The $\textit{plcR}$ regulon is involved in the opportunistic properties of $\textit{Bacillus thuringiensis}$ and $\textit{Bacillus cereus}$ in mice and insects

Sylvie Salamitou, François Ramisse, Michel Brehélin, Denis Bourguet, Nathalie Gilois, Myriam Gominet, Eric Hernandez, and Didier Lereclus

$\textit{Bacillus thuringiensis}$ 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 $\textit{B. thuringiensis}$ $\text{Cry}^-$ and $\textit{Bacillus cereus}$ strains were investigated in an insect species, $\textit{Galleria mellonella}$, and in a mammal, $\textit{BALB/c}$ mice. In both animal models, the pathogenicity of the two bacterial species was similar. Mutant strains were constructed in which the $\textit{plcR}$ gene, encoding a pleiotropic regulator of extracellular factors, was disrupted. In larvae, co-infection of $10^8$ 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 $\Delta\textit{plcR}$ mutant strain were used. In mice, nasal instillation of $10^8$ spores of the parental strain caused 100% mortality whereas instillation with the same number of $\Delta\textit{plcR}$ 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 $\textit{B. thuringiensis}$ supernatant in infected mice suggested that haemolytic toxins were involved. The cytolytic properties of strains of $\textit{B. thuringiensis}$ and $\textit{B. cereus}$, using sheep, horse and human erythrocytes and $\textit{G. mellonella}$ haemocytes, were therefore investigated. The level of cytolytic activity is highly reduced in $\Delta\textit{plcR}$ strains. Together, the results indicate that the pathogenicity of $\textit{B. thuringiensis}$ strain 407 and $\textit{B. cereus}$ strain ATCC 14579 is controlled by PlcR.

Keywords: $\textit{Bacillus}$, cytolysin, haemolysin, insect pathogen, $\textit{plcR}$ regulon

INTRODUCTION

$\textit{Bacillus thuringiensis}$ and $\textit{Bacillus cereus}$ are spore-forming Gram-positive bacteria commonly found in soil and many other sources. In the light of genomic data, $\textit{B. thuringiensis}$ can be seen as a $\textit{B. cereus}$ that produces crystals (Carlson & Kolsto, 1993; Helgason et al., 2000); the key characteristic of $\textit{B. thuringiensis}$ is the production of insecticidal crystaline 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 toxemia may lead, by itself, to the death of the insect larvae. However, in some cases, $\textit{B. thuringiensis}$ and $\textit{B. cereus}$ 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 toxemic 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. $\textit{B. cereus}$,
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 phospholipases C, enterotoxins and haemolysins, which are putative virulence factors (Beecher et al., 1995a; Drobniewski, 1993). A transcriptional activator that could be stored at 4°C Spores and vegetative cells were counted before bioassays by monia and endophthalmitis (Beecher et al., 1989). The *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 (10,410 g for 10 min) and the supernatants were sterilized by filtration using filters with 0.22 µm pores (Sartorius).

**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 acrycystalliferous mutant strain belonging to the *thuringiensis* subspecies (serotype 1) (Lereclus 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.

**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 (Smal/HindIII) into the pKS vector. The resulting plasmid isolated from *E. coli* transformants was designated pKS0B1. The KmR cassette of pDG783, conferring resistance to kanamycin (Km, 250 µg ml–1), was isolated as a 1.5 kb EcoRI fragment carrying the aphA3 gene from *Enterococcus faecalis* (Trieu-Cuot & Courvalin, 1983). This KmR cassette was introduced into this strain, resulting in the production of Cry1C crystals in a Spo+ background (Sanchis et al., 1996). The resulting 407 Cry–Aphp0A, pHT3-1C 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 10,410 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.
PlcR controls pathogenicity

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 OA-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 vegetative 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 species (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. 1. Synergism of spores and crystals as an evaluation of pathogenicity in G. mellonella larvae. Cyr1C crystals (1 µg per larva) were added in all assays except when B. thuringiensis spores alone were used as a control. Cyr1C 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.
The pathogenicity of the
Black bars, Bt wild-type; hatched bars, Bt

times after infection (24, 48 or 72 h) from living and dead
B. thuringiensis
to the larvae.

Mean c.f.u. per larva (log scale)

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Fig. 2. Dynamics of infection in G. mellonella larvae. Cry1C
crystals (1 µg per larva) were added in all assays. (a) Mono-
infection: 2 × 10^6 407 Cry− spores (Bt) or 2 × 10^6 407 Cry− ΔplcR
spores (Bt ΔplcR) were fed to the larva. (b) Co-infection: 10^6
spores 407 Cry− strain and 10^6 spores 407 Cry− ΔplcR 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 ΔplcR.

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

The pathogenicity of the B. thuringiensis parental strain,
407 Cry−, and of the 407 Cry− ΔplcR 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: χ^2 = 0.68, d.f. = 5,
P > 0.98; the ratio of the LD_{50} included the value 1). The
LD_{50} (and the confidence intervals), determined 2 d after
injection of the spores, were 1.7 × 10^3 (8.4 × 10^2–
3.7 × 10^4) for the parental strain, 407 Cry−, and 1.8 × 10^4
(8.5 × 10^3–4.1 × 10^5) for the 407 Cry− ΔplcR 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; Ökstad et al., 1999). Thus, the 407 Cry− ΔplcR
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− ΔplcR mutant
strain in G. mellonella larvae, following mono-infestation
experiments with spore/crystal preparations (Fig. 2a).
At 24 h after infection, the two strains performed
similarly with bacterial counts of about 9 × 10^3 c.f.u.
larva−1 and 10^4 c.f.u. larva−1, respectively. Two days
later, substantial multiplication (10^6 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 ΔplcR mutant. After 72 h, 2 × 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− ΔplcR 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−
ΔplcR 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 ΔplcR 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 ΔplcR 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 bacterial can multiply, or, conversely,
(2) the plcR regulon may be required to create favourable

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PlcR controls pathogenicity

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− ∆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 septicemia.

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− ∆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− ∆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) (Brehelin & 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− ∆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− ∆plcR mutant. The vacuole formation observed with the supernatant of the 407 Cry− ∆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
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 (Agaisse 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 pX02− 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− ΔplcR and B. cereus ΔplcR strains were also assessed. Instillation of 10⁸ or 5 × 10⁷ spores or vegetative cells per mouse of the 407 Cry− ΔplcR mutant strain caused few or no deaths. The B. cereus ΔplcR 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 ΔplcR 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 (Agaisse et al., 1999). These results suggest that pathogenesis in mice and insects requires common

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

<table>
<thead>
<tr>
<th>Dose</th>
<th>B. thuringiensis 407 Cry−</th>
<th>B. thuringiensis 407 Cry− ΔplcR</th>
<th>B. cereus ATCC 14579</th>
<th>B. cereus ATCC 14579 ΔplcR</th>
<th>B. anthracis Sterne</th>
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<td>Spores per mouse</td>
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<td>10⁸</td>
<td>100 (20/20)</td>
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<tr>
<td>5 × 10⁷</td>
<td>31 (11/35)</td>
<td>0 (0/10)</td>
<td>90 (9/10)</td>
<td>22 (2/9)</td>
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<tr>
<td>10⁷</td>
<td>0 (0/25)</td>
<td>0 (0/5)</td>
<td>90 (9/10)</td>
<td>0 (0/10)</td>
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<td>Vegetative cells per mouse</td>
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<td>10⁸</td>
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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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