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The Role of Pili in *Bacillus cereus* Intraocular Infection

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Abstract

Bacterial endophthalmitis is a potentially blinding intraocular infection. The bacterium *Bacillus cereus* causes a devastating form of this disease which progresses rapidly, resulting in significant inflammation and loss of vision within a few days. The outer surface of *B. cereus* incites the intraocular inflammatory response, likely through interactions with innate immune receptors such as TLRs. This study analyzed the role of *B. cereus* pili, adhesion appendages located on the bacterial surface, in experimental endophthalmitis. To test the hypothesis that the presence of pili contributed to intraocular inflammation and virulence, we analyzed the progress of experimental endophthalmitis in mouse eyes infected with wild type *B. cereus* (ATCC 14579) or its isogenic pilus-deficient mutant (*bcpA-srtD-bcpB* or Pil). One hundred CFU were injected into the mid-vitreous of one eye of each mouse. Infections were analyzed by quantifying intraocular bacilli and retinal function loss, and by histology from 0 to 12 h postinfection. *In vitro* growth and hemolytic phenotypes of the infecting strains were also compared. There was no difference in hemolytic activity (1:8 titer), motility, or *in vitro* growth ($p > 0.05$, every 2 h, 0-18 h) between wild type *B. cereus* and the Pil mutant. However, infected eyes contained greater numbers of wild type *B. cereus* than Pil during the infection course ($p = 0.05$, 3-12 h). Eyes infected with wild type *B. cereus* experienced greater losses in retinal function than eyes infected with the Pil mutant, but the differences were not always significant. Eyes infected with Pil or wild type *B. cereus* achieved similar degrees of severe inflammation. The results indicated that the intraocular growth

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of pilus-deficient *B. cereus* may have been better controlled, leading to a trend of greater retinal function in eyes infected with the pilus-deficient strain. Although this difference was not enough to significantly alter the severity of the inflammatory response, these results suggest a potential role for pili in protecting *B. cereus* from clearance during the early stages of endophthalmitis, which is a newly described virulence mechanism for this organism and this infection.

Keywords

eye; infection; endophthalmitis; inflammation; virulence; *Bacillus*; pili

1. Introduction

Bacillus cereus causes a form of severe intraocular infection (endophthalmitis) which progresses rapidly, resulting in significant inflammation and loss of vision within a few days. In severe cases, *B. cereus* endophthalmitis may lead to enucleation or evisceration of the infected eye (Chan et al., 2003, Chen and Roy, 2000, Cowan et al., 1997, David et al., 1994, O'Day et al., 1981, and Roy et al., 1997). This devastating outcome may occur regardless of antimicrobial and anti-inflammatory therapies that may be successful against staphylococcal or streptococcal endophthalmitis (Astley et al., 2016, Callegan et al., 2007, and Sadaka et al., 2012). Therefore, identifying the specific factors of *B. cereus* which contribute to its unique intraocular virulence is important in devising better treatment strategies for this disease.

During experimental endophthalmitis, the outer envelope of *B. cereus* incites an intraocular inflammatory response (Callegan et al., 1999). This acute response is likely mediated by interactions between peptidoglycan found on the surface of *B. cereus* and Toll-like receptor 2 (TLR2), an innate immune receptor which recognizes Gram-positive peptidoglycan (Novosad et al., 2011). TLR4 and its adaptor molecules MyD88 and TRIF are also important in the acute intraocular inflammatory response to *B. cereus* (Parkunan et al., 2015), but the ligand(s) initiating this response have not yet been identified. Flagella also contribute to the intraocular virulence of *B. cereus*, primarily through their function in propelling the organism throughout the eye during infection (Callegan et al., 2005 and Callegan et al., 2006), but not via interactions with TLR5 (Parkunan et al., 2014).

In an effort to identify factors associated with the unique virulence of this organism in the eye, we analyzed the role of *B. cereus* pili, which are adhesion appendages, in experimental endophthalmitis. A number of bacterial species rely on pili as a virulence determinant for adherence to mucosal tissues, to tissues in areas of high fluid flow, and in establishment of biofilms (Axner et al., 2011, Danne and Dramsi, 2012, Mandlik et al., 2008, Melville and Craig, 2013, Romero, 2013, Scheewind and Missiakas, 2012, and Vengadesan and Narayana, 2011). Engagement of pili with components of innate immunity has also been suggested, potentially contributing to the acute inflammatory response (Barrochi et al., 2006, Bassett et al., 2013, Crotty Alexander et al., 2010, and Vargas Gardia et al., 2015). Budzik *et al.* (2007, 2008, 2009a, and 2009b) have reported on the structure and assembly of pili on the surface of vegetative *B. cereus*, but a specific role for pili in *B. cereus* infections has not yet been ascribed. In the present study, we tested the hypothesis that *B. cereus* pili

contributed to intraocular virulence in a well-established mouse model of *B. cereus* endophthalmitis.

2. Materials and Methods

2.1. Bacterial Strains

Bacterial strains used to initiate experimental endophthalmitis in mice included *B. cereus* ATCC 14579 or its isogenic pilus-deficient mutant (*bcpA-srtD-bcpB* or Pil) (Budzik et al., 2007). The isogenic pilus-deficient mutant was generated by allelic replacement mutagenesis as previously described (Budzik et al., 2007). The *in vitro* growth, hemolytic titers, and motility of these strains were compared. Each strain was cultured for 18 h at 37°C with aeration in brain heart infusion (BHI; VWR, Radnor PA), then subcultured to an approximate concentration of 10³ CFU/mL in fresh BHI. Subcultures were incubated for 18 h as described above. Every two hours, aliquots were removed to quantify bacteria by track dilution (see 2.3 below) and hemolytic activity. Aliquots were filter sterilized (0.22 µm; EMD Millipore, Darmstadt, Germany) and supernatants were serially diluted 1:2 in PBS (pH 7.4). Supernatants were then mixed 1:1 with 4% (vol/vol) sheep erythrocytes (Rockland Immunochemicals, Pottstown PA) and incubated for 30 min at 37°C. The hemolytic titer was determined to be the lowest dilution of supernatant exhibiting 50% hemolysis at an optical density of 540 nm. Strains were also incubated on tryptic soy agar (TSA) with 5% sheep blood agar for 18 h at 37°C for colony morphology and hemolytic phenotype comparisons, and on motility agar (0.75% agar in BHI) for motility phenotype comparisons. Strains were also compared by transmission electron microscopy (Oklahoma Medical Research Foundation Imaging Core, Oklahoma City OK). Lawns of *B. cereus* were scraped from BHI agar plates following 18 h growth at 37°C. Bacteria were suspended in PBS, fixed with 5% paraformaldehyde/4% glutaraldehyde, washed with water, and stained with 4% uranyl acetate. Samples were viewed with a Hitachi H-7600 transmission electron microscope.

2.2. Mice and Intraocular Infection

The *in vivo* experiments described herein involved the use of mice. All animal procedures were conducted according to guidelines and recommendations from the National Institutes of Health Guide for the Care and Use of Laboratory Animals, the University of Oklahoma Health Sciences Center Institutional Animal Care and Use Committee (IACUC), and the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. The OUHSC IACUC approved these studies under protocol 13-086.

C57BL/6J mice were purchased from commercially available stock colonies (Stock No. 000664, Jackson Labs, Bar Harbor ME). All mice were housed in microisolation conditions on a 12 h on/12 h off light cycle for at least two weeks before use, then under biosafety level 2 conditions during experiments. Mice were 8–10 weeks of age at the time of experiment.

Mice were anesthetized with a combination of ketamine (85 mg/kg body weight; Ketathesia, Henry Schein Animal Health, Dublin, OH) and xylazine (14 mg/kg body weight; AnaSed; Akorn Inc., Decatur, IL). Experimental *B. cereus* endophthalmitis was induced in mouse

eyes by injecting approximately 100 CFU *B. cereus*/0.5 µl BHI into the mid-vitreous, as previously described (Moyer et al., 2009, Novosad et al., 2011, Parkunan et al., 2014, Parkunan et al., 2015, and Ramadan et al., 2008). The contralateral eye served as the uninjected/uninfected control. Mice were euthanized by CO₂ inhalation, which occurred before eyes were removed for analysis.

2.3. Bacterial Quantitation

Intraocular bacteria were quantified as previously described (Moyer et al., 2009, Novosad et al., 2011, Parkunan et al., 2014, Parkunan et al., 2015, and Ramadan et al., 2008). Whole eyes were removed from euthanized mice at 3, 6, 9, and 12 h postinfection. Eyes were homogenized in PBS with sterile 1mm glass beads (BioSpec Products, Inc., Bartlesville, OK). Homogenates were plated and bacterial numbers were quantified by the track dilution method. Values represent the mean ± SEM for N = 6 eyes per time point. Two independent experiments were performed.

2.4. Electroretinography

Electroretinography (ERG) was used to analyze retinal function, as previously described (Moyer et al., 2009, Novosad et al., 2011, Parkunan et al., 2014, Parkunan et al., 2015, and Ramadan et al., 2008). Scotopic ERGs were performed at 6, 9, and 12 h postinfection (Espion E2, Diagnosys LLC, Lowell MA). Following infection, mice were dark-adapted for at least 6 h. Mice were anesthetized with the ketamine/xylazine cocktail and pupils were dilated with topical phenylephrine (Akorn, Inc., IL). Retinal function was recorded with gold-wire electrodes placed on each cornea and a reference electrode. Retinal responses were stimulated by five white light flashes (1,200 cd·s/m²). The average of the resulting A- and B-wave amplitudes following each flash was calculated. A- and B-wave amplitudes were recorded for infected and contralateral uninfected eyes in the same animal. A- and B-wave amplitudes were measured from the initiation of the light flash to the trough of the A-wave, and the trough of the A-wave to the peak of the B-wave. The percentage of retinal function retained in the infected eye was calculated in comparison with uninfected left eye controls as: $100 - \{[1 - (\text{experimental A- or B-wave amplitude}/\text{control A- or B-wave amplitude})] \times 100\}$. Values represent the mean ± SEM for N = 6 eyes per time point. At least 3 independent experiments were performed.

2.5. Histology

Eyes were harvested at 3, 6, 9, and 12 h postinfection. Harvested eyes were incubated in buffered zinc formalin or Davidson's fixative for 24 h at room temperature (Moyer et al., 2009, Novosad et al., 2011, Parkunan et al., 2014, Parkunan et al., 2015, and Ramadan et al., 2008). Eyes were then transferred to 70% ethanol, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Images are representative of at least 3 eyes per time point from at least 2 independent experiments.

2.6. Statistics

Data are the arithmetic means ± the standard errors of the mean (SEM) of all samples in the same experimental group in replicate experiments. Statistical significance was $p < 0.05$. The

Mann Whitney Rank Sum test was used to compare experimental groups to determine the statistical significance for all assays (Parkunan et al., 2015, and Parkunan et al., 2016). All statistical analyses were performed using GraphPad Prism 6.05 (GraphPad Software, Inc., La Jolla CA).

3. Results

3.1. Bacterial Growth and Phenotypes

B. cereus strains ATCC 14579 and Pil grew at similar rates in BHI (Figure 1A). Numbers of ATCC 14579 and Pil were statistically similar at each time point tested ($P = 0.05$). Both strains reached stationary phase in BHI at approximately 8 h postinfection. Figure 1B depicts inoculation of TSA with 5% sheep blood with *B. cereus* ATCC 14579 and Pil. Four areas on each blood agar plate were spot-inoculated and incubated at 37°C for 18 h. Spreading colonies of *B. cereus* were observed at each inoculation point, each with the characteristic double zone of hemolysis. On sheep blood agar (Figure 1B) and motility agar (ATCC 14579, 31.7 ± 9.5 mm; Pil, 31.3 ± 3.2 mm at 48 h, $P=0.97$), these strains were phenotypically indistinguishable. Hemolytic assays confirmed this observation, as hemolytic titers of 18-h supernatants of ATCC 14579 and Pil were similar (titer = 1:8, $N=3$). These results suggested that the absence of pili (Figure 1C) did not affect the *in vitro* growth, motility, or hemolytic phenotype and that, based on these similarities, *in vivo* infection with these strains might be equivalent.

3.2. Intraocular Growth and Retinal Function

The intraocular growth of *B. cereus* ATCC 14579 and Pil in C57BL/6J mice is depicted in Figure 2. Eyes were inoculated with 112 ± 3 CFU/eye ATCC 14579 and 107 ± 4 CFU/eye Pil ($P=0.26$). The bacterial load in eyes infected with ATCC 14579 was significantly greater than the bacterial load in eyes infected with Pil at 3, 6, 9, and 12 h postinfection ($P = 0.03$). At 12 h postinfection, eyes infected with ATCC 14579 had 8.8×10^6 CFU/eye, while eyes infected with Pil had 5.2×10^5 CFU/eye ($P=0.0003$). The significant differences in CFU suggested that ocular infection by Pil might be less severe than that of infection with ATCC 14579.

The retained retinal function of eyes infected with *B. cereus* ATCC 14579 or Pil and representative waveforms of these eyes are depicted in Figure 3. The retained A-wave function of eyes infected with ATCC 14579 decreased from 6 to 12 h postinfection, to a retained response of approximately 26%. The retained B-wave function in these eyes decreased over the same time period to approximately 39%. The retained A-wave function of eyes infected with Pil also decreased from 6 to 12 h postinfection, to a retained response of approximately 35%, while the retained B-wave function declined to approximately 48%. Although there was an observed trend of higher retained A-wave and B-wave function in eyes infected with Pil compared to that of eyes infected with ATCC 14579 at each time point, this difference was not statistically significant ($P = 0.29$).

3.3. Ocular Pathology

Pathologic changes resulting from intraocular infection with *B. cereus* ATCC 14579 and

Pil are depicted in Figure 4. At 3 h postinfection, eyes infected with each strain presented with fibrin infiltrate into the anterior chamber and posterior segments. Corneas were clear, retinas were intact, and retinal layers were distinguishable at this time. *B. cereus* were noted in the vitreous of eyes infected with both strains. At 6 h postinfection, significant fibrin and infiltrating cells were visible in the anterior segments of eyes infected with each strain. *B. cereus* were noted in the mid-vitreous and along the inner limiting membrane (Figure 4B), as well as along the posterior capsule and near the ciliary body in eyes infected with either strain. Corneas were slightly edematous, but retinal layers in these infected eyes remained intact. By 9 h postinfection, there was significant inflammation in the anterior and posterior segments of eyes infected with each strain. In addition to the areas described at 6 h, *B. cereus* were also noted in the anterior chamber of eyes infected with either strain (Figure 4B). Retinal detachments were present in approximately half of eyes from each infection group, but generally, retinal layers remained intact. By 12 h postinfection, all eyes infected with *B. cereus* ATCC 14579 and Pil exhibited severe inflammation, with significant corneal edema, anterior and posterior segment infiltration, and fully detached retinas with indistinguishable retinal layers. *B. cereus* organisms were noted in the same areas as described for 9 h postinfection. In summary, the gross pathological changes developing in eyes infected with *B. cereus* ATCC 14579 and Pil were indistinguishable from one another.

Taken together, these results suggest that the growth of nonpiliated *B. cereus* may have been better controlled during the early stages of intraocular infection, but not to the extent that it significantly altered the decline of retinal function, the development of damaging inflammation, or the migration of *B. cereus* within the eye.

4. Discussion

Up to two-thirds of eyes intraocularly infected with *B. cereus* lose significant vision, experiencing severe retinal damage and inflammation that may also result in loss of the eye. The unique virulence of *B. cereus* in the eye is attributed to a dangerous combination of rapid replication, migration throughout the eye, toxin production, and a resulting robust inflammatory response. Efforts to treat this blinding disease rely on identifying bacterial and host targets that can be exploited to significantly alter the outcome of infection and prevent vision loss.

Pili are proteinaceous appendages on the bacterial surface that perform a myriad of functions related to virulence. Pili on the surface of Gram-positive bacteria are comprised of covalently-linked pilin subunits which are anchored to the cell wall by a sortase/transpeptidase enzyme. The link between pili and the virulence of Gram-negative bacteria has been widely reported, as has the role of fimbriae in the attachment and virulence of both Gram-negative and Gram-positive bacteria. For the eye, the corneal virulence of nonpiliated *Pseudomonas* was highly attenuated, but attenuation was strain dependent (Alarcon et al., 2009, Hazlett et al., 1991, and Zolghahar et al., 2003). A recent report also highlighted an association between greater production of fimbriae, which are pili-like adhesion appendages, and increased severity of *Pseudomonas* corneal ulcers (Hammond et al., 2016), so it is

reasonable to hypothesize that pili/fimbriae may be involved in bacterial ocular virulence. Since the first report of the presence pili in *Corynebacterium* (Yanagawa et al., 1968) and the subsequent dissection of pathways involved in pili assembly in *Corynebacterium* (Ton-That and Schneewind, 2003) and other Gram-positive pathogens (Budzik et al., 2008, Dramsi et al., 2006, Falker et al., 2008, Kline et al., 2009, Lazzarin et al., 2015, LeMieux et al., 2008, Mazmanian et al., 2001, Okura et al., 2011, and Ton-That and Schneewind, 2004), including *B. cereus* (Budzik et al., 2007, 2008, and 2009a), the importance of pili to bacterial virulence and its potential as a vaccine target has become more appreciated.

Adhesins, including pili and fimbriae, are found on the surface of a number of Gram-positive ocular pathogens, including *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Enterococcus faecalis*, and *B. cereus*. *S. aureus* adhesins contribute to virulence in a number of infection models (Heilmann 2011), but the presence or importance of pili for this pathogen in the eye has not been determined. *S. pneumoniae* possesses at least two types of pili which promote *in vivo* virulence and interact with the host immune response (Bagnoli et al., 2008, Barrochi et al., 2006, and Nelson et al., 2007), but a role for pili in *S. pneumoniae* eye infections has not been ascribed. Similarly, *E. faecalis* pili have not been investigated in the context of eye infections, but their role in the pathogenicity of endocarditis and urinary tract infections and the formation of biofilms has been reported (Nallapareddy et al., 2006, Pinkston et al., 2014, and Sillanpaa et al., 2013). To our knowledge, this is the first analysis of the role of *B. cereus* pili in mammalian infection, including infection of the eye.

Our results demonstrated that during experimental endophthalmitis, *B. cereus* with pili grew to a greater intraocular burden than *B. cereus* without pili. Although these strains replicated at similar rates *in vitro*, the intraocular growth of pili-deficient *B. cereus* was significantly less compared to that of wild type *B. cereus* from 3-12 h postinfection. These results suggested that the growth of pili-deficient *B. cereus* in the eye may have been better controlled during the course of infection. Experimental endophthalmitis initiated by *B. cereus* deficient in hemolysin BL (Callegan et al., 1999), phosphatidylinositol-specific phospholipase C (Callegan et al., 2002), phosphatidylcholine-specific phospholipase C (Callegan et al., 2002), quorum sensing (Callegan et al., 2003), or swarming (Callegan et al., 2006) were not controlled to the same degree as observed with pili-deficient *B. cereus*. Studies reported that the presence of pili enhanced the uptake of pneumococci and lactobacilli by macrophages (Orrskog et al., 2012 and Vargas Garcia et al., 2015). If this were the case in our model, one would expect the bacterial burden to be greater in eyes infected with pili-deficient *B. cereus*. However, uptake and killing of Group B streptococci by macrophages was reported to occur regardless of the presence or absence of pili (Papaserghi et al., 2011), suggesting that pili-macrophage interactions may be species-dependent. Macrophages and monocytes comprised <5% of cells infiltrating into the eye during *B. cereus* endophthalmitis (Ramadan et al., 2006), so the interactions of these cells with *B. cereus* would likely not impact bacterial burden.

Polymorphonuclear neutrophils (PMN) are the primary infiltrating cell type recruited to the site of infection during *B. cereus* endophthalmitis (Ramadan et al., 2006). The majority of studies on pili and neutrophil function have involved Gram-negative bacteria such as *Neisseria*, *E. coli*, and *Moraxella*. Pili-deficient mutants of *Neisseria gonorrhea* were killed

more readily by PMN than piliated strains (McNeil and Virgi, 1997 and Stohl et al., 2013). Also, the interaction of this organism directly with the uropods of PMN was pili-dependent (Soderholm et al., 2011). In experimental endophthalmitis, the pili-deficient *B. cereus* burden was lower than that of the pili-containing *B. cereus* burden, suggesting that the latter strain was better controlled by the intraocular immune response. Conversely, *Moraxella*-containing pili were cleared from a mouse otitis media model more readily than pili-deficient strains (Kawano et al., 2013). It has also been suggested that pili-deficient bacteria escape phagocytosis because they are less adherent. This apparently did not occur in our endophthalmitis model, as pili-containing *B. cereus* grew better in the eye than pili-deficient *B. cereus*, suggesting that any neutrophil interaction with pili on the surface of *B. cereus* may have had little effect on clearance or that pili protected *B. cereus* from interaction with neutrophils.

We reported that TLR2 and TLR4 are important in the severe intraocular inflammation observed during *B. cereus* endophthalmitis (Novosad et al., 2012 and Parkunan et al., 2015). TLR2 is also important in mediating inflammation in *Staphylococcus aureus* endophthalmitis (Kochan et al., 2012 and Talreja et al., 2015). We do not know whether the pili of *Bacillus* interact with these innate immune receptors and are, in fact, TLR ligands. Bassett *et al.* (2013) reported that inflammatory responses to the RgrA pneumococcal pilus type 1 protein were mediated by TLR2. Fimbriae have also been reported to activate TLR2 in the context of *Porphyromonas gingivalis* and *Salmonella enterica* virulence (Hajishengallis et al., 2009, Tukel et al., 2005, and Wang et al., 2007). Further studies showed that the initial inflammatory response to *P. gingivalis* in macrophages required cooperative signaling of TLR2 and TLR4 (Davey et al., 2008 and Papadopoulos et al., 2013). Reports on the activation of TLR4 by adhesins have primarily involved the FimH adhesin of type 1 fimbriae in Gram-negative bacteria (Abdul-Careem et al., 2011, Fischer et al., 2006, Hedlund et al., 2001, and Mossman et al., 2008). Whether *B. cereus* pili serve as a TLR ligand is an open question.

Our results demonstrated that the absence of pili resulted in a lower bacterial burden and a trend of less retinal function loss in eyes infected with the pili-deficient mutant. Retinal function loss in *B. cereus* endophthalmitis is, in part, directly proportional to the number of organisms in the eye in wild type mice (Ramadan et al., 2006), so a lower intraocular bacterial burden should have resulted in a greater degree of retained retinal function. Logically, local toxin production would be decreased in an environment with a lower bacterial burden. However, it is only when *B. cereus* are quorum sensing-deficient (Callegan et al., 2003 and Callegan et al., 2005) or motility-deficient (Callegan et al., 2005 and Callegan et al., 2006) or mice are immunodeficient (Novosad et al., 2012, Parkunan et al., 2015, and Parkunan et al., 2016) that we have noted stark contrasts in infection severity with equivalent bacterial numbers in control and experimental groups. Taken together, these results highlight the sensitivity of the eye to *B. cereus* burden and the importance of infection control during its earliest stages.

Tsilia *et al.* (2015) analyzed the adhesion of pathogenic *B. cereus* to synthetic intestinal mucin, and suggested that adhesion may contribute to virulence by providing close contact to nutrient sources. While the authors reported that extensive attachment to mucin was not

required for virulence, adhesion may provide a stable surface for growth and disruption of the protective mucin layer. Although the role of pili in this scenario was not examined, in the posterior segment, pili or fimbriae of any organism could effectively adhere to intraocular tissues or surfaces of intraocular lenses or suture material, making these infections more difficult to treat. This is an interesting concept to consider in the context of non-motile piliated Gram-positive ocular pathogens such as *S. aureus*, streptococci, or *Enterococcus* (Bagnoli et al., 2008, Barocchi et al., 2006, Heilmann 2011, Nallapareddy et al., 2006, Nelson et al., 2007, Pinkston et al., 2014, and Sillanpaa et al., 2013). Indeed, Jett *et al.* (1998) reported that the localization of *E. faecalis* to distinct tissues in the eyes of rabbits was likely due to adhesins other than aggregation substance, but this study did not examine pili. For *B. cereus*, migration throughout the eye occurs within hours of infection (Callegan et al., 1999), suggesting that adhesion may not be an important phenotype in the early stages of this disease.

5. Conclusions

Our findings suggested a potential role for pili in protecting *B. cereus* from early intraocular clearance, potentially contributing to its virulence during the early stages of endophthalmitis. The growth of nonpiliated *B. cereus* may have been better controlled during the early stages of intraocular infection, leading to lower numbers of *B. cereus* and greater retinal function in infected eyes. These data underscore the importance of early infection control. Left untreated, however, infections with piliated and nonpiliated *B. cereus* resulted in significant vision loss, suggesting that other factors are involved in virulence to a more significant degree. The pathogenicity of *B. cereus* endophthalmitis is multifactorial, and because of its explosively blinding course, the identification of bacterial and host factors which do and do not contribute to disease pathogenesis is critical in formulating plausible strategies for preventing blindness.

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Highlights

- *Bacillus cereus* endophthalmitis is a potentially blinding intraocular infection.
- The hypothesis that *B. cereus* pili contributed to intraocular virulence was tested.
- Intraocular growth of pilus-deficient *B. cereus* was better controlled.
- Results suggest a role for pili in protecting *B. cereus* from intraocular clearance.

Figure 1. *In vitro* growth of piliated and nonpiliated *B. cereus*

(A) *B. cereus* ATCC 14579 or its isogenic pilus-deficient mutant (Δ Pil) were cultured in BHI for 18 h at 37°C, then subcultured to an approximate concentration of 10³ CFU/mL in fresh BHI. Aliquots were removed every two hours to quantify bacteria. Numbers of ATCC 14579 and Δ Pil were statistically similar at each time point tested ($P > 0.05$). Both strains reached stationary phase in BHI at approximately 8 h postinfection. Values represent the mean $\pm$ SD for N=3 samples per time point. (B) *B. cereus* ATCC 14579 or its isogenic pilus-deficient mutant (Δ bcpA-srtD-bcpB) were spotted onto TSA + 5% sheep blood agar and incubated for 18 h at 37°C. Spreading colonies of *B. cereus* were observed at each inoculation point and colonies had the characteristic double zone of hemolysis. On blood agar, these strains were phenotypically indistinguishable. (C) Transmission electron microscopy (TEM) of ATCC 14579 (WT) and Δ Pil. Pili are denoted by white arrows, while black arrows denote flagella

or flagellar bundles. Pili were noted surrounding ATCC 14579, but not Pil. Magnification, 50,000X.

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Figure 2. Intraocular growth of piliated and nonpiliated *B. cereus*

Eyes of C57BL/6J mice were infected with 100 CFU of *B. cereus* ATCC 14579 (WT) or its isogenic pilus-deficient mutant ($\Delta\Pil$). Eyes were harvested and *B. cereus* were quantified. Greater numbers of WT *B. cereus* were recovered from infected eyes than $\Delta\Pil$ *B. cereus* at all postinfection time points (* P 0.03). Values represent mean $\pm$ SEM of $N = 6$ eyes at each time point.

Figure 3. Retinal function of eyes infected with pilated or nonpilated *B. cereus*

Eyes of C57BL/6J mice were infected with 100 CFU of *B. cereus* ATCC 14579 (WT) or its isogenic pilus-deficient mutant (Δ Pil). Retinal function was assessed by electroretinography at 6, 9, and 12 h postinfection. **(A)** In general, retinal function declined in all infected eyes by 12 h postinfection. Although there was an observed trend of higher retained A-wave and B-wave function in eyes infected with Δ Pil compared to that of eyes infected with WT at each time point, these differences were not statistically significant ($P = 0.29$). Values represent mean $\pm$ SEM of $N = 6$ eyes each time point. **(B)** Representative waveforms of eyes infected with WT and Δ Pil *B. cereus* and uninfected contralateral eyes at 12 h postinfection.

Figure 4. Retinal damage and intraocular inflammation in eyes infected with pilated or nonpilated *B. cereus*

Eyes of C57BL/6J mice were infected with 100 CFU of *B. cereus* ATCC 14579 (WT) or its isogenic pilus-deficient mutant (Δ Pil). Infected globes were harvested at 3, 6, 9, and 12 h postinfection and processed for hematoxylin and eosin staining. Uninfected C57BL/6J eyes had no inflammation and were architecturally and morphologically similar. At 3 h postinfection, eyes infected with each strain had fibrin infiltrate in the anterior chamber and posterior segments. Corneas were clear, retinas were intact, and retinal layers were distinguishable at this time. *B. cereus* were noted in the vitreous of eyes infected with both strains. At 6 and 9 h postinfection, significant fibrin and infiltrating cells were visible in the anterior segments of eyes infected with each strain. *B. cereus* were noted in the mid-vitreous, as well as along the posterior capsule and near the ciliary body in eyes infected with both strains. Retinal layers in these infected eyes remained intact. At 9 h postinfection, *B. cereus* were also noted in the anterior chamber of eyes infected with either strain. By 12 h postinfection, all infected eyes had severe inflammation, anterior and posterior segment infiltration, and fully detached retinas. *B. cereus* were noted in the same areas as described

for 9 h postinfection. The gross pathological changes developing in eyes infected with each strain were indistinguishable from one another. Sections are representative of three eyes per time point. *B. cereus* are denoted by white arrows in the higher magnification images in 4B. In 4B: Ret, retina; Vit, vitreous; AqH, aqueous humor; Ir, iris; L, lens. Magnification in A, 10×. Magnification in B, 40×.