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Original article

Adhesion and cytotoxicity of *Bacillus cereus* and *Bacillus thuringiensis* to epithelial cells are FlhA and PlcR dependent, respectively

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Abstract

Some bacteria of the *Bacillus cereus* group are enteropathogens. The first cells encountered by bacteria following oral contamination of the host are epithelial cells. We studied the capacity of these bacteria to adhere to epithelial cells and the consequences of this interaction. We found that cell adhesion is strain dependent and that a strain mutated in *flhA*, which encodes a component of flagellum-apparatus formation, is impaired in adhesion, suggesting that flagella are important virulence factors. The bacteria are cytotoxic to epithelial cells and induce substantial cytoplasmic and membrane alterations. However, direct contact between cells and bacteria is not required for cytotoxicity. The determinants of this cytotoxicity are secreted and their expression depends on the pleiotropic regulator PlcR. Adhesion and cytotoxicity of *B. cereus* to epithelial cells might explain the diarrhea caused by these pathogens. Our findings provide further insight into the pathogenicity of *B. cereus* group members.

Keywords: *Bacillus cereus*; Adhesion; Cytotoxicity; Virulence factors; FlhA; PlcR

1. Introduction

The *Bacillus cereus* group comprises several species of sporulating bacteria, which are found ubiquitously. The species *Bacillus anthracis*, *Bacillus thuringiensis* and *B. cereus* are pathogens [1]. *B. anthracis* and *B. thuringiensis* produce specific toxins conferring the ability to colonize hosts as diverse as insects and mammals [2,3]. The genes encoding the insecticidal toxins of *B. thuringiensis* and the *B. anthracis* toxins have been extensively studied and are now well characterized. They are plasmid-borne and might have been transferred between species by conjugation. In contrast, the genetic determinants of other aspects of the infectious process (i.e. for adhesion, invasion and colonization) might be common to the three species and located on the chromosome [4]. However, no general and non species-specific virulence factors have been identified. Symptoms associated with *B. cereus* gastroenteritis and opportunistic infections (endophthalmitis, periodontitis, pneumonia) might be the consequence of the production of various extracellular factors including enterotoxins Hbl and Nhe, and cytotoxin CytK, although no direct link has been demonstrated [5]. The genes encoding these potential virulence factors belong to the PlcR regulon. Their transcription is activated at the onset of the stationary phase of growth by a quorum-sensing mechanism [6,7]. Deletion of *plcR* from *B. cereus* and *B. thuringiensis* reduces, but does not abolish, the virulence of the bacteria in insects, in mice and during endophthalmitis in rabbits [8,9]. Moreover, *B. anthracis* does not express *plcR*, and addition of a functional copy does not increase virulence of the bacteria in a murine model [10]. Thus, the PlcR-regulated genes are necessary but not sufficient to account for the pathogenicity of *B. cereus* in mammals, suggesting that other factors are required for pathogenicity. It has consistently been shown that flagella are important in the early stages of infection [11,12]: they confer motility, and work with a strain mutated in *flhA,* which encodes a component of flagellum-apparatus formation, suggests that they are essential bacterial virulence factors [13].

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Adhesion of bacteria to eukaryotic cells is often the first key event during infection of susceptible hosts. Microorganisms of the genus *Bacillus* are known to interact with epithelial cells [14–16], but the bacterial factors responsible for adhesion have not been identified. The role of the adhesion of *B. cereus* to epithelial cells in virulence is unknown but it may prevent elimination of the bacteria by the cleansing mechanisms of the intestine. Adhesion and cytotoxicity to epithelial cells might be important steps, although not necessarily related, as diarrhea presumably occurs through the destruction of epithelial cells by bacterial toxins produced in the small intestine [17]. Although PlcR may contribute to cytotoxicity, no detailed quantitative assays have been done [8]. Therefore, we studied adhesion of *B. cereus* and *B. thuringiensis* to epithelial cells, and invasion, to determine whether they contribute to virulence. Strains were also tested for cytotoxicity to epithelial cells, and bacterial factors involved in adhesion and cytotoxicity to epithelial cells were identified.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The acrystalliferous *B. thuringiensis* strain 407 Cry^- (Bt 407), the sequenced *B. cereus* strains ATCC 10987 and ATCC 14579, and the *B. cereus* diarrheic strains Bc D1, Bc D17 and Bc D23 were used [18–21]. The mutant strains Bt 407 Δ*plcR*, Bt 407 Δ*inhA2*, Bt 407 Δ*plcA*, Bt 407 Δ*flhA* and the complemented strain Bt 407 Δ*flhA*/*flhA* have been described previously [8,13,22,23]. The *B. subtilis* strain 168 and *Escherichia coli* TG1 were used as controls.

Bt 407 Δ*hblC*, Bt 407 Δ*cytK* and Bt 407 Δ*tlo* were constructed in the laboratory. The *cytK* and *hblC* genes were disrupted, through homologous recombination with pRN5101, a derivative of the thermo-sensitive plasmid $pE194^{ts}$ [24]. DNA fragments corresponding to internal regions of the *cytK* and *hblC* genes (290 and 284 bp, respectively) were generated from the Bt 407 chromosome by PCR using the primer pairs CytB (5′-CGGGATCCGAGCGCTGTTATCTTGAAGGTT-3′), CytH (5′-GCCGAAGCTTTCGGGCAAAATGCAAAAACAC-3′), and HblB (5′-CGGGATCCGTGGCAACTGCGCAAGGCATA-3′), HblH (5′-GCCGAAGCTTCTCAGCTTCTAGAATAGAGTT-3′). These DNA fragments were inserted between the *Bam*HI and *Hin*dIII sites of pRN5101, and the resulting plasmids were introduced into Bt 407 by electroporation. Transformants resistant to erythromycin were grown at 30 °C and transferred to non-permissive temperature (39 °C) for about 10 generations. The bacterial cells were then plated onto LB agar plates supplemented with erythromycin and incubated for 24 h at 39 °C. Integration of the recombinant plasmid was confirmed by PCR using primers mapping at the ends of pRN5101 and primers external to the insertion site. The insertion mutant strains were designated Bt 407 Δ*hblC* and Bt 407 Δ*cytK*.

The *tlo* gene encoding thuringiolysin 0 (a cholesterol-binding cytolysin) was disrupted as follows. *Hin*dIII–*Xba*I (450 bp) and *Pst*I–*Bam*HI (453 bp) DNA fragments were generated by PCR using Bt 407 chromosomal DNA as the template and primer pairs Tlo7 (5′-CCCAAGCTTATTAGTATAGACTTACCTGGC-3′), Tlo8 (5′-CCCTCTAGAATAAACCGTTCTACCATAAGCTAC-3′), and Tlo9 (5′-CCCGAATTCTTTACTGCTGTCGTATTAGGTGGA-3′), Tlo10 (5′-CCCGGATCCCCATTCCCATGCAAGACCTGTACA-3′), respectively. A Km^R cassette carrying the *aphA3* gene was purified from pDG783 (laboratory stock) as a 1.5-kb *Xba*I–*Pst*I fragment. The amplified DNA fragments were digested with the appropriate enzymes and inserted between the *Hin*dIII and *Bam*HI sites of pRN5101. The resulting plasmid was introduced into Bt 407 by electroporation, and the *tlo* gene was deleted by a double crossover event as described previously [25]. Chromosomal allele exchange was confirmed by PCR with oligonucleotide primers located upstream from Tlo7 and downstream from Tlo10.

All strains were grown in LB medium at 37 °C under agitation until the OD_{600nm} reached 2. The growth curves of the various strains were indistinguishable. Bacteria were harvested by centrifugation for 10 min at 3500 rpm. Pellets were washed two times with phosphate-buffered saline (PBS) and resuspended in PBS at 10^8 bacteria/ml. The culture supernatant was filtered through a 0.2-μm pore-size filter.

2.2. Cell cultures

HeLa and CaCo2 epithelial cells were maintained in Dulbeco's modified Eagle's minimum essential medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Invitrogen). Cells were incubated at 37 °C under a 5% CO_2 atmosphere and saturating humidity. Cells were detached using 0.02% trypsin, counted with a hematocytometer and seeded into multiwell disposable trays containing DMEM + FBS at a density of 2×10^5 cells per well. HeLa cells in the trays were infected the day after seeding, and CaCo2 cells 15–20 days after confluence was reached.

2.3. Cell infection assays

Cells were covered with fresh DMEM and infected with bacterial suspension at a multiplicity of infection (m.o.i.) of 10 or 50 for 30 min. Non-attached bacteria were removed by carefully washing with PBS. Infected cells were fixed in 3.7% paraformaldehyde (PFA) overnight at 4 °C and then washed in PBS. The cells were stained using eosin, or fluorescently immunostained (green) using FITC-phalloidin (Sigma) to stain the actin. Bacteria were either fluorescently labeled (red) before infection using 5-carboxytetramethylrhodamine succinimidyl ester (5-TAMRA, SE) (Molecular Probes) for 20 min at room temperature according to the manufacturer's instructions or visualized by Gram staining. At least 100 cells in four different experiments were observed under the microscope and the percentage of cells in contact with at least one bacterium was scored as the percentage of infected cells.