The *plcR* regulon is involved in the opportunistic properties of *Bacillus thuringiensis* and *Bacillus cereus* in mice and insects

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***Bacillus thuringiensis* has been widely used for 40 years as a safe biopesticide for controlling agricultural pests and mosquitoes because it produces insecticidal crystal proteins. However, spores have also been shown to contribute to overall entomopathogenicity. Here, the opportunistic properties of acrySTALLIFEROUS *B. thuringiensis* Cry[−] and *Bacillus cereus* strains were investigated in an insect species, *Galleria mellonella*, and in a mammal, BALB/c mice. In both animal models, the pathogenicity of the two bacterial species was similar. Mutant strains were constructed in which the *plcR* gene, encoding a pleiotropic regulator of extracellular factors, was disrupted. In larvae, co-ingestion of 10⁶ spores of the parental strain with a sublethal concentration of Cry1C toxin caused 70% mortality whereas only 7% mortality was recorded if spores of the Δ *plcR* mutant strain were used. In mice, nasal instillation of 10⁸ spores of the parental strain caused 100% mortality whereas instillation with the same number of Δ *plcR* strain spores caused much lower or no mortality. Similar effects were obtained if vegetative cells were used instead of spores. The cause of death is unknown and is unlikely to be due to actual growth of the bacteria in mice. The lesions caused by *B. thuringiensis* supernatant in infected mice suggested that haemolytic toxins were involved. The cytolytic properties of strains of *B. thuringiensis* and *B. cereus*, using sheep, horse and human erythrocytes and *G. mellonella* haemocytes, were therefore investigated. The level of cytolytic activity is highly reduced in Δ *plcR* strains. Together, the results indicate that the pathogenicity of *B. thuringiensis* strain 407 and *B. cereus* strain ATCC 14579 is controlled by *PlcR*.**

Keywords: *Bacillus*, cytolysin, haemolysin, insect pathogen, *plcR* regulon

INTRODUCTION

Bacillus thuringiensis and *Bacillus cereus* are spore-forming Gram-positive bacteria commonly found in soil and many other sources. In the light of genomic data, *B. thuringiensis* can be seen as a *B. cereus* that produces crystals (Carlson & Kolstø, 1993; Helgason *et al.*, 2000); the key characteristic of *B. thuringiensis* is the production of insecticidal crystalline inclusions during the stationary phase (Schnepf *et al.*, 1998). Generally, the Cry toxins that constitute the crystal inclusion are sufficient to kill infected insect larvae. When ingested by susceptible insect larvae, these crystal proteins are

dissolved and activated in the insect gut. They bind to specific receptors located on the midgut epithelial cells, forming transmembrane pores and causing cell lysis. This specific toxæmia may lead, by itself, to the death of the insect larvae. However, in some cases, *B. thuringiensis* and *B. cereus* spores have been shown to be involved in pathogenicity in insects (Dubois & Dean, 1995; Johnson & McGaughey, 1996; Li *et al.*, 1987). It has been suggested that the toxæmic effect of the crystal proteins creates conditions favourable for the development of the bacteria in the gut of weakened insect larvae (Schnepf *et al.*, 1998). The bacteria can then invade the haemocoel and cause septicaemia. *B. cereus*,

although commonly regarded as a relatively non-pathogenic opportunist associated with diarrhoeal food poisoning, has been increasingly isolated from serious and sometimes fatal cases including non-gastrointestinal infections such as endocarditis, wound infections, pneumonia and endophthalmitis (Beecher *et al.*, 1995a; Drobniewski, 1993; Miller *et al.*, 1997).

B. thuringiensis and *B. cereus* cells produce several extracellular degradative enzymes such as phospholipase C, enterotoxins and haemolysins, which are putative virulence factors (Beecher *et al.*, 1995a; Drobniewski, 1993). A transcriptional activator that positively regulates the expression of phospholipase C genes, during the late vegetative growth of *B. thuringiensis* cells, has been identified and named PlcR (Lereclus *et al.*, 1996). This regulator governs the expression of a large regulon encoding extracellular proteins including degradative enzymes, cell-surface proteins and enterotoxins (Agaisse *et al.*, 1999). Hence, PlcR appears to be a pleiotropic regulator of genes encoding extracellular factors potentially involved in pathogenicity. In this paper, we report experiments with two animal models (a lepidopteran insect, *Galleria mellonella*, and a mammal, BALB/c mice) to evaluate the opportunistic properties of *B. thuringiensis* and *B. cereus*. In both models, the pathogenicity of *B. thuringiensis* and *B. cereus* was similar and was highly reduced or abolished by the disruption of the *plcR* gene.

METHODS

Bacterial strains and culture conditions. *Escherichia coli* K-12 strain TG1 (Gibson, 1984) was used as intermediary host for the cloning experiments. The *B. thuringiensis* 407 Cry⁻ strain used throughout this study is an acrySTALLIFEROUS mutant strain belonging to the *thuringiensis* subspecies (serotype 1) (Lereclus *et al.*, 1989). The *B. cereus* strain was the reference strain ATCC 14579. For insect bioassays, spores of the various *Bacillus* species were obtained by culturing cells in HCT medium (Lecadet *et al.*, 1980) at 30 °C for 4 d. Spores were harvested by centrifugation (10410 g, 15 min) and washed twice with sterile distilled water (2 × 100 ml). The spore pellets were resuspended in 10 ml sterile distilled water. Spore preparations were heated for 20 min at 80 °C.

For nasal instillation in mice, the vegetative cells were prepared from cultures in Tryptic Soy Broth (Difco) at 37 °C. The cells were recovered after culture for 18 h (late stationary phase) by centrifugation at 7000 g for 15 min. The pellets were washed and suspended in PBS (phosphate-buffered saline, pH 7.2; Sigma). Spore suspensions used for nasal instillation were prepared from a 10-d-old culture on agar medium containing 10 g yeast extract l⁻¹, 5 g NaCl l⁻¹ and 20 g agar l⁻¹. Spores were washed and suspended in sterile water, and incubated for 1 h at 65 °C to kill the vegetative forms.

Spores and vegetative cells were counted before bioassays by plating serial dilutions on LB agar plates. Spore preparations could be stored at 4 °C for 10 d; vegetative cells were prepared for each experiment.

The *B. thuringiensis* 407 Cry⁻ *Δspo0A* mutant strain has been described elsewhere (Lereclus *et al.*, 1995). This strain is an asporogenic acrySTALLIFEROUS mutant. pHTF3-1C, carrying the *cry1C* gene fused to the *cry3A* promoter region, was intro-

duced into this strain, resulting in the production of Cry1C crystals in a Spo⁻ background (Sanchis *et al.*, 1996). The resulting 407 Cry⁻ [*Δspo0A*, pHTF3-1C] strain was designated 407 Cry⁻ 0A-1C. Cry1C toxins were prepared from the 407 Cry⁻ 0A-1C strain grown in LB medium at 30 °C for 4 d. The culture was centrifuged at 10410 g for 20 min and the pellet was washed twice in sterile distilled water (2 × 100 ml). It was resuspended in 10 ml sterile distilled water. The Cry1C crystal preparation was briefly sonicated before use. Crystal protein was determined with the Bio-Rad protein assay procedure (Bradford), using bovine serum albumin as the standard.

For evaluation of the cytolytic activity of the culture supernatants, *B. thuringiensis* strains were grown at 30 °C in LB medium for the tests with insect haemocytes or in Tryptic Soy Broth (Difco) for the tests with human erythrocytes. The culture supernatants were recovered 2 h after the end of the exponential-growth phase. Cells were pelleted by centrifugation (10410 g for 10 min) and the supernatants were sterilized by filtration using filters with 0.22 µm pores (Sartorius).

In vivo experiments. *G. mellonella* eggs were hatched at 30 °C and the larvae reared on beeswax and pollen (Naturalim). Last instar larvae (150–350 mg) were force-fed using 0.5 × 25 mm needles (Burkard Manufacturing) and a microinjector (Automatic Microapplicator; Burkard Manufacturing) with spore/crystal suspensions in sterile water (10 µl per larva). Thirty larvae were used for each dose. Infection of *G. mellonella* larvae (last instar) by intrahaemocoelic injection was performed as follows. Groups of 30 larvae were injected at the base of last proleg with 10 µl spore preparation using the Burkard microinjector with a 1 ml hypodermic syringe and 0.45 × 12 mm needles (Terumo). After force-feeding and injection experiments, the larvae were kept individually in boxes containing beeswax and pollen at 25 °C. They were checked daily and casualties were recorded over 7 d. For injection experiments, mortality data were analysed using the log-probit program of Raymond *et al.* (1993). This program tests the linearity of dose-mortality curves, provides lethal doses (LD₅₀) and the slope of each dose-mortality curve. Mortality curves were considered identical when their parallelism was not rejected at the 0.05 level and the 95% confidence limits of the susceptibility ratio included the value 1. To count *B. thuringiensis* cells in the living and dead insects, 10 larvae were crushed and homogenized in 10 ml sterile water; dilutions were plated onto LB agar plates containing appropriate antibiotics. The parental strain, *B. thuringiensis* 407 Cry⁻, is resistant to oxacillin (10 µg ml⁻¹) and the mutant strain, *B. thuringiensis* 407 Cry⁻ *ΔplcR*, is resistant to oxacillin and kanamycin (250 µg ml⁻¹).

We used 5-week-old female BALB/c mice (Charles River) kept in a biosafety containment facility in groups of five, with sterile water and food. For each strain, groups of 5–10 mice were infected intranasally under slight ether anaesthesia with 50 µl of the appropriate suspension. The spore or bacterial suspension was carefully deposited at the corner of the nostril. The mouse inhaled the inoculum naturally by breathing, so there was therefore no mechanical traumatism.

Construction of mutant strains. To obtain the *ΔplcR* strains, the B1 insert cloned in pHT304 (Lereclus *et al.*, 1996) was inserted (*Sma*I/*Hind*III) into the pKS vector. The resulting plasmid isolated from *E. coli* transformants was designated pKSΩB1. The Km^R cassette of pDG783, conferring resistance to kanamycin (Km, 250 µg ml⁻¹), was isolated as a 1.5 kb *Eco*RI fragment carrying the *aphA3* gene from *Enterococcus faecalis* (Trieu-Cuot & Courvalin, 1983). This Km^R cassette

was then inserted into pKSΩB1, between the internal *EcoRI* sites of *plcR*, yielding pKSΩB1Km^R. The disrupted *plcR* gene was then inserted between the *Bam*HI and *Hind*III sites of the thermosensitive plasmid pRN5101, conferring resistance to erythromycin (Em, 5 µg ml⁻¹). The resulting plasmid, pRNB1Km^R, was introduced into *B. thuringiensis* and *B. cereus* by electroporation (Lereclus *et al.*, 1989) and the chromosomal wild-type copy of *plcR* was replaced by the disrupted copy as previously described (Lereclus *et al.*, 1995). Mutant colonies had an Em^S, Km^R phenotype. The genotype was checked by PCR.

Degradative enzyme activity measurements. Columbia medium agar plates (BioMérieux) containing either 5% horse blood or 5% sheep blood, and 5% human blood agar plates (Sanofi Pasteur) were used to evaluate the haemolytic activity of the *B. thuringiensis* and *B. cereus* strains. Tests for cytotoxic activity were performed as previously described (Ribeiro *et al.*, 1999). Briefly, chilled 2-d-old last-instar larvae of *G. mellonella* were surface sterilized before collection of haemolymph in anticoagulant buffer (62 mM NaCl, 100 mM glucose, 10 mM EDTA, 30 mM trisodium citrate, 26 mM citric acid, pH 4.5). The haemolymph was centrifuged and the haemocyte pellet was rinsed and resuspended in PBS. Haemocyte suspension (20 µl) was layered on heat-sterilized (220 °C, 2 h) coverslips and haemocytes were allowed to spread and to adhere to the glass for 15 min in a wet chamber at room temperature. The monolayers were rinsed, 20 µl of the solution under study was added and the monolayers were incubated for 1 h at 23 °C for microscopy and counting of dead cells. Haemocyte mortality was checked by adding 2 µl Trypan Blue dye (0.4% in PBS) and incubating for a further 5 min.

RESULTS AND DISCUSSION

Pathogenicity of *B. thuringiensis* and *B. cereus* in insects

To assess the pathogenicity of *B. thuringiensis* and *B. cereus* against insects, spores were either fed to the larvae in association with the insecticidal toxin Cry1C or injected alone into the haemocoel. The lepidopteran species *G. mellonella* is a useful model because the larvae are susceptible to the ingestion of *B. thuringiensis* spore/crystal mixtures but only weakly susceptible to the ingestion of crystals alone (Li *et al.*, 1987). To prevent the cross-contamination of crystals with spores, we used engineered *B. thuringiensis* strains that produce either Cry1C crystals and no spores (strain 407 0A-1C), or spores but no crystals (strain 407 Cry⁻).

A clear pattern of synergism was observed in analysis of the force-feeding assays (Fig. 1). Very low mortality levels (<10%) were obtained with crystals or spores alone. In contrast, mixing *B. thuringiensis* spores and crystals strongly increased mortality, demonstrating synergism (70% mortality). Killing the spores by autoclaving before force-feeding them to *G. mellonella* abolished synergism (not shown), suggesting that either heat-labile toxic components are associated with the spores or the spores germinate. As co-ingested crystals and vegetative cells of *B. thuringiensis* were also synergistic (47% mortality, not shown), the spores are almost certainly living and able to develop into veg-

Fig. 1. Synergism of spores and crystals as an evaluation of pathogenicity in *G. mellonella* larvae. Cry1C crystals (1 µg per larva) were added in all assays except when *B. thuringiensis* spores alone were used as a control. Cry1C indicates Cry1C crystals without spores (control); Spores Bt, 10⁶ 407 Cry⁻ spores without crystals per larva (control). Bt, Bc, Bs and Bm indicate the results obtained when 10⁶ spores of *B. thuringiensis* 407 Cry⁻, *B. cereus*, *B. subtilis* and *B. megaterium* were used, respectively. Bt ΔplcR and Bc ΔplcR indicate the results obtained with 10⁶ spores of the *B. thuringiensis* 407 Cry⁻ ΔplcR and *B. cereus* ΔplcR mutant strains, respectively. All experiments were repeated twice, except those with the *B. cereus* ΔplcR strain (one assay). Bars indicate standard error.

etative cells. This is consistent with the increase in bacterial count observed on collection of the contents of the haemocoel from living larvae when, due to transit elimination, a decrease in spore count was expected (Fig. 2a).

We investigated whether synergism with Cry1C crystals could be achieved with any *Bacillus* species using a fixed dose of spores (Fig. 1). There was a significant difference in the synergism with *Bacillus megaterium* and *Bacillus subtilis* spores (Fisher's test; $P < 0.0001$). In contrast, the level of synergism with *B. cereus* spores was of the same order as that obtained with *B. thuringiensis*. This is consistent with the taxonomic identity of *B. thuringiensis* and *B. cereus* (Carlson & Kolstø, 1993; Helgason *et al.*, 2000). The two species have extensive genome identity, including a complete copy of the *plcR* gene (Agaisse *et al.*, 1999; Økstad *et al.*, 1999).

We assessed the extent to which the PlcR-regulated genes are involved in pathogenicity by evaluating the entomopathogenic properties of *B. thuringiensis* 407 Cry⁻ ΔplcR and *B. cereus* ΔplcR mutant strains in *in vivo* experiments. Spores from these two mutant strains did not have a synergistic effect against the *G. mellonella* larvae (Fig. 1): the lethality was similar to that of Cry1C crystals alone. On agar plates, the germination rate of the 407 Cry⁻ ΔplcR strain did not appear to differ from that of the parental strain, thus excluding a major defect

Fig. 2. Dynamics of infection in *G. mellonella* larvae. Cry1C crystals ($1 \mu\text{g}$ per larva) were added in all assays. (a) Mono-infection: 2×10^6 407 Cry⁻ spores (Bt) or 2×10^6 407 Cry⁻ $\Delta plcR$ spores (Bt $\Delta plcR$) were fed to the larvae. (b) Co-infection: 10^6 spores 407 Cry⁻ strain and 10^6 spores 407 Cry⁻ $\Delta plcR$ were fed to the larvae. *B. thuringiensis* cells were counted at various times after infection (24, 48 or 72 h) from living and dead larvae. Where standard error (bars) is not indicated, the results were obtained from the mean of a single pool of 10 larvae. Black bars, Bt wild-type; hatched bars, Bt $\Delta plcR$.

in germination. This is consistent with the co-infection experiments (see below and Fig. 2a, b), showing that the $\Delta plcR$ mutants are able to germinate in insect larvae.

The pathogenicity of the *B. thuringiensis* parental strain, 407 Cry⁻, and of the 407 Cry⁻ $\Delta plcR$ mutant was also assessed by introducing the spores into the larval haemocoel by injection. The mortality curves for the parental and mutant strains were not significantly different (parallelism not rejected: $\chi^2 = 0.68$, d.f. = 5, $P > 0.98$; the ratio of the LD₅₀ included the value 1). The LD₅₀ (and the confidence intervals), determined 2 d after injection of the spores, were 1.7×10^4 (8.4×10^3 – 3.7×10^4) for the parental strain, 407 Cry⁻, and 1.8×10^4 (8.5×10^3 – 4.1×10^4) for the 407 Cry⁻ $\Delta plcR$ mutant strain. In addition, mortality kinetics were similar for the two *B. thuringiensis* strains: in both cases, the larvae began to die 16 h after infection and mortality reached a

plateau 2 d later. These results indicate that the *plcR* regulon is not required for pathogenicity if the spores are mechanically introduced into the haemocoel. Hence, PlcR appears to play a key role in the regulation of the synergistic properties of *B. thuringiensis* (and *B. cereus*) if spores enter the larvae via the digestive tract, suggesting a role in the early stages of infection (for example, by allowing the cells to gain access to the haemocoel from the gut).

The role of the *plcR* regulon in the entomopathogenicity of *B. thuringiensis*

PlcR-regulated genes encode proteins containing sequences similar to secretory signal peptides (Agaisse *et al.*, 1999; Økstad *et al.*, 1999). Thus, the 407 Cry⁻ $\Delta plcR$ strain may be less able to cause opportunistic infections due to its inability to produce one or several of these secreted factors. Thus, the parental strain may provide the mutant with extracellular factors, enabling it to multiply in co-infected larvae. To test this hypothesis, we first compared the fate of the *B. thuringiensis* parental strain, 407 Cry⁻, and of the 407 Cry⁻ $\Delta plcR$ mutant strain in *G. mellonella* larvae, following mono-infection experiments with spore/crystal preparations (Fig. 2a). At 24 h after infection, the two strains performed similarly with bacterial counts of about 9×10^6 c.f.u. larva⁻¹ and 10^7 c.f.u. larva⁻¹, respectively. Two days later, substantial multiplication (10^9 c.f.u. larva⁻¹) had occurred in dead larvae infected with the parental strain. No dead larvae were obtained from the insects infected with the $\Delta plcR$ mutant. After 72 h, 2.4×10^6 c.f.u. larva⁻¹ were isolated from living larvae infected with the parental strain and 3.7×10^5 c.f.u. larva⁻¹ were isolated from larvae infected with 407 Cry⁻ $\Delta plcR$ spores. This suggests that bacterial multiplication essentially occurs in the dead larvae. Thus, both the wild-type and mutant cells are slowly eliminated from larvae surviving the infection (compare bacterial counts from living larvae at 24 h and 72 h, in Fig. 2a).

We next assessed the multiplication of the parental and mutant strains following co-infection with spore/crystal preparations. Larvae were fed with a 50% initial ratio of the two types of spores (Fig. 2b). At 24 h after ingestion, 5.1×10^6 of the recovered c.f.u. were of the 407 Cry⁻ $\Delta plcR$ type and 1.5×10^6 were of the 407 Cry⁻ type. The ratio (77% versus 23%) is significantly different from the expected 50% ratio (Student's *t*-test; $P < 0.01$). This may indicate that the $\Delta plcR$ mutant has a selective advantage during the first few hours of infection. The ratio significantly decreased over time (one-way ANOVA; $P = 0.0073$) and similar amounts of both strains were recovered from dead larvae at 48 h and from living larvae at 72 h. These results show that the parental *B. thuringiensis* 407 Cry⁻ strain may provide extracellular factors enabling the $\Delta plcR$ mutant to multiply in the infected larvae. Thus, the results for mono- and co-infections suggest two possible roles for PlcR: (1) the *plcR* regulon may be required to cause the death of the larvae so that the bacterium can multiply, or, conversely, (2) the *plcR* regulon may be required to create favourable

Fig. 3. Cytolytic activity of culture supernatants on *G. mellonella* haemocytes. (a) Sterile LB (control). Note the shape of plasmatocytes, which look like fibroblasts. The refractive rounded cells are granular haemocytes I. (b) *B. subtilis* supernatant. (c) 407 Cry⁻ supernatant. Most haemocytes are dead, swollen cells stained with Trypan Blue (dark nucleus). The arrow indicates a live granular haemocyte I. Plasmatocytes have lost their fibroblastic shape. (d) 407 Cry⁻ Δ plcR supernatant. Dead cells are very few in number (arrow). The haemocytes of both types show numerous small rounded vacuoles (compare with a and b).

conditions for bacterial multiplication, resulting in the death of the larvae by septicaemia.

Cytotoxic and haemolytic activities are controlled by PlcR

Previous studies have indicated a possible correlation between phospholipase activity and the entomopathogenic properties of *B. thuringiensis* (Heimpel, 1955; Krieg, 1971; Stephens, 1952). Two phosphatidylcholine esterases are known to be encoded by PlcR-regulated genes (Agaisse *et al.*, 1999). They are phospholipase C and sphingomyelinase, which together form cereolysin AB (Gilmore *et al.*, 1989). We checked that the 407 Cry⁻ Δ plcR strain did not form a halo on lecithin-agar plates, consistent with the genetic data (results not shown). In addition, the gene encoding the haemolytic HblC component of the Hbl enterotoxin of *B. thuringiensis* and *B. cereus* is also regulated by PlcR (Agaisse *et al.*, 1999; Økstad *et al.*, 1999). As HblC is enterotoxigenic and haemolytic (Lindbäck *et al.*, 1999), and phospholipases C are known to degrade the phospholipid constituents of cell membranes, we investigated the activity of culture supernatants from the 407 Cry⁻ parental strain and from the 407 Cry⁻ Δ plcR mutant strain against *G. mellonella* haemocytes.

Four haemocyte types are present in the haemolymph of *G. mellonella* larvae. Two of these cell types (Fig. 3a and 3b), plasmatocytes (Pl) and granular haemocytes I

(GH1) (Brehélin & Zachary, 1986), are directly involved in defence reactions such as phagocytosis. They account for more than 80% of the total haemocyte population. The supernatant of the parental 407 Cry⁻ strain was strongly cytolytic to Pl and GH1 (Fig. 3c). Both cell types lost their shape and were stained with Trypan Blue, indicating cell death on a large scale. The supernatant of the 407 Cry⁻ Δ plcR mutant strain had a different activity: cells were clearly not killed but extensive vacuole formation was observed (Fig. 3d). There was a highly significant difference in the number of dead cells between these two haemocyte preparations (Student's *t*-test; $P < 10^{-17}$). Indeed, 99.0% of the cells were dead after incubation with supernatant from the parental strain, 407 Cry⁻, whereas only 5.3% of cells were dead after treatment with a supernatant from the 407 Cry⁻ Δ plcR mutant. The vacuole formation observed with the supernatant of the 407 Cry⁻ Δ plcR mutant was not dependent on PlcR-regulated functions and may have been due to other compounds, such as the *B. cereus* emetic toxins, which are known to cause vacuole formation in eukaryotic cells (Agata *et al.*, 1995). In contrast, sterile LB and the supernatant of a *B. subtilis* strain, used as controls, had no cytolytic effect on *G. mellonella* haemocytes (Fig. 3a and 3b).

The effect of *plcR* disruption was also tested on agar plates containing sheep, human or horse erythrocytes. *B. thuringiensis* 407 Cry⁻ and *B. cereus* grown on sheep-blood agar produced intense haloes of degradative

Table 1. Opportunistic properties of *B. thuringiensis* and *B. cereus* in mice

Percentage mouse mortality (number of dead mice/number of infected mice) at 24 h after nasal instillation with spores or vegetative cells of wild-type and $\Delta plcR$ *Bacillus* strains. NT, not tested.

Dose	<i>B. thuringiensis</i> 407 Cry ⁻	<i>B. thuringiensis</i> 407 Cry ⁻ $\Delta plcR$	<i>B. cereus</i> ATCC 14579	<i>B. cereus</i> ATCC 14579 $\Delta plcR$	<i>B. anthracis</i> Sterne
Spores per mouse					
10 ⁸	100 (20/20)	5 (1/20)	100 (10/10)	100 (10/10)	0 (0/10)
5 × 10 ⁷	31 (11/35)	0 (0/10)	90 (9/10)	22 (2/9)	0 (0/10)
10 ⁷	0 (0/25)	0 (0/5)	90 (9/10)	0 (0/10)	0 (0/5)
Vegetative cells per mouse					
10 ⁸	100 (10/10)	0 (0/10)	NT	NT	NT
2 × 10 ⁷	100 (5/5)	NT	NT	NT	NT
6 × 10 ⁶	NT	NT	100 (6/6)	0 (0/5)	0 (0/5)

activity (not shown). Similar results were obtained with colonies grown on human-blood agar and if culture supernatants, harvested 2 h after the end of the exponential-growth phase, were loaded on human-blood agar (not shown). On sheep-blood agar, a complex ring pattern was visible after a 36 h incubation. In contrast, colonies grown on horse-blood agar gave a simple faint halo. Therefore, various haemolytic components with different host specificities are probably involved in the formation of this pattern. The disruption of *plcR* markedly decreased activity on sheep- and human-blood agar plates for both the 407 Cry⁻ and *B. cereus* strains, but activity was not completely abolished for *B. cereus* (not shown). No such effect was observed with horse-blood agar plates. It has been shown that the HblC component of *B. cereus* is partly responsible for the haemolytic activity against sheep erythrocytes, but not against human erythrocytes (Callegan *et al.*, 1999; Lindbäck *et al.*, 1999). Thus, the specific activity of *B. thuringiensis* and *B. cereus* against human erythrocytes is probably due to the cereolysin AB component, which is known to cause the haemolysis of human erythrocytes (Gilmore *et al.*, 1989).

The opportunistic properties of *B. thuringiensis* and *B. cereus* in mice

Hernandez *et al.* (1998, 1999) recently showed that some *B. thuringiensis* strains are pathogenic to mice by nasal instillation. We first determined whether the *B. thuringiensis* 407 Cry⁻ strain (subspecies *thuringiensis*) and *B. cereus* ATCC 14579 belonged to this group. Nasal instillation of 10⁸ or 5 × 10⁷ spores of the 407 Cry⁻ strain and of the *B. cereus* strain per mouse rapidly resulted in high mortality rates (Table 1). Mice died within 24 h, with syndromes similar to those described for the *B. thuringiensis* strain *konkukian* (Hernandez *et al.*, 1999). However, instillation of 10⁷ spores per mouse in the same volume (50 µl) resulted in no deaths with 407 Cry⁻ spores and a high mortality rate with the *B. cereus* strain, which suggests that the two strains differ in

pathogenicity. Similar assays were performed with the *Bacillus anthracis* vaccine strain, Sterne, which lacks the virulence plasmid, pXO2. *B. anthracis* is a member of the *B. cereus* group, in which *plcR* is present in a truncated inactive form (Agaïsse *et al.*, 1999). *B. anthracis* Sterne, instilled at the same concentration, did not kill mice (Table 1). This indicates the existence in *B. thuringiensis* and *B. cereus* of specific opportunistic traits, not functional in a pXO2⁻ strain of *B. anthracis* (i.e. the *plcR* regulon). *B. thuringiensis* vegetative forms instilled in mice at a dose of 10⁸ or 2 × 10⁷ cells per mouse caused 100% mortality within 4–6 h, with haemorrhagic symptoms (nose bleeding and lungs clearly haemorrhagic on autopsy).

The properties of the 407 Cry⁻ $\Delta plcR$ and *B. cereus* $\Delta plcR$ strains were also assessed. Instillation of 10⁸ or 5 × 10⁷ spores or vegetative cells per mouse of the 407 Cry⁻ $\Delta plcR$ mutant strain caused few or no deaths. The *B. cereus* $\Delta plcR$ strain killed mice, but was less lethal than the parental strain (Table 1). This suggests that *B. cereus* strain ATCC 14579 possesses additional factors, not regulated by PlcR, which may potentiate its opportunistic properties.

Conclusions

These results show that both *B. thuringiensis* and *B. cereus* possess opportunistic properties that may lead to rapid death of the host animals if large doses of vegetative or sporulated cells are used. The cause of death is unknown, and, in mice, it is unlikely to be due to the growth of the bacteria. However, as previously described (Hernandez *et al.*, 1999), the syndromes observed in infected mice suggest that haemolysin production may be involved in death. These opportunistic properties have been lost (or highly reduced) in the $\Delta plcR$ mutants of the *B. thuringiensis* and *B. cereus* strains used in this study, and they are also absent from a *B. anthracis* vaccine strain known to be defective in PlcR activity (Agaïsse *et al.*, 1999). These results suggest that pathogenesis in mice and insects requires common

extracellular factors that depend on the *plcR* regulon. Thus, PlcR-regulated genes, such as those encoding the enterotoxins Hbl (Beecher *et al.*, 1995b) and Nhe (Granum *et al.*, 1999; Lund & Granum, 1997), the phospholipase PI-PLC (Kuppe *et al.*, 1989; Lechner *et al.*, 1989) and the cereolysin AB component (Gilmore *et al.*, 1989), may be responsible for pathogenicity. These four genes were found in the *B. thuringiensis* 407 and *B. cereus* ATCC 14579 strains used in this study (Agaisse *et al.*, 1999; Økstad *et al.*, 1999).

Here we show that in specific infection conditions, mammals may act as alternative hosts for insect pathogens. This is reminiscent of the multi-host system of the opportunistic bacterium *Pseudomonas aeruginosa* (Finlay, 1999; Jander *et al.*, 2000; Rahme *et al.*, 1997; Tan *et al.*, 1999). Similarly, the *B. thuringiensis*/insect pathogenesis model can be used to determine the factors involved in the opportunistic properties of bacilli.

In practical terms, these results clearly show that *B. thuringiensis* and *B. cereus* are opportunistic pathogens. As with *B. cereus*, *B. thuringiensis* strains produce various food poisoning toxins (Granum & Lund, 1997) and a *B. thuringiensis* strain has been found associated with an outbreak of food poisoning (Jackson *et al.*, 1995). However, by comparison with chemical pesticides, *B. thuringiensis* is generally recognized as a safe product and there is no evidence to suggest that its use for insect control should be reduced. Nevertheless, more detailed knowledge of the pathogenicity of each commercial strain may be required to improve the safety of *B. thuringiensis*-based biopesticides. It is therefore important to identify the PlcR-regulated functions specifically responsible for the opportunistic properties of *B. thuringiensis* and *B. cereus*.

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