

Role of Swarming Migration in the Pathogenesis of *Bacillus* Endophthalmitis

Michelle C. Callegan,^{1,2} Billy D. Novosad,¹ Raul Ramirez,¹ Emilia Ghelardi,³ and Sonia Senesi³

PURPOSE. *Bacillus cereus* causes one of the most rapidly blinding forms of bacterial endophthalmitis. Migration of *B. cereus* throughout the eye during endophthalmitis is a unique aspect of this disease that may contribute to intraocular virulence. This study was conducted to analyze the contribution of swarming and intraocular migration to the pathogenesis of experimental endophthalmitis.

METHODS. Eyes were injected intravitreally with 100 colony-forming units (CFU) of either wild-type, nonswarming, or swarming-complemented strains of *B. cereus*. Pathogenicity was compared throughout the course of infection by biomicroscopy, histology, electroretinography, and bacterial and inflammatory cell quantitation.

RESULTS. Wild-type, nonswarming, and swarming-complemented *B. cereus* strains grew to a similar number in the vitreous throughout the course of infection. Unlike the wild-type and swarming-complemented strains, the nonswarming mutant did not migrate to the anterior segment during infection. The rate of decrease in retinal responses of eyes infected with the all strains was similar, resulting in near complete elimination of retinal function by 12 hours. All *Bacillus* strains caused similar degrees of posterior segment inflammation and retinal destruction. However, the accumulation of inflammatory cells in the anterior chamber, hyphema, and corneal ring abscesses did not occur in eyes infected with the nonswarming mutant.

CONCLUSIONS. The deficiency in swarming had little effect on retinal function loss or the overall course or severity of experimental *B. cereus* endophthalmitis. However, a deficiency in swarming prevented *Bacillus* from migrating to the anterior segment, leading to less severe anterior segment disease. (*Invest Ophthalmol Vis Sci.* 2006;47:4461–4467) DOI:10.1167/iovs.06-0301

Endophthalmitis is a potentially blinding infection caused by the introduction of bacteria into the posterior segment of the eye. *Bacillus* is a leading cause of endophthalmitis after

penetrating injury, during which the globe is infected with organisms from a contaminated foreign body. Once inside the eye, *B. cereus* causes a rapidly destructive form of the infection that, despite aggressive therapeutic and surgical intervention, often results in significant vision loss, if not loss of the eye itself, within 24 to 48 hours. Estimates in clinical reviews show that approximately two thirds of *Bacillus* patients with endophthalmitis lose significant functional vision, and almost half of such cases result in loss of the infected eye.^{1–3}

In experimental models of endophthalmitis, *Bacillus* migrates from the initial site of inoculation throughout the entire eye within a short period. Bacilli can be found within the layers of the retina and in the anterior segment within 12 hours.⁴ The correlation between the ability of a bacterium to migrate within the eye and its virulence potential has been addressed, but only to a limited extent. We recently determined that nonmotile *B. cereus* and *B. thuringiensis* are less virulent in the eye than are wild-type motile strains. Infection of the eye with isogenic nonmotile *B. cereus* or *B. thuringiensis* resulted in slower evolution of retinal function loss and intraocular inflammation than did motile wild-type strains.^{5,6} However, the strains analyzed were also deficient in toxin secretion, and so the specific contribution of motility to the virulence of *Bacillus* endophthalmitis remains in question.

Swarming migration is an aspect of bacterial motility that may contribute to the pathogenicity of *Bacillus* infection. Movement of bacteria across a moist, solid surface or through a viscous environment involves a change in the morphology of short, motile, vegetative rods into elongated, multinucleated, hyperflagellated swarmer cells that form structured rafts and rapidly migrate away from the colony in a coordinated fashion. For some Gram-negative organisms, the differentiation of vegetative cells into swarmer cells was accompanied by a notable increase in virulence factor expression.^{7–11} There is also evidence that swarmer cells may initiate the interaction between the pathogen and host cell during invasion and may be the primary virulence form of the organism.¹² Elevated antibiotic resistance in differentiated *Salmonella* swarm cells has also been reported,¹³ conferring on these organisms a survival advantage during infection.

For *Bacillus* spp., motility, swarming, and virulence factor production may be closely associated. Motile, nonswarming mutants of *B. cereus* have been shown to be defective in the production of the lytic L₂ component of hemolysin BL.¹⁴ Nonswarming *B. subtilis* mutants have been shown to be deficient in extracellular protease production and/or surfactin, deficiencies that appear to contribute to the inability of the mutants to form biofilms. These results suggested that swarming, extracellular protease production, and biofilm formation are also linked.¹⁵ However, the contribution of swarming to virulence during *Bacillus* infection has not yet been determined.

In this study, we analyzed the potential contribution of swarming migration to the pathogenesis of *Bacillus* intraocular infection. Differences in the virulence of wild-type and nonswarming *B. cereus* were analyzed in an experimental in vivo model of endophthalmitis. Our results demonstrated that although a defect in swarming prevented migration of *Bacillus*

From the Departments of ¹Ophthalmology and ²Microbiology and Immunology, University of Oklahoma Health Sciences Center, Dean A. McGee Eye Institute, Oklahoma City, Oklahoma; and ³Dipartimento di Patologia Sperimentale, Biotechnologie Mediche, Infettivologia ed Epidemiologia, Università di Pisa, Pisa, Italy.

Supported by National Institutes of Health Grants R01EY12985 (MCC) and P30EY12191 (NIH CORE grant for the Health Sciences Center), Fight for Sight (MCC), and an unrestricted Career Development Award from Research to Prevent Blindness, Inc. (MCC).

Submitted for publication March 20, 2006; revised June 19, 2006; accepted August 17, 2006.

Disclosure: M.C. Callegan, None; B.D. Novosad, None; R. Ramirez, None; E. Ghelardi, None; S. Senesi, 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: Michelle C. Callegan, Department of Ophthalmology DMEI 419, 608 Stanton L. Young Boulevard, Oklahoma City, OK 73104; michelle-callegan@ouhsc.edu.

throughout all parts of the eye and resulted in attenuation of anterior chamber inflammation, this defect did not prevent rapid retinal function loss or severe posterior segment inflammation, detrimental events that ultimately lead to blindness during endophthalmitis.

METHODS

Bacterial Strains and Media

The wild-type *B. cereus* strain NCIB 8122, its *fliY*[−] nonswarming mutant (MP01), and the *fliY*-complemented swarming strain (MP04) have been described.¹⁴ Briefly, *B. cereus* strain MP01 was isolated as a spontaneous nonswarming mutant of NCIB 8122. MP01 harbors a deletion in *fliY*, which encodes a component of the flagellar motor-switch complex (C-ring). Strain MP04 was constructed by complementation of MP01 in trans with *fliY*, as previously described.¹⁴ Strains were propagated in brain-heart infusion (BHI; Difco, Detroit, MI) medium or agar, unless otherwise specified.

Phenotypic Analysis of *Bacillus* Strains

Phenotypic analysis involved measurement of toxins and/or their activities, motility, and swarming during growth in vitro. Culture supernatants were prepared and analyzed for phosphatidylinositol-specific phospholipase C (PI-PLC), phosphatidylcholine-specific phospholipase C (PC-PLC), sphingomyelinase, and protease activities, and motility as previously described.^{16,17} Hemolysin BL activity was determined by the formation of a discontinuous zone of hemolysis on sheep blood agar.^{18,19} The presence of the hemolysin BL component L₂ was detected by Western immunoblot analysis with polyclonal antisera specific to these components, as previously described.¹⁴ Cereolysin O activity was quantified by hemolytic assay, essentially as described by Limbago et al.²⁰ Swarming on tryptone agar (TrA) plates (0.5% wt/vol) was determined by measuring the diameter of developing colonies after a 24-hour incubation at 37°C.¹⁴

Student's *t*-test was used for statistical comparisons of phenotypic data between strains. Values represent the mean \pm SD for $n \geq 3$ samples per strain, unless otherwise specified. $P \leq 0.05$ was considered significant.

Experimental *Bacillus* Endophthalmitis

Experimental *Bacillus* endophthalmitis was induced in New Zealand White rabbits as previously described.^{4–6,16,17,21} Briefly, rabbits were anesthetized by intramuscular injection of ketamine (Ketavet, 35 mg/kg body weight; Phoenix Scientific Inc., St. Joseph, MO) and xylazine (Rompun, 5 mg/kg of body weight; Bayer Corp., Shawnee Mission, KS). Topical anesthetic (0.5% proparacaine HCl; Ophthetec; Allergan, Hormigueros, Puerto Rico) was applied to each eye before injection. After aqueous humor paracentesis, 100- μ L BHI containing 100 CFU of *B. cereus* was injected into the midvitreal. Contralateral eyes were injected with either BHI (surgical control) or were left undisturbed (absolute control). At various times after injection, infection courses were analyzed as described later. Rabbits were maintained in accordance with Institutional Animal Care and Use Committee guidelines and the ARVO Statement for the Use of Laboratory Animals in Ophthalmic Research.

Analysis of *Bacillus* Endophthalmitis

Electroretinography. Scotopic electroretinography (ERG) was used to measure retinal responsiveness, as previously described.^{4–6,16,17,21} ERGs were measured in terms of a-wave function (corresponding to photoreceptor cell activity) and b-wave function (corresponding to Müller, bipolar, and amacrine cell activity). After dilation and dark adaptation, scotopic a- and b-wave amplitudes and latencies of implicit time (τ) were recorded for each eye (EPIC2000 and UTAS3000; LKC Technologies, Inc., Gaithersburg, MD). The percentage of retinal function retained was calculated as either

$100 - \{[1 - (\text{experimental a-wave amplitude/absolute control a-wave amplitude})] \times 100\}$ or $100 - \{[1 - (\text{experimental b-wave amplitude/absolute control b-wave amplitude})] \times 100\}$. Latency of retinal responses corresponding to percentages of latency of implicit time (τ) were calculated as $[1 - (\text{experimental } \tau/\text{control } \tau)] \times 100$.

Bacterial and Inflammatory Cell Quantitation. Quantitation of *Bacillus* in aqueous and vitreous humor and inflammatory cells in aqueous humor have been described.^{4–6,16,17,21} Retention of motility and swarming phenotypes was confirmed by light microscopy and replica plating onto TrA plates.

Thin-Section Histology. Globes recovered for histologic analysis were fixed in 10% formalin for 24 hours. Eyes were sectioned and stained with hematoxylin and eosin.^{4–6,16,17,21}

Statistical Analysis

Data for parameters used to analyze progressive infection were expressed as the mean \pm SEM of results in four or more eyes per time point, unless otherwise specified. Wilcoxon's rank sum test was used for statistical comparison between infection groups. Student's *t*-test was used for statistical comparison of phenotypic data. $P \leq 0.05$ was considered significant.

RESULTS

Phenotypic Analysis of *Bacillus* Strains

When grown in liquid media, the replication rates and phenotypic profiles of wild-type *B. cereus* NCIB 8122, MP01, and MP04 were similar (data not shown), as was the production of several toxins (Table 1). However, on TrA plates, colony sizes of NCIB 8122 and MP04 were significantly greater than that of MP01 at 24 hours of growth ($P \leq 0.006$), indicating that in strain MP01, a deficiency in swarming¹⁴ is associated with a reduction in colony size.

Experimental Endophthalmitis: Bacterial Growth and Disease

The explosive nature of experimental *B. cereus* endophthalmitis has been described.^{5,17,21} In the present study, reproducible endophthalmitis was achieved with the following *B. cereus* strains: wild-type NCIB 8122, $2.20 \pm 0.05 \log_{10}$ CFU/eye; *fliY*-MP01, $2.30 \pm 0.06 \log_{10}$ CFU/eye; and *fliY*-complemented MP04, $2.20 \pm 0.03 \log_{10}$ CFU/eye ($P = 0.95$). The intravitreal growth rates of NCIB 8122 and MP01 were similar throughout the infection course ($P \geq 0.34$; Fig. 1). At 6 hours, the number of MP04 in the vitreous was less than that of NCIB 8122 ($P = 0.005$) and MP01 ($P = 0.02$), but greater than that of MP01 at 12 hours ($P = 0.01$). The migration and growth of the *fliY*-mutant in the anterior segment was significantly less than that of the wild-type strain and the *fliY*-complemented strain at 9 and 12 hours ($P \leq 0.001$) after infection (Fig. 1). At 18 hours, all eyes infected with MP04 perforated during harvest and accurate bacterial counts were not available.

Ocular pathologic changes caused by wild-type *B. cereus* NCIB 8122 and its companion strains occurred more rapidly than that observed with other wild-type *B. cereus* strains tested in this model.^{5,17,21} During infections with each strain, mild inflammatory symptoms (posterior segment inflammatory cell influx, increasing vitreous haze, decreased fundus reflex) were observed at 6 hours after infection. In eyes infected with wild-type *B. cereus* NCIB 8122 and *fliY*-complemented MP04, inflammation reached moderate to severe levels by 12 to 18 hours after infection, including significant influx of inflammatory cells into the anterior segment (hypopyon) and cornea (corneal ring abscess), and influx of erythrocytes into the eye (hyphema). In eyes infected with the *fliY*-mutant MP01, inflammation reached moderate to severe levels in the posterior

TABLE 1. Phenotypic Analysis of *B. cereus* Wild-Type Strain NCIB 8122, Nonswarming MP01, and *fliY*-Complemented MP04

Component	Assay Used	<i>B. cereus</i> NCIB 8122 Wild Type	<i>B. cereus</i> MP01 <i>fliY</i> -Mutant	<i>B. cereus</i> MP04 <i>fliY</i> -Complemented
Cereolysin O*	Hemolytic titer	1:4	1:2	1:2
Hemolysin BL	Immunoblot for L2 component; discontinuous hemolytic zone	Detected	Not detected	Detected
Motility	Light microscopy	Not detected	Not detected	Not detected
PC-PLC	Agar well diffusion assay	Detected	Detected	Detected
PI-PLC	Chromogenic assay	29.3 ± 1.15 U/mL	32.0 ± 1.73 U/mL	32.0 ± 1.73 U/mL
Protease	Hide azure blue assay	1.60 ± 0.05 µg/mL	1.49 ± 0.05 µg/mL	1.45 ± 0.05 µg/mL
Sphingomyelinase	Hide azure blue assay	0.18 ± 0.02 U/mL	0.13 ± 0.04 U/mL	0.14 ± 0.004 U/mL
Swarming†	TNPAL-sphingomyelin hydrolysis assay	0.64 ± 0.05 µg/mL	0.58 ± 0.33 µg/mL	0.53 ± 0.10 µg/mL
	Migration on TrA agar	13.7 ± 1.5 mm	4.7 ± 0.6 mm	12.3 ± 1.2 mm

For all phenotypic analyses, three or more separate assays were performed.

* Cereolysin O titer was determined by hemolytic assay²⁰ as the dilution of supernatant exhibiting 50% hemolysis or less based on an H₂O lysis control. A fourfold difference in titer was considered significant.

† Diameters of individual NCIB 8122 and MP04 colonies were similar ($P = 0.19$) and were significantly greater than that of MP01 at 24 hours ($P = 0.006$). Evaluation of intracolony swarm cell differentiation of NCIB 8122 has been reported.¹⁸

segment only. The presence of fibrin and a few inflammatory cells in the anterior chamber was noted, but neither hyphema nor corneal ring abscesses were observed in eyes infected with the nonswarming mutant. Because of the pending panoph-

thalmitis in the majority of infected eyes in each group at 18 hours, infections were not allowed to progress further (data not shown).

Experimental Endophthalmitis: Retinal Function Analysis

Retinal function analyses are summarized in Figures 2 and 3. The retinal function of all surgical and absolute control eyes was similar to the preoperative retinal function throughout the experiment (data not shown).

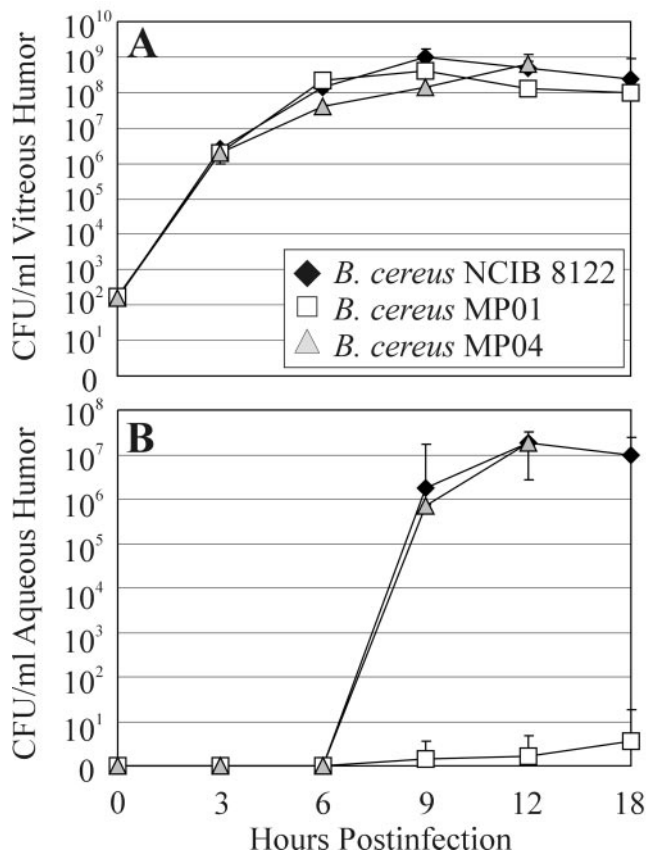


FIGURE 1. Intraocular growth during experimental *B. cereus* endophthalmitis. Approximately 100 CFU of *B. cereus* wild-type NCIB 8122, nonswarming *fliY*-mutant MP01, or *fliY*-complemented MP04 were injected intravitreally. Bacteria were quantified from the vitreous (A) and aqueous (B) humor every 3 hours throughout 18 hours. All strains grew to similar concentrations in the vitreous throughout the infection course, but the nonswarming *fliY*-mutant was unable to reach the aqueous humor. At 18 hours, all eyes infected with MP04 perforated during harvest and accurate bacterial counts were not available. The data are expressed as the mean ± SEM of results in four or more eyes per group.

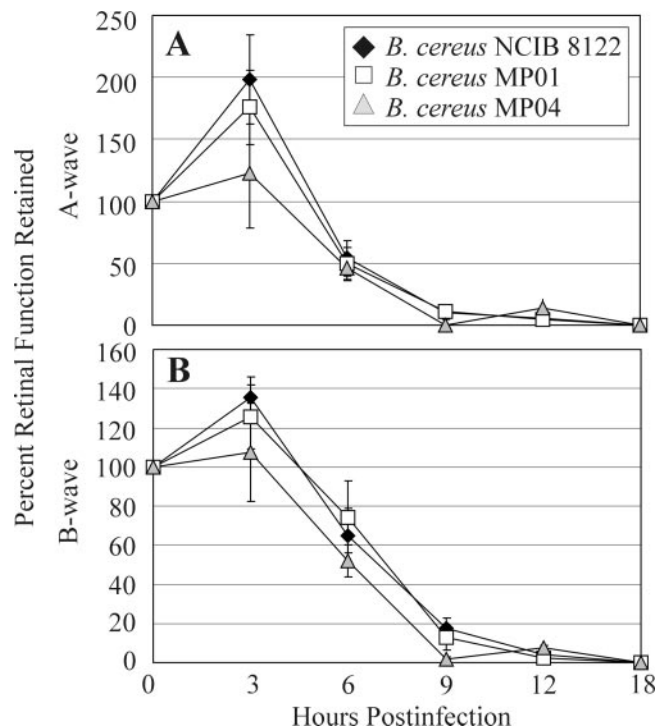


FIGURE 2. Analysis of a- and b-wave amplitudes in experimental *B. cereus* endophthalmitis. Electroretinography was performed on eyes injected with wild-type *B. cereus* NCIB 8122, nonswarming *fliY*-mutant MP01, or *fliY*-complemented MP04, and amplitudes were recorded every 3 hours throughout the infection course. Rapid decreases in (A) a- and (B) b-wave amplitudes were observed in eyes infected with either strain throughout 18 hours. The data are expressed as the mean ± SEM of results in four or more eyes per group.

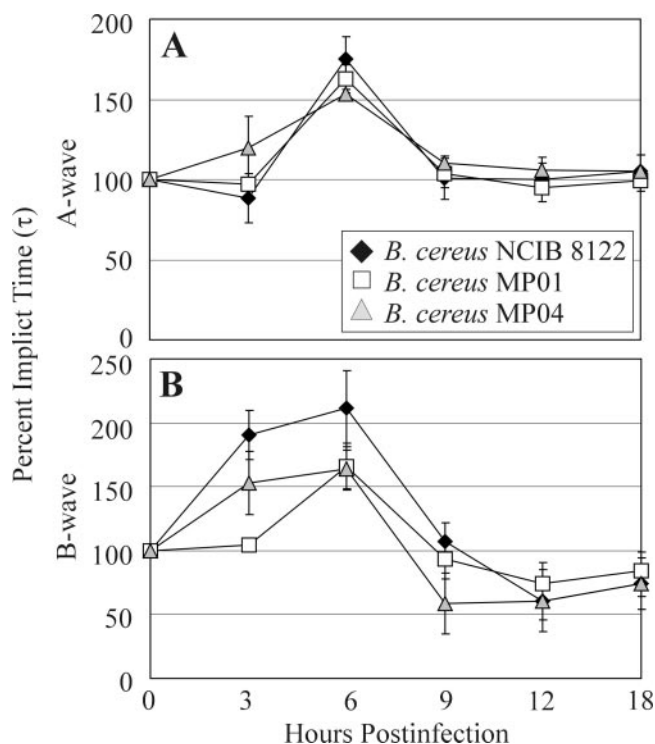


FIGURE 3. Analysis of latent a- and b-wave responses in experimental *B. cereus* endophthalmitis. Electroretinography was performed on eyes injected with wild-type *B. cereus* NCIB 8122, nonswarming *fliY*-mutant MP01, or *fliY*-complemented MP04, and implicit times were recorded every 3 hours throughout the infection course. In general, increases in (A) a- and (B) b-wave implicit time latencies were observed by 6 hours. The data are expressed as the mean \pm SEM of results in four or more eyes per group.

The rapid loss of retinal function in eyes infected with each strain was similar to that observed in previous studies.^{4–6,16,17,21} In eyes infected with each strain, super-ERG responses were recorded for both a- and b-wave amplitudes at 3 hours after infection ($P \geq 0.15$). Although the retinal responsiveness of eyes infected with MP04 were significantly different from that of NCIB 8122 and MP01 at 9 and 12 hours after infection ($P \leq 0.03$), loss of retinal function in all infected eyes corresponded collectively to $>95\%$ by 12 hours and nearly 100% by 18 hours.

Changes in the latency of retinal responses are summarized in Figure 3. Significant increases in latency of the a-wave implicit times were detected in eyes infected with each strain at 6 hours after infection only. At this time, the a-wave implicit times for eyes infected with each strain were similar ($P \geq 0.25$). For b-waves, increases in latency of implicit times were detected in eyes infected with wild-type *B. cereus* NCIB 8122 and the *fliY*-complemented mutant at 3 hours and 6 hours after infection. Increases in latency of implicit times of eyes infected with the nonswarming mutant were detected at 6 hours only. At 6 hours, the b-wave implicit time latencies for eyes infected with each strain were similar ($P \geq 0.14$). The implicit time latencies in eyes infected with each *Bacillus* strain were similar to preoperative values, to control latencies at 12 and 18 hours after infection, and to each other throughout the infection course ($P \geq 0.08$).

Anterior Segment Inflammation

Figure 4 summarizes the migration of inflammatory cells into the anterior segment in eyes infected with *B. cereus*. A signif-

icant number of inflammatory cells were recovered from eyes infected with wild-type *B. cereus* and the *fliY*-complemented mutant from 6 to 12 hours after infection. In contrast, few inflammatory cells were recovered from eyes infected with the nonswarming *B. cereus* mutant MP01 from 3 to 18 hours after infection. The number of inflammatory cells recovered from eyes infected with wild-type *B. cereus* and the *fliY*-complemented mutant was greater than that recovered from eyes infected with the *fliY*-mutant MP01 from 6 to 12 hours after infection ($P \leq 0.02$). At 18 hours, all eyes infected with MP04 had perforated during harvest, and accurate inflammatory cell counts were not available. No inflammatory cells were recovered from the aqueous humor of control eyes.

Histologic Analysis

Immediately after intravitreal injection, eyes in all infection and control groups had intact retinal layers, no anterior or posterior segment inflammation, and few bacilli in the vitreous (data not shown).

Eyes infected with all *B. cereus* strains exhibited mild to moderate inflammatory cell influx into the posterior segment at 6 hours after infection (Fig. 5). Mild to moderate disruption of retinal layers was also observed (Fig. 6). At this time, inflammatory cells were observed in the vitreous in close proximity to the optic nerve head and near the iris and ciliary body. Mild to moderate disruption of retinal layers was also observed (Fig. 6). By 12 hours after infection, a significant number of inflammatory cells were present throughout the posterior segment, and retinal layers were severely disrupted in eyes infected with swarming strains (Figs. 5, 6). By 18 hours after infection, retinal layers were indistinguishable in these eyes, and a significant number of fibrin, inflammatory cells, and erythrocytes was present throughout the vitreous, surrounding the iris and ciliary body, and in the anterior chamber (Figs. 5, 6). In eyes infected with the *fliY*-nonswarming mutant MP01, significant numbers of inflammatory cells were present throughout the vitreous at 12 and 18 hours after infection, but few were present in the anterior chamber or cornea (Fig. 5). Bacilli were not seen in the anterior chamber or cornea on higher magnification of these sections (data not shown), correlating with

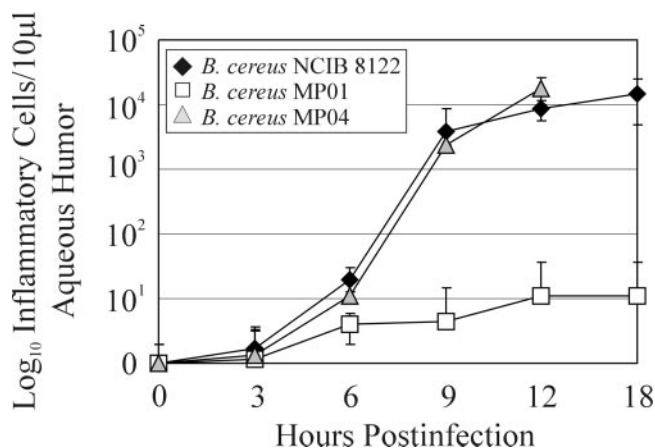


FIGURE 4. Infiltration of inflammatory cells into the anterior segment during experimental *B. cereus* endophthalmitis. Inflammatory cells were quantified from aqueous humor of eyes injected with wild-type *B. cereus* NCIB 8122, nonswarming *fliY*-mutant MP01, or *fliY*-complemented MP04, every 3 hours for 18 hours. A significant number of inflammatory cells were present in eyes infected with wild-type *B. cereus* and the *fliY*-complemented strain. At 18 hours, all eyes infected with MP04 perforated during harvest, and accurate inflammatory cell counts were not obtainable. The data are expressed as the mean \pm SEM of results in four or more eyes per group.

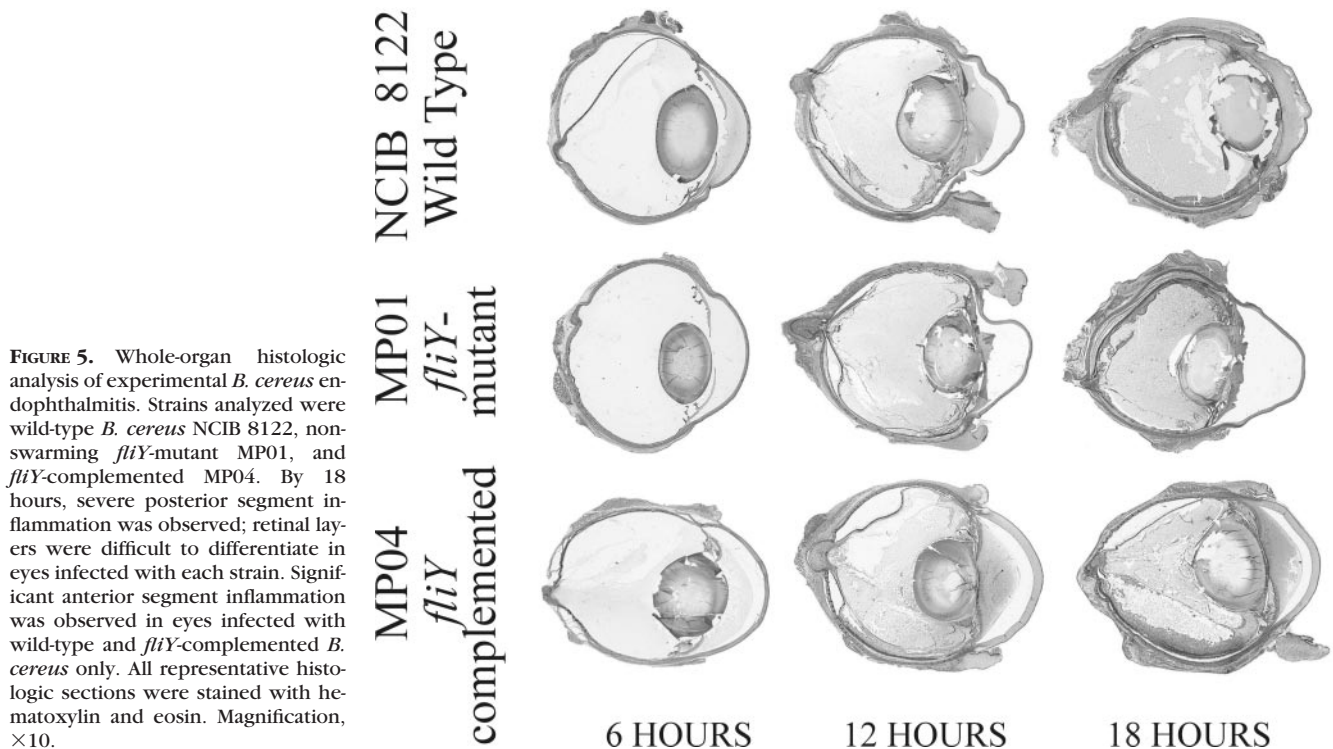


FIGURE 5. Whole-organ histologic analysis of experimental *B. cereus* endophthalmitis. Strains analyzed were wild-type *B. cereus* NCIB 8122, non-swarming *fliY*-mutant MP01, and *fliY*-complemented MP04. By 18 hours, severe posterior segment inflammation was observed; retinal layers were difficult to differentiate in eyes infected with each strain. Significant anterior segment inflammation was observed in eyes infected with wild-type and *fliY*-complemented *B. cereus* only. All representative histologic sections were stained with hematoxylin and eosin. Magnification, $\times 10$.

bacterial counts from the aqueous humor. The course of retinal layer disruption in eyes infected with the nonswarming mutant was similar to that of the wild-type and *fliY*-complemented *Bacillus* strains (Fig. 6). No erythrocytes were seen in eyes infected with the *fliY*-nonswarming strain.

DISCUSSION

Bacillus is one of the most virulent pathogens in the eye, causing explosive inflammation and significant vision loss that, in most cases, cannot be improved by the most aggressive of therapies. The ability of *Bacillus* to spread throughout the eye has been shown to contribute to its unique virulence during endophthalmitis.⁴ In a comparison of *Bacillus* with other Gram-positive pathogens in the eye, *Bacillus* was able to migrate throughout the eye during infection, whereas *Staphylococcus aureus* and *Enterococcus faecalis* remained and grew in the vitreous. Although *S. aureus* and *E. faecalis* caused significant inflammation and retinal function loss within 3 days, endophthalmitis caused by these toxigenic organisms did not achieve the explosive severity that *B. cereus* did, in part, because of the inability of *S. aureus* and *E. faecalis* to spread throughout the eye.⁴ Additional studies investigating the importance of motility and toxin production to explosive intraocular virulence demonstrated that nonmotile *Bacillus* were less virulent than their wild-type parental strains. Intraocular infection with isogenic nonmotile *B. cereus* or *B. thuringiensis* resulted in slower evolution of retinal function loss and intraocular inflammation than motile wild-type strains.^{5,6} However, because motility and toxin production appeared to be closely linked in the nonmotile strains used, the contribution of motility alone to infection of the eye remains in question.

With respect to toxin production, the parental strain used in the present study was similar to that of *B. cereus* and *B. thuringiensis* strains used in previous studies,^{5,6,16,17,21} with the exception that NCBI 8122 did not produce hemolysin BL. The differences in toxin production between nonmotile mutants and their parental strains generated in previous studies

have been reported.^{5,6} The nonmotile *flbA* insertional mutant^{5,6} and transposon mutant⁵ were defective in toxin secretion, indicating a global effect on toxin output, probably resulting from these specific mutations. In the present study, the *fliY* mutation did not alter the toxin production profile of the mutant strain, altering only its ability to swarm.

During the early stages of experimental *B. cereus* endophthalmitis, detectable increases in ERG amplitude (super-ERG) and latencies occurred. Super-ERG responses were recorded for both a- and b-wave amplitudes at 3 hours after infection in all infected eyes, indicating a possible change in the retinal cells responsible for these functions during the earliest stages of infection. Increases in latency of the a- and b-wave implicit times were detected in infected eyes before or 6 hours after infection, further indicating potential changes in retinal cells at this time. There was a significantly greater b-wave latency detected at 3 hours in eyes infected with the wild-type and *fliY*-complemented *B. cereus*. Because a deficiency in swarming rendered MP01 unable to reach the anterior segment during infection, MP01 may also not have been able to migrate easily toward the retina. Hypothetically, if the change in b-wave latency at 3 hours was due to bacilli or its toxins in close proximity to retinal cells responsible for the b-wave (i.e., Müller cells, bipolar cells, ganglion cells), MP01 may not have affected these cells at this time, because MP01 simply may not have been near the retina. Toxin-dependent differences in retinal function latencies have been reported in experimental *B. cereus* endophthalmitis.¹⁷ There were no strain-specific differences in ERG amplitudes, suggesting a potential difference in the mechanisms of amplitude and latency alterations during the infection that may not be due to a close association of *Bacillus* or its toxins with the retina. Nevertheless, our recent studies strongly suggest that early changes in retinal responses during endophthalmitis could result from bacteria- or toxin-induced retinal dysfunction. The detrimental effects of *Bacillus* and its toxins on specific cells of the retina are presently being analyzed.

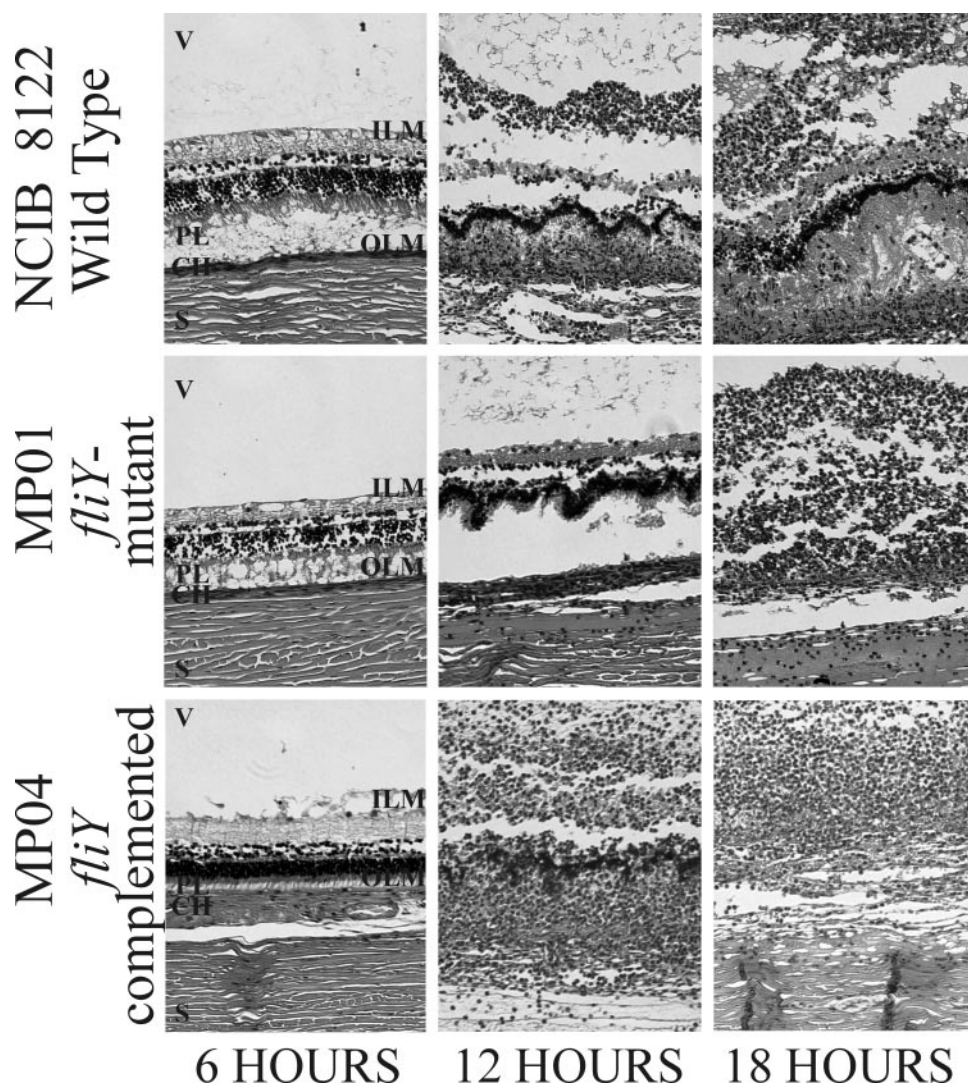


FIGURE 6. Retinal histologic analysis of experimental *B. cereus* endophthalmitis. Strains analyzed were wild-type *B. cereus* NCIB 8122, non-swarming *fliY*-mutant MP01, and *fliY*-complemented MP04. By 12 hours, significant inflammation and photoreceptor layer folding was observed in eyes infected with each strain. By 18 hours, specific retinal cell layers in eyes infected with either strain were virtually indistinguishable. Photographs were taken of the inferior aspect of eyes shown in Figure 5. V, vitreous; ILM, inner limiting membrane; PL, photoreceptor cell layer; OLM, outer limiting membrane; CH, choriocapillaris; S, sclera. Magnification, $\times 200$.

During infection of the posterior segment, bacteria are deposited within the semisolid vitreous humor. The vitreous humor is a transparent, gelatinous medium through which light must travel to the retina to form an image and is composed primarily of water, hyaluronic acid, and a network of collagen fibrils. During endophthalmitis, *Bacillus* traverses and grows within the vitreous, reaching nearly all parts of the eye. In the present study, the nonswarming mutant was unable to escape from the vitreous and reach the anterior segment, resulting in a notable lack of anterior segment inflammation. However, the explosive posterior segment inflammation and retinal dysfunction were similar, regardless of the infecting strain, suggesting that swarming did not contribute significantly to overall intraocular virulence. *Bacillus* toxins, which are collectively essential for endophthalmitis virulence,¹⁷ were probably produced by each strain in the posterior segment and may have contributed to inciting the explosive inflammation observed in that area.

These studies also confirmed the lack of a significant role for hemolysin BL in endophthalmitis. We demonstrated in an earlier study that the intraocular virulence of wild-type *B. cereus* and its hemolysin BL-deficient isogenic mutant are similar.²¹ In the present study, wild-type NCIB 8122 and its companion strains produced only the L2 component of hemolysin BL, not a functional lytic toxin. Yet, the virulence of the wild-type, *fliY*-nonswarming mutant, and the *fliY*-complemented *B.*

cereus strains were comparatively more virulent than the *B. cereus* or *B. thuringiensis* strains previously analyzed in this model. These findings correlated with our report of a limited role for hemolysin BL in endophthalmitis.

As reported previously, the *fliY*-nonswarming mutant is unable to produce the L2 component of hemolysin BL, suggesting the potential for swarming-dependent production of this protein.¹⁴ Although we did not detect differences in the number of different strains or the quantity secreted by the wild-type, *fliY*-, and *fliY*-complemented *B. cereus* strains in vitro, the possibility exists that production of these virulence factors in vivo is associated with swarming. Associations between swarming and virulence have been reported for *Proteus*, *Salmonella*, and *Clostridium*.^{7,8,10-12} These organisms exhibit increased virulence characteristics, such as invasion or toxin production, when in the swarmer cell state. Recent studies by Kim and Surette²² demonstrated the coordinate regulation of cell signaling systems exclusively in *Salmonella* swarming cells. The unique physiological environment of the interior of the eye may trigger the transformation of *Bacillus* into a hypervirulent migrating organism that could be the cause for its explosive intraocular virulence.

Migration of *Bacillus* during endophthalmitis may result from an as yet unknown biochemical or physiological stimulus within the eye. Lack of sufficient nutrients, oxygen, or other necessary growth factors in the interior of the eye may trigger

Bacillus to migrate to a more favorable environment. Entry of inflammatory cells or their products into the posterior segment during the early stages of infection may also prompt *Bacillus* to navigate away from a hostile environment. Chemotaxis of an organism toward or away from specific environmental stimuli provides bacteria with an adaptation and survival advantage. Chemotaxis, swarming, motility, and the virulence potential of *Bacillus* and other motile organisms appear to be closely associated within complex regulatory networks, but these links are not well-defined.²³⁻²⁷ Senesi et al¹⁴ demonstrated the inability of the nonswarming *B. cereus* mutant MP01 to move toward a nutrient stimulus. It is not clear whether this defect in chemotaxis resulted in the inability of MP01 to navigate toward or away from specific intraocular stimuli, thus preventing migration into the anterior segment. The biochemical and physiological triggers for migration of *Bacillus* and other motile organisms within the eye during endophthalmitis are presently being investigated.

Acknowledgments

The authors thank Mark Dittmar and Andrea Mauer (DMEI Animal Resources Facility) for technical assistance and Paula Pierce (Excalibur Pathology, Oklahoma City, OK) for preparation of the histology specimens.

References

- Affeldt JC, Flynn HW Jr, Forster RK, et al. Microbial endophthalmitis resulting from ocular trauma. *Ophthalmology*. 1987;94:407-413.
- Davey RT Jr, Tauber WB. Posttraumatic endophthalmitis: the emerging role of *Bacillus cereus* infection. *Rev Infect Dis*. 1987; 9:110-123.
- David DB, Kirkby GR, Noble BA. *Bacillus cereus* endophthalmitis. *Br J Ophthalmol*. 1994;78:577-580.
- Callegan MC, Engelbert M, Parke DW 2nd, Jett BD, Gilmore MS. Bacterial endophthalmitis: epidemiology, therapeutics, and bacterium-host interactions. *Clin Microbiol Rev*. 2002;15:111-124.
- Callegan MC, Kane ST, Cochran DC, Gilmore MS. Molecular mechanisms of *Bacillus* endophthalmitis pathogenesis. *DNA Cell Biol*. 2002;21:367-373.
- Callegan MC, Kane ST, Cochran DC, et al. *Bacillus* endophthalmitis: roles of toxins and motility during infection. *Invest Ophthalmol Vis Sci*. 2005;46:3233-3238.
- Allison C, Coleman N, Jones PL, Hughes C. Ability of *Proteus mirabilis* to invade human urothelial cells is coupled to motility and swarming differentiation. *Infect Immun*. 1992;60:4740-4746.
- Allison C, Lai HC, Hughes C. Co-ordinate expression of virulence genes during swarm-cell differentiation and population migration of *Proteus mirabilis*. *Mol Microbiol*. 1992;6:1583-1591.
- Eberl L, Christiansen G, Molin S, Givskov M. Differentiation of *Serratia liquefaciens* into swarm cells is controlled by the expression of the *flhD* master operon. *J Bacteriol*. 1996;178:554-559.
- Macfarlane S, Hopkins MJ, Macfarlane GT. Toxin synthesis and mucin breakdown are related to swarming phenomenon in *Clostridium septicum*. *Infect Immun*. 2001;69:1120-1126.
- Wang Q, Frye JG, McClelland M, Harshey RM. Gene expression patterns during swarming in *Salmonella typhimurium*: genes specific to surface growth and putative new motility and pathogenicity genes. *Mol Microbiol*. 2004;52:169-187.
- Allison C, Emody L, Coleman N, Hughes C. The role of swarm cell differentiation and multicellular migration in the uropathogenicity of *Proteus mirabilis*. *J Infect Dis*. 1994;169:1155-1158.
- Kim W, Killam T, Sood V, Surette MG. Swarm-cell differentiation in *Salmonella enterica* serovar *typhimurium* results in elevated resistance to multiple antibiotics. *J Bacteriol*. 2003;185:3111-3117.
- Senesi S, Celandroni F, Salvetti S, Beecher DJ, Wong AC, Ghelardi E. Swarming motility in *Bacillus cereus* and characterization of a *fltY* mutant impaired in swarm cell differentiation. *Microbiology*. 2002;148:1785-1794.
- Connelly MB, Young GM, Sloma A. Extracellular proteolytic activity plays a central role in swarming motility in *Bacillus subtilis*. *J Bacteriol*. 2004;186:4159-4167.
- Callegan MC, Cochran DC, Kane ST, Gilmore MS, Gominet M, Lereclus D. Contribution of membrane-damaging toxins to *Bacillus* endophthalmitis pathogenesis. *Infect Immun*. 2002;70:5381-5389.
- Callegan MC, Kane ST, Cochran DC, Gilmore MS, Gominet M, Lereclus D. Relationship of *plcR*-regulated factors to *Bacillus* endophthalmitis virulence. *Infect Immun*. 2003;71:3116-3124.
- Ghelardi E, Celandroni F, Salvetti S, et al. Requirement of *flhA* for swarming differentiation, flagellin export, and secretion of virulence-associated proteins in *Bacillus thuringiensis*. *J Bacteriol*. 2002;184:6424-6433.
- Beecher DJ, Olsen TW, Somers EB, Wong AC. Evidence for contribution of tripartite hemolysin BL, phosphatidylcholine-prefering phospholipase C, and collagenase to virulence of *Bacillus cereus* endophthalmitis. *Infect Immun*. 2000;68:5269-5276.
- Limbago B, Penumalli V, Weinrick B, Scott JR. Role of streptolysin O in a mouse model of invasive group A streptococcal disease. *Infect Immun*. 2000;68:6384-6390.
- Callegan MC, Jett BD, Hancock LE, Gilmore MS. 1999. Role of hemolysin BL in the pathogenesis of extraintestinal *Bacillus cereus* infection assessed in an endophthalmitis model. *Infect Immun*. 1999;67:3357-3366.
- Kim W, Surette MG. Coordinated regulation of two independent cell-cell signaling systems and swarmer differentiation in *Salmonella enterica* serovar Typhimurium. *J Bacteriol*. 2006;188:431-440.
- Boin MA, Austin MJ, Hase CC. Chemotaxis in *Vibrio cholerae*. *FEMS Microbiol Lett*. 2004;239:1-8.
- Dons L, Eriksson E, Jin Y, et al. Role of flagellin and the two-component CheA/CheY system of *Listeria monocytogenes* in host cell invasion and virulence. *Infect Immun*. 2004;72:3237-3244.
- Foyne S, Dorrell N, Ward SJ, et al. *Helicobacter pylori* possesses two CheY response regulators and a histidine kinase sensor, CheA, which are essential for chemotaxis and colonization of the gastric mucosa. *Infect Immun*. 2000;68:2016-2023.
- Merrell DS, Butler SM, Qadri F, et al. Host-induced epidemic spread of the cholera bacterium. *Nature*. 2002;417:642-645.
- O'Toole R, Milton DL, Wolf-Watz H. Chemotactic motility is required for invasion of the host by the fish pathogen *Vibrio anguillarum*. *Mol Microbiol*. 1996;19:625-637.