis*, *B. pumilus*, and *P. polymyx*. The data demonstrated clearly that ciprofloxacin was the most potent antibiotic, to which all isolates tested were sensitive. We also identified two *B. cereus* strains with intermediate sensitivity to vancomycin (MIC$_{100}$ = 7.8 µg/ml) and two *P. polymyx* strains with intermediate sensitivity to vancomycin and amikacin (MIC$_{100}$ = 7.8 µg/ml). In descending order of susceptibility of all ocular isolates: ciprofloxacin > vancomycin > amikacin = clindamycin > ceftazidime.

PCR Analysis of Toxin Genes in *Bacillus* Ocular Isolates

Chromosomal DNA from ocular isolates was used to amplify *plcA*, *plcB*, *sphA*, and *hblCA* as indicated. Representative positive amplifications are shown in Figure 2. PCR products were of the predicted sizes, as listed in Table 1.

The results from PCR amplification of toxin genes is summarized in Figure 3. The majority of *B. cereus* and *B. thuringiensis* isolates possessed *plcA*, *plcB*, and *sphA*. Isolates of *B. subtilis*, *B. pumilus*, and *B. mycoides* did not contain *plcA*, *plcB*, *sphA*, or *hblCA*. The single *B. flexus* isolate was positive for amplification of *plcA*. The two *Paenibacillus* isolates were positive for amplification of *sphA* and *plcB*.

![Figure 2: PCR amplification of toxin genes in Bacillus and Paenibacillus isolates. Representative positive amplifications are shown. PCR products were of the predicted sizes, as listed in Table 1.](image-url)
positive strains were similar (p ≥ 0.160). Data were analyzed based on strain species (top panel) or infection type (bottom panel)."}

**Phenotypic Analysis of Virulence Factors in Bacillus Ocular Isolates**

Phenotypic analysis of virulence factors produced by *Bacillus* ocular isolates is summarized in Table 3. Isolates of *B. subtilis*, *B. pumilus*, and *B. mycoides* did not harbor *plcA*, *plcB*, *sphA*, or *hblCA* and therefore did not produce these corresponding toxins.

*B. cereus*, *B. thuringiensis*, and *Paenibacillus* isolates produced similar PI-PLC and PC-PLC activities (p ≥ 0.373). *B. flexus* isolates that were not positive for *plcA* by PCR produced PI-PLC at concentrations similar to that of *B. cereus*, *B. thuringiensis*, and *P. polymyxa* (p ≥ 0.160). PC-PLC activity was detected in *B. cereus* and *B. thuringiensis* strains found to be deficient in *plcB* by PCR, suggesting lecithinase activity independent of *plcB* in these strains. In these *plcB*-negative strains, the lecithinase activity was significantly less than that produced by strains found to harbor *plcB* (p = 0.011). Isolates of *B. flexus* did not harbor *plcB* and did not produce PC-PLC. When PI-PLC- or PC-PLC positive *B. cereus* and *B. thuringiensis* or all PI-PLC- or PC-PLC-positive isolates were compared by infection source, the PI-PLC and PC-PLC concentrations were similar (p ≥ 0.219).

*B. cereus* isolates demonstrated significantly greater SPH and protease activities than the other *Bacillus* and *Paenibacillus* isolates (p ≤ 0.041). *B. subtilis* and *B. thuringiensis* isolates had significantly higher protease activities than the other *Bacillus* and *Paenibacillus* isolates (excluding *B. cereus*, p ≤ 0.003). *B. thuringiensis* isolates also had significantly higher SPH activities than the other *Bacillus* and *Paenibacillus* isolates (excluding *B. cereus*, p ≤ 0.026). When compared by infection type, toxin activities of all positive strains were similar (p ≥ 0.161).

**TABLE 3**

<table>
<thead>
<tr>
<th>Comparison</th>
<th>PI-PLC (µg/ml)</th>
<th>PC-PLC (µg/ml)</th>
<th>SPH (µg/ml)</th>
<th>Protease (Units/ml)</th>
<th>Motility (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Comparison by strain</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>B. cereus</em></td>
<td>11.5 ± 4.6</td>
<td>209.9 ± 101.2</td>
<td>0.30 ± 0.10</td>
<td>0.28 ± 0.12</td>
<td>22.1 ± 12.9</td>
</tr>
<tr>
<td><em>B. thuringiensis</em></td>
<td>11.9 ± 3.2</td>
<td>225.2 ± 75.7</td>
<td>0.10 ± 0.06</td>
<td>0.16 ± 0.08</td>
<td>21.5 ± 10.2</td>
</tr>
<tr>
<td><em>B. flexus</em></td>
<td>10.2 ± 1.3</td>
<td>ND</td>
<td>0.09 ± 0.05</td>
<td>0.04 ± 0.02</td>
<td>2.5 ± 0.7</td>
</tr>
<tr>
<td><em>B. pumilus</em></td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.06</td>
<td>36</td>
</tr>
<tr>
<td><em>B. subtilis</em></td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.17 ± 0.03</td>
<td>32.3 ± 3.9</td>
</tr>
<tr>
<td><em>B. mycoides</em></td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.1</td>
<td>15.1</td>
</tr>
<tr>
<td><em>P. polymyxa</em></td>
<td>10.2 ± 0.2</td>
<td>116.4 ± 51.5</td>
<td>0.06 ± 0.02</td>
<td>0.06 ± 0.01</td>
<td>8.5 ± 0.3</td>
</tr>
<tr>
<td><strong>Comparison by infection type</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blepharitis</td>
<td>11.7 ± 2.3</td>
<td>212.6 ± 81.9</td>
<td>0.13 ± 0.12</td>
<td>0.31 ± 0.13</td>
<td>22.0 ± 14.2</td>
</tr>
<tr>
<td>Keratitis/cornea</td>
<td>11.7 ± 3.8</td>
<td>207.8 ± 129.1</td>
<td>0.16 ± 0.11</td>
<td>0.21 ± 0.18</td>
<td>15.9 ± 10.7</td>
</tr>
<tr>
<td>Endophthalmitis</td>
<td>10.7 ± 6.4</td>
<td>114.6 ± 75.9</td>
<td>0.19 ± 0.16</td>
<td>0.17 ± 0.14</td>
<td>26.4 ± 12.6</td>
</tr>
</tbody>
</table>

PI-PLC, phosphatidylinositol-specific phospholipase; PC-PLC, phosphatidylcholine-specific phospholipase; SPH, sphingomyelinase.

aFor all phenotypic analyses, n ≥ 2 separate assays of triplicate samples per strain.

bChromogenic PI-PLC assay. Values represent mean ± standard deviation (SD) in filtered supernatants of 10-h Bacillus cultures. PI-PLC activities among positive strains were similar (p ≥ 0.160).

cEgg yolk agar well diffusion assay for PC-PLC activity. Values represent mean ± SD in filtered supernatants of 10-h Bacillus cultures. PC-PLC activities among positive strains were similar (p ≥ 0.373).

dChromogenic SPH assay and CAMP test. Nondetectable SPH activities were confirmed with a negative CAMP test. SPH activities of *B. cereus* isolates were significantly greater than that of the other *Bacillus* and *Paenibacillus* isolates (p ≤ 0.041).

eHide azure blue protease assay. Values represent mean ± SD in filtered supernatants of 8-h Bacillus cultures. SPH activities of *B. cereus* isolates were significantly greater than that of the other *Bacillus* and *Paenibacillus* isolates (p ≤ 0.030).

fSwarming on motility agar. Values represent mean diameter ± SD after an 8-h incubation at 37°C. Diameters of *B. cereus*, *B. thuringiensis*, *B. mycoides*, *B. pumilus*, and *B. subtilis* were similar (p ≥ 0.623).

gFor *B. pumilus* and *B. mycoides*, only one strain was analyzed and statistics were not performed.

hND, not detected.

iWhen compared by infection type, toxin activities of all positive strains were similar (p ≥ 0.161).
FIGURE 4 LDH release from retinal and corneal cells after infection with Bacillus ocular isolates. Retinal Muller cells or corneal keratocytes were incubated with $10^4$ cfu/ml of ocular Bacillus isolates for 6 h. Significant LDH release was considered to be $\geq 50\%$ LDH release compared with that of uninfected or sham-infected monolayers. These data are combined results from infections with strains that resulted in $>50\%$ LDH release (“toxic strains”). Bc = B. cereus, Bt = B. thuringiensis.

source, however, the protease and SPH activities of Bacillus and Paenibacillus ocular isolates were not significantly different ($p \geq 0.161$).

B. cereus, B. thuringiensis, B. mycoides, B. pumilus, and B. subtilis were all motile in liquid media and produced colonies with diameters significantly larger than that of B. flexus and Paenibacillus ($p \leq 0.029$). Colony sizes of the aforementioned five Bacillus isolates were similar ($p \geq 0.623$). The motility of Bacillus and Paenibacillus ocular isolates was also compared with that of a non-motile isogenic mutant of B. cereus.$^{14}$ Colonies of the nonmotile isogenic Bacillus mutant were similar in size to that of B. flexus and Paenibacillus (data not shown).

Ocular Cell Toxicity

Retinal Muller cells and corneal keratocytes were exposed to viable Bacillus for 6 h, and LDH release (Fig. 4) and trypan blue exclusion were analyzed to determine the extent of membrane damage and cell death, respectively. B. flexus, B. pumilus, B. subtilis, B. mycoides, or Paenibacillus isolates were not cytotoxic to retinal Muller cells or keratocytes. Trypan blue staining indicated that
<5% of Muller cells or keratocytes were dead after 6-h incubation with these Bacillus or Paenibacillus isolates.

In contrast, the majority of B. cereus and B. thuringiensis isolates were toxic to ocular cells: 66.7% of B. cereus and 75.0% of B. thuringiensis endophthalmitis isolates caused significant LDH release and death of Muller cells within 6 h; 55.6% of B. cereus and 50.0% of B. thuringiensis anterior segment infection isolates caused significant LDH release and death of keratocytes within 6 h. As demonstrated in Figure 4, the toxic B. cereus or B. thuringiensis isolates killed a significant percentage of retinal and corneal cells, as measured by LDH release after a 6-h incubation. Keratocyte and Muller cell cytopathology included cell rounding, membrane blebbing, and loss of adherence (Fig. 5). Trypan blue staining indicated that Muller cells and keratocyte death paralleled the LDH release during infection.

CONCLUSIONS

B. cereus is one of the most virulent bacterial pathogens for the eye and has been reported to cause the vast majority of post-traumatic and endogenous endophthalmitis cases.10−13 To our knowledge, this is the first report of B. thuringiensis, a close genetic relative to B. cereus, as a significant cause of Bacillus endophthalmitis or anterior segment infections. Identification by riboprint analysis and parasporal crystal detection demonstrated that three strains originally identified as B. cereus were actually B. thuringiensis. Because B. thuringiensis produces several of the same toxins as B. cereus, B. thuringiensis may have been misidentified on the basis of Gram stain and blood agar hemolysis alone. Although B. thuringiensis is rarely considered an important human pathogen, because of its widespread use as an organic pesticide, infections with B. thuringiensis may be more common than once thought.

The recommended management of Bacillus intraocular infections includes intravitreal injection of antibiotics. Commonly used antibiotics include ceftazidime, amikacin, clindamycin, or vancomycin, often in combination. We report here that for Bacillus spp., ciprofloxacin and vancomycin were the most potent antibiotics in vitro. We previously reported that the fourth-generation quinolones, moxifloxacin and gatifloxacin, were highly effective against B. cereus ocular isolates in vitro,45 highlighting the potential for the use of quinolones in treating Bacillus ocular infections. Unfortunately, in the majority of Bacillus endophthalmitis cases, vision loss occurs regardless of therapeutic and surgical intervention, suggesting that tissue damage and vision loss occurs even though the infected eye is sterilized by antibiotics. Therapeutic failures may be due to delayed or inadequate therapeutic regimens, toxin production by the infecting strain, migration and/or sequestration of viable bacilli out of antibiotic reach, or from bystander damage resulting from the explosive inflammatory response. It is likely a combination of these factors. To date, no single therapeutic regimen stands out as being superior for the treatment of severe forms of endophthalmitis. However, these results suggest that for Bacillus, the use of quinolones and/or vancomycin may offer a more viable therapeutic option.

B. cereus and B. thuringiensis produce a variety of proteins and enzymes that could be toxic to ocular cells during infection. Our findings correlate with previous reports identifying similar toxin profiles in members of the B. cereus taxonomic group.5,24−26 Diarrheal, nongastrointestinal, and food-borne isolates of B. cereus and B. thuringiensis have been shown to produce the entero toxins and membrane-damaging toxins identified here.24,25,46,47 As in this study, enrichment of a particular phenotype in isolates from a specific infection type was not found. In addition, with the exception of B. flexus and Paenibacillus, most ocular Bacillus isolates were motile, demonstrating their potential for migration in the eye during infection. Considering the significance of intraocular motility in endophthalmitis pathogenesis,48,49 the motility of these isolates is an important virulence trait.

We previously reported that three Bacillus toxins (PI-PLC, PC-PLC, and HBL) contributed little to the overall course and severity of experimental Bacillus endophthalmitis.41,42 However, these toxins and others may individually affect specific ocular cell types, leading to detrimental effects that may be masked when analyzed in the context of infection and inflammation. B. cereus and B. thuringiensis were able to kill retinal Muller cells and corneal keratocytes in vitro within 6 h, indicating the susceptibility of these cell types to Bacillus growth and toxin production. Previous reports have also demonstrated the ability of Bacillus spp. to adhere to, invade, and kill mammalian cells in vitro.25,26,46,47 Because keratocytes and Muller cells contribute significantly to the structure of the cornea and retina, respectively, cytotoxicity and death of these cells may result in the detrimental changes observed during Bacillus corneal infections (ulcer formation) and
endophthalmitis (retinal detachment and destruction). We reported that an atoxigenic plcR-deficient B. cereus strain was able to kill Muller cells, but at a significantly slower rate than wild-type B. cereus (R. Ramadan, A.L. Moyer, A.H. Hibbard, A. Sharp, M. Balkis, J. Ash, M. C. Callegan, Abstr. Assoc. Res. Vis. Ophthalmol., abstr. 4015/B476, 2004). These in vitro data correlate with our in vivo findings of a less rapid course of vision loss and inflammation after intraocular injection of plcR-deficient B. cereus and B. thuringiensis. The extent to which Bacillus toxin–induced Muller cell damage or death is related to vision loss during endophthalmitis is currently under investigation.

These studies identified B. cereus and B. thuringiensis as significant pathogens associated with infections of the eye and confirmed the potential ocular toxicity of these strains. The relationship of these findings to the pathogenic mechanisms of Bacillus ocular infections is currently under investigation. Antibiotic susceptibility studies also highlighted the superior efficacy of ciprofloxacin and vancomycin for Bacillus ocular isolates, a finding that may translate to improvements in therapy of blinding Bacillus infections of the eye.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health grants R01EY12985 (M.C.C.) and P30EY12191 (NIH CORE grant for Dr. Robert E. Anderson, OUHSC), an unrestricted Career Development Award from Research to Prevent Blindness, Inc. (M.C.C.), and a Physician/Scientist Merit Award from Research to Prevent Blindness, Inc. (J.C.). We thank Dr. Astrid Jester for the kind gift of corneal keratocytes. We thank Dr. Darlene Miller (Bascom Palmer Eye Institute, Miami, FL, USA) and Dr. Regis Kowalski (University of Pittsburgh Medical Center, Pittsburgh, PA, USA) for supplying ocular isolates. We also thank Beryl Ojwang (Oklahoma School for Science and Math, Oklahoma City, OK, USA) and Andrea Moyer (OUHSC Ophthalmology) for technical assistance.

REFERENCES


