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A quadruple-enterotoxin-deficient mutant of *Bacillus thuringiensis* remains insecticidal

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Bacillus thuringiensis is the leading biopesticide used to control insect pests worldwide. Although they have a long record of safe use, under certain conditions commercial strains of *B. thuringiensis* have the ability to produce numerous putative enterotoxins that have been associated with food poisoning attributed to *Bacillus cereus*. Therefore, we designed a strategy to delete the genes encoding these toxins. *B. thuringiensis* strain VBTS 2477 contained genes encoding NHE, CytK-2 and three homologues of haemolysin BL (HBL, HBL_{a1} and HBL_{a2}). This is the first report, to our knowledge, of a strain of *B. cereus* or *B. thuringiensis* containing three sets of *hbl* operons. The genes encoding HBL_{a1} and HBL_{a2} were 96–97 % identical to each other and 76–84 % identical to those encoding HBL. The hbl_{a2} operon was detected by PCR amplification only after hbl_{a1} was deleted. We used sequential gene replacement to replace the wild-type copies of the NHE and three HBL operons with copies that contained internal deletions that span the three genes in each operon. The insecticidal activity of the quadruple-enterotoxin-deficient mutant was similar to that of the wild-type strain against larvae of *Trichoplusia ni*, *Spodoptera exigua* and *Plutella xylostella*. This demonstrates that the genes for enterotoxins can be deleted, eliminating the possibility of enterotoxin production without compromising the insecticidal efficacy of a strain of *B. thuringiensis*.

INTRODUCTION

Bacillus thuringiensis has been used globally for over 50 years as a bioinsecticide for the control of insect pests in agriculture, forestry and public health. *B. thuringiensis* produces one or more crystal proteins during sporulation that are toxic to insect larvae upon ingestion. The toxins exhibit a narrow host spectrum, with an individual toxin typically affecting a subset of species within an insect order (Lepidoptera, Diptera, Coleoptera). Numerous studies show *B. thuringiensis* to be harmless to humans and mammals (Fisher & Rosner, 1959; US Environmental Protection Agency, 1998) and it has become increasingly popular as a method of insect control that is more environmentally friendly than synthetic chemical insecticides.

B. thuringiensis is a Gram-positive spore-forming bacterium that is a member of the *Bacillus cereus* group. Phylogenetic studies and whole-genome sequencing reveal that *B. thuringiensis* and *B. cereus* are members of the same species group (Carlson *et al.*, 1994; Helgason *et al.*, 2000; Hill *et al.*, 2004; Rasko *et al.*, 2005). Both *B. thuringiensis* and *B. cereus* are found in soils, in the guts of invertebrates and associated with plants (Smith & Couche, 1991; Margulis *et al.*, 1998; Martin & Travers, 1989; Jensen *et al.*, 2003; Jara *et al.*, 2006; Swiecicka & Mahillon, 2006; Bizzarri & Bishop, 2007), and the only distinguishing phenotype is the production in *B. thuringiensis* of plasmid-encoded crystal toxins upon sporulation. Some strains of *B. cereus* are known to be food-poisoning agents and opportunistic pathogens (Kotiranta *et al.*, 2000). Pathogenic strains of *B. cereus* are responsible for two foodborne illnesses: an emetic syndrome, caused by a small cyclic peptide cereulide (Agata *et al.*, 1995), and a diarrhoeal syndrome, caused by one or more putative enterotoxins (Beecher & MacMillan, 1991; Lund & Granum, 1996; Granum *et al.*, 1999; Lund *et al.*, 2000).

Many commercial strains of *B. thuringiensis* carry the genes encoding putative diarrhoeal enterotoxins. This has generated

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Abbreviations: BCET-RPLA, *Bacillus cereus* enterotoxin-reverse passive latex agglutination; BDE, *Bacillus* diarrhoeal enterotoxin; LC_{50}, 50 % lethal concentration; SOE, gene splicing by overlap extension.

The GenBank/EMBL/DDBJ accession numbers for the near-full-length enterotoxin operon sequences of *B. thuringiensis* are EU925141–EU925144.

A supplementary table, listing the primers used for detection of enterotoxin genes and generation of deletion constructs by SOEing PCR, is available with the online version of this paper.

debate in the scientific community about whether *B. thuringiensis* on produce could cause food poisoning (Damgaard, 1995; Hansen & Hendriksen, 2001; Frederiksen *et al.*, 2006, Rosenquist *et al.*, 2005; Kyei-Poku *et al.*, 2007). Early toxicology studies of *B. thuringiensis* included tests on human volunteers who ingested 1 g (3×10^9 *B. thuringiensis* spores) of the commercial product Thuricide daily for 5 days without any adverse health effects (Fisher and Rosner, 1959). More recently, greenhouse workers exposed to *B. thuringiensis*-based sprays were found to carry 10^2–10^3 c.f.u. *B. thuringiensis* g^{-1} in faecal samples but the presence of *B. thuringiensis* did not correlate with any gastrointestinal symptoms (Jensen *et al.*, 2002), suggesting that it was unlikely that *B. thuringiensis* exposure would be implicated in human illness. However, food poisoning outbreaks caused by a *B. thuringiensis* strain could be mistakenly attributed to *B. cereus*, since clinical laboratories have not routinely examined putative *B. cereus* isolates for the presence of crystal toxins. Moreover, heat treatment of food can result in plasmid loss, and therefore a *B. thuringiensis* strain that caused disease would no longer carry *B. thuringiensis*-specific genes at the time of diagnosis (Granum, 2002; European Food Safety Authority, 2005). A recent report re-examined 155 *B. cereus*-group-like species collected from 39 foodborne outbreaks in British Columbia, Canada, between 1991 and 2005 (McIntyre *et al.*, 2008). Of these *B. thuringiensis* isolates, 23 were identified as the only *Bacillus* sp. associated with four of the outbreaks for which *B. cereus* was originally reported. Due to the concern about food safety, some scientists have advocated deleting the genes for the enterotoxins from commercial strains (Damgaard, 1995; Gaviria Rivera *et al.*, 2000). Others have suggested that the use of *B. thuringiensis* is unlikely to pose a public health risk but that removal of the enterotoxin genes would be a prudent precaution that would allay public concerns (Kyei-Poku *et al.*, 2007).

In this study, we examined a strain of *B. thuringiensis* for the presence of genes encoding three enterotoxins that have been implicated in diarrhoeal illness: three-component enterotoxin complexes haemolysin BL (HBL) and NHE and cytotoxin K (CytK) (Beecher & MacMillan, 1991; Lund & Granum, 1996; Lund *et al.*, 2000; reviewed by Stenfors Arnesen *et al.*, 2008). HBL has haemolytic, vascular permeability and dermonecrotic activities, is cytotoxic and causes fluid accumulation in the rabbit ileal loop (RIL) assay (Beecher & MacMillan, 1991; Beecher & Wong, 1994b; Beecher *et al.*, 1995; Lund & Granum, 1997), considered the definitive assay for diarrhoeal toxins (Bergdoll, 1988). The enterotoxigenicity of NHE and CytK are inferred from their cytotoxicity toward different cell lines, including human intestinal Caco-2 cells, and their ability to form pores in planar lipid bilayers (Hardy *et al.*, 2001; Lindbäck *et al.*, 2004; Fagerlund *et al.*, 2008).

HBL and NHE are each comprised of three proteins encoded by genes that are arranged in operons (Heinrichs *et al.*, 1993; Ryan *et al.*, 1997; Granum *et al.*, 1999). The respective HBL and NHE proteins share 23–40 % sequence identity and they may be part of a family of pore-forming toxins (Fagerlund *et al.*, 2008). A homologue of HBL, HBL_a, was isolated from a strain of *B. cereus* that produces two distinct sets of HBL proteins (Beecher & Wong, 2000). The HBL_a set exhibited biological activity similar to the prototypic HBL set, and the homologous proteins functioned interchangeably, suggesting that HBL_a may also contribute to food poisoning.

Cytotoxin K is a single-protein enterotoxin that was originally isolated from a strain of *B. cereus* that caused a food poisoning outbreak that resulted in three fatalities (Lund *et al.*, 2000). Two versions have been characterized: the original version, CytK-1, which appears to be limited to a genetically remote cluster of *B. cereus* strains (Fagerlund *et al.*, 2007) and a second version, CytK-2, which exhibits lower toxicity toward human intestinal epithelial cells (Fagerlund *et al.*, 2004). It is not known whether CytK-2 contributes to the diarrhoeal illness.

In this study we screened a strain of *B. thuringiensis* subsp. *kurstaki* for the presence of genes that encode HBL, HBL_a, NHE and CytK. We performed a series of gene replacements, in which we replaced four enterotoxin operons with copies that contain large deletions, and assessed the activity of the quadruple mutant in insect bioassays.

METHODS

Bacterial strains, plasmids and growth conditions. The strains and plasmids used in the present study are listed in Table 1. *Escherichia coli* was grown in Luria–Bertani (LB) medium at 37 °C. *B. thuringiensis* was grown in either LB or half-strength tryptic soy broth (TSB) or on half-strength tryptic soy agar (TSA) at 28, 37 or 40.5 °C. For conjugation, *B. thuringiensis* was grown in brain heart infusion (BHI) medium. Antibiotics were used at the following concentrations: for *E. coli*, 200 μg ampicillin ml^{-1}; 10 μg chloramphenicol ml^{-1}; for *B. thuringiensis*, 3 μg erythromycin ml^{-1} for selection of pMAD or 5 μg erythromycin ml^{-1} for selection of pBKJ236; 60 μg polymixin B ml^{-1} for conjugations with pBKJ236 and10 μg tetracycline ml^{-1} for selection of pBKJ223.

DNA isolation. Genomic DNA was isolated from cultures of *B. thuringiensis* that were grown with shaking overnight. DNA was isolated either by the boiling-cell-lysis method (Raffel *et al.*, 1996) or by Protocol no. 3 in the Easy-DNA kit (Invitrogen), except that prior to the addition of solution A, the cells were pelleted, resuspended in sterile water and vortexed for 2–3 min with 50 μl 0.1 mm diameter silica beads to enhance cell lysis. Plasmid DNA was isolated from *E. coli* using the Qiagen Spin Miniprep kit (Qiagen).

Transformation and conjugation. Competent cells of *E. coli* were electroporated in 0.2 cm cuvettes with a Gene Pulser apparatus (Bio-Rad Laboratories) set at 2.5 kV, 200 Ω and 25 μF. Cells were transferred to 1 ml LB, allowed to recover for 1 h at 37 °C with shaking and then plated on selective media. Competent cells of *B. thuringiensis* were prepared as described previously (Silo-Suh *et al.*, 1994) or by the method described by Janes & Stibitz (2006). Because *B. thuringiensis* restricts methylated DNA, recombinant plasmids isolated from *E. coli* DH5α were passed through *E. coli* GM2929 (methylation-deficient strain) before being introduced into *B. thuringiensis*. pBKJ236::Δhbl_{a2} was introduced into the *B. thuringiensis* triple mutant by conjugation as described by Janes & Stibitz (2006).

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
B. thuringiensis		
VBTS 2477	*B. thuringiensis* subsp. *kurstaki* wild-type strain; *cry1Aa cry1Ab cry1Ac cry2Aa cry2Ab*	Valent Biosciences
AK7701	VBTS 2477 Δhbl_{a1}, single mutant	This study
AK7702	VBTS 2477 Δhbl_{a1} Δ*nhe*, double mutant	This study
AK7703	VBTS 2477 Δhbl_{a1} Δ*nhe* Δ*hbl*, triple mutant	This study
AK7704	VBTS 2477 Δhbl_{a1} Δ*nhe* Δ*hbl* Δhbl_{a2}, quadruple mutant	This study
E. coli		
DH5α	General purpose strain	Hanahan (1983)
GM2929	*dcm-6 dam-13*::Tn*9*, Cm^r	*E. coli* Genetic Stock Center
SS1827	Helper strain for conjugation into *B. thuringiensis*, Amp^r	Janes & Stibitz (2006)
Plasmids		
pMAD	Temperature-sensitive gene replacement vector, Ery^r, expresses β-galactosidase gene	Arnaud *et al.* (2004)
pBKJ236	Temperature-sensitive gene replacement vector, Ery^r, contains 18 bp recognition site for I-*Sce*I	Janes & Stibitz (2006)
pBKJ223	Facilitator plasmid, Tet^r, encodes I-*Sce*I	Janes & Stibitz (2006)

Screening for presence of enterotoxin genes. Gene sequences for HBL (*hblC*, *hblD*, *hblA*), NHE (*nheA*, *nheB*, *nheC*) and *cytK* were obtained from strains of *B. cereus* and *B. thuringiensis* that were available in the GenBank database at the onset of this study, and from the unpublished *B. cereus* UW85 partial genome sequence (D. Rasko, J. Ravel, J. Handelsman, unpublished data). Sequences were aligned using the DNASTAR program MEGALIGN and regions of high conservation were selected for PCR primer sequences (see Supplementary Table S1, available with the online version of this paper). Primers for *cytK* were designed to amplify either variant of the gene (*cytK-1* or *cytK-2*). The HBL_a primers were based on the UW85 genome and differed from the corresponding HBL region by 2–6 nt. Primers were synthesized at Integrated DNA Technologies. Typical PCRs contained 1 μl genomic DNA, 2 μl 10× *Taq* buffer, 0.5 μM of each primer, 0.2 mM of each dNTP, 0.2 μl *Taq* DNA polymerase (Promega) in a final volume of 20 μl. PCR cycle conditions consisted of an initial 1 min denaturation at 94 °C, followed by 35 cycles of 30 s at 94 °C, 1.5 min at 55 °C, 2 min at 72 °C, and a final extension of 5 min at 72 °C. PCR products were analysed on 0.8 % agarose gels.

Sequence analysis of enterotoxin operons in VBTS 2477. To obtain near-full-length sequences of the *hbl*, hbl_{a1} and *nhe* enterotoxin operons in VBTS 2477, primers to the ends of each operon were used to amplify each one (i.e. hblC-F/hblA-R; hblCa-F/hblAa-R, nheA-F/nheC-R), the products were purified using AMPure magnetic beads (Agencourt Bioscience), and the full sequence was obtained by primer walking. For hbl_{a2}, sequence was obtained from the PCR products generated with the following primer pairs using genomic DNA from the Δhbl_{a1} mutant: hblCa-F/hblDa-R, and hblDa-F/hblAa-R. Typical sequencing reactions consisted of 1 μl BigDye Terminator v. 3.1 mix (Applied Biosystems), 1.5 μl sequencing buffer v. 3.1 (Applied Biosystems), 0.5 μM of each primer and 5 μl template DNA in a final reaction volume of 20 μl. Cycle conditions were an initial 3 min denaturation at 95 °C, followed by 35 cycles of 10 s at 96 °C, 3.5 min at 58 °C, and a final extension of 7 min at 72 °C. Excess dye terminators were removed using the CleanSeq magnetic bead sequencing reaction clean up kit (Agencourt Bioscience). Sequencing gels were run on an Applied Biosystems 3730xl automated DNA sequencing instrument at the University of Wisconsin Biotechnology Center. Data were analysed using PE-Biosystems version 3.7 of Sequencing Analysis. Contigs were assembled using the DNASTAR software SeqMan. Nucleotide sequences of the near-full-length enterotoxin operons were deposited in GenBank under the accession numbers EU925141–EU925144.

Generation of deletion constructs by gene splicing by overlap extension (SOE)ing PCR. The deletion constructs were created by SOEing PCR (Horton *et al.*, 1989). The SOEing primers used in this study are presented in Supplementary Table S1. To generate the Δhbl_{a1} and Δhbl_{a2} constructs, the same set of outer primers was used (hblCa_Bam-F, hblAa_Bam-R) as the sequences of the two homologues in these regions were identical, but different overlapping primers were selected so that different-sized deletions was introduced. This enabled us to discriminate between the two mutations by PCR.

Typical conditions for the first round of PCRs were 1 μl genomic DNA, 5 μl 10× *Pfu* buffer, 0.5 μM of each primer, 0.4 mM dNTPs and 0.5 μl *Pfu* DNA polymerase (Stratagene) in a total volume of 50 μl. For the Δhbl_{a2} construct, the template consisted of the PCR fragments obtained with the hblCa-F/hblDa-R and hblDa-F/hblAa-R primer sets used with genomic DNA from the Δhbl_{a1} mutant. PCR cycle conditions were 30 cycles of 30 s at 94 °C, 30 s at 55 °C and 1 min at 72 °C. The PCR fragments were purified using AMPure magnetic beads (Agencourt Biosciences). Reaction conditions for the second round of PCR were the same as for the first round except the template consisted of 0.5 μl of the PCR fragments of the 5′ and 3′ regions of the operon, and *Taq* DNA polymerase (Promega) was used instead of *Pfu*. The same PCR program was used for the second round of amplification. The spliced PCR product was gel-purified using the QIAEX II gel purification kit (Qiagen).

The resulting deletion constructs were digested with *Bam*HI (Promega) and ligated to either pMAD (Δhbl_{a1}, Δ*nhe*, Δ*hbl*) or pBKJ236 (Δhbl_{a2}) that had been *Bam*HI-digested and treated with shrimp alkaline phosphatase (Promega). The recombinant vectors were confirmed by restriction digest analysis and the inserts were sequenced.

Gene replacement using pMAD or pBKJ236/pBKJ223. Gene replacement with the pMAD constructs was carried out in a manner similar to the method described by Arnaud *et al.* (2004). For construction of the first mutant (Δhbl_{a1}) of the series, pMAD::Δhbl_{a1} was electroporated into *B. thuringiensis* 2477 and transformants were selected on half-strength TSA with erythromycin (3 μg ml^{-1}) and X-Gal (50 μg ml^{-1}) after two days of incubation at 28 °C, the permissive temperature for plasmid replication. The gene replacement was conducted in two steps by first selecting for a single recombination event resulting in integration of the plasmid at the enterotoxin locus, and then screening for excision of the plasmid by a second recombination event and subsequent loss of the plasmid.

Transformants were grown on plates containing erythromycin at 40.5 °C, the nonpermissive temperature for replication of pMAD, to select for clones in which the plasmid had integrated into the chromosome via a single crossover event. Integrants were then grown at the permissive temperature in nonselective media to allow for a second crossover event, and then diluted into fresh medium and grown at the nonpermissive temperature to cure remaining freely replicating plasmid. Cultures were plated for single colonies on half-strength TSA with X-Gal at 40.5 °C and screened for white colonies, which were putative double recombinants. PCR analysis was performed on genomic DNA to determine whether the double recombinants had reverted to wild-type hbl_{a1} or had undergone a successful gene replacement and to verify the absence of the plasmid. The *nhe* and *hbl* operons were replaced with the Δ*nhe* and Δ*hbl* deletion constructs in an iterative manner to obtain the triple mutant.

We were unable to obtain the quadruple mutant using the pMAD::Δhbl_{a2} construct due to an unexpected low frequency of recombination in the integrant. Therefore, we used the pBKJ236/pBKJ223 gene replacement system (Janes & Stibitz, 2006) which enhances the frequency of the second crossover event. pBKJ236::Δhbl_{a2} was introduced into the triple mutant by conjugation, and integrants were selected on BHI with erythromycin at 37 °C, the non-permissive temperature for replication. Integration at the hbl_{a2} locus was verified by PCR analysis (1 min at 94 °C followed by 35 cycles of 30 s at 94 °C, 1.5 min at 55 °C and 2 min at 72 °C, and final extension of 5 min at 72 °C), using one primer specific to the chromosome and one to the vector (hblDa2-F, 5′-GCT GCT AAA CAA AGT TGG AAT G-3′, pBKJ236-R, 5′-CGT AAT ACG ACT CAC TAT AGG G-3′). pBKJ223 was electroporated into the integrant and selected on media containing tetracycline. A resulting transformant was grown in half-strength TSB with tetracycline overnight at 28 °C and plated for single colonies on half-strength TSA with tetracycline and incubated at 37 °C. Colonies were screened for sensitivity to erythromycin to identify putative double recombinants that had lost pBKJ236 via a second crossover event. The double recombinants were screened by PCR with hblCa_Bam-F/hblAa_Bam-R primers to identify clones that had retained the Δhbl_{a2} locus. The quadruple mutant was grown in half-strength TSB at 37 °C and single colonies were patched onto plates with and without tetracycline to identify isolates that had been cured of pBKJ223.

Commercial assays for detection of enterotoxin proteins. Two commercial immunoassay kits were used for the detection of the L_2 component of HBL and the NheA protein of NHE. Cultures of *B. thuringiensis* strain VBTS 2477, the single, double, triple and quadruple mutants were grown for 18 h in 125 ml flasks containing 12 ml BHI with 0.1 % glucose. The cultures were centrifuged and the supernatant was filter-sterilized through a 0.22 μm pore filter (Millipore). The cell-free culture supernatants were then assayed with the Oxoid *Bacillus cereus* enterotoxin reverse passive latex agglutination (BCET-RPLA) kit (Fisher Scientific) and the Tecra *Bacillus* diarrhoeal enterotoxin (BDE) visual immunoassay (VIA) (3M) according to the manufacturer's instructions, with the exception that in the Oxoid assay, four additional dilutions were included for each sample. The assays were performed on two independent sets of cultures.

Preparation of fermentation broths. Culture broths were prepared in 7.5 l fermenters under proprietary manufacturing conditions for production of *B. thuringiensis*-based pesticides. Media comprised an organic nitrogen source (such as flours, yeast extract, fish meal, etc.) and glucose with typical salts used in fermentation processes. Cultures were grown under aerobic conditions at 28 °C with agitation until sporulation was complete. The same cultures were used for both bioassays and protein analysis.

Protein analysis of *B. thuringiensis* crystal toxins. Approximately 100 mg of each fermentation broth was transferred into microfuge tubes, weighed and washed in 1 M NaCl–5 mM EDTA pH 8.0, then once in 5 mM EDTA pH 8.0, followed by centrifugation at 14 000 ***g*** after each wash. The pellets were resuspended in 1 ml 5 mM EDTA pH 8.0. Aliquots (100 μl) were transferred to fresh microfuge tubes and centrifuged as before. The pellets were resuspended in 150 μl sample buffer pH 10.0 [8 M urea, 0.05 M CAPS, 2 % (w/v) SDS, 20 mM DTT], boiled for 8 min, followed by addition of 150 μl sample buffer pH 8.8 [0.25 M Tris, 2 %(w/v) SDS, 40 % (v/v) glycerol, 20 mM DTT]. Aliquots of a DiPel technical powder (a proprietary fermentation broth of the DiPel strain, which has been concentrated and spray-dried) quantitative standard were prepared in a similar manner. After a brief centrifugation, 5 μl of each sample and standard were loaded onto an 8–16 % Tris–glycine denaturing gel (Novex; Invitrogen). The gel was electrophoresed for approximately 2 h, stained overnight with Colloidal Blue (Invitrogen), and destained to produce a clear background. The gel was scanned using a Bio-Rad GS-710 scanning densitometer. The 135 and 60 kDa bands (Cry1 and Cry2 protoxins respectively) were quantified by comparison to the DiPel standard curve using Bio-Rad Quantity One 4.2.3 software.

Insect bioassays. Bioassays were carried out using 4-day-old *Trichoplusia ni* (cabbage looper), 4-day-old *Plutella xylostella* (diamondback moth) or 2-day-old *Spodoptera exigua* (beet armyworm) from laboratory-reared colonies (Valent Biosciences Corporation's quality assurance facility). All bacterial treatments were incorporated into the diet. Two or three replications were included in each study. In each replicate, we tested seven doses of *B. thuringiensis* whole culture and an untreated control. For *T. ni* and *S. exigua*, 30 larvae were tested per dose. For *P. xylostella*, 40 larvae were tested per dose. Insects were incubated at 28 ± 2 °C for *T. ni* and *S. exigua*, and at 25 ± 2 °C for *P. xylostella* with a 12 h light/12 h dark cycle for 3 days. Larval mortality values from all of the replications were pooled and using log-probit analysis, a single regression line was used to estimate the 50 % lethal concentration (LC_{50}).

RESULTS

Detection and sequence analysis of enterotoxin genes in *B. thuringiensis* VBTS 2477

The goal of this work was to identify and delete the enterotoxin genes present in a strain of *B. thuringiensis*, and assess the efficacy of the resulting mutant as a bioinsecticide. PCR screening indicated that all 10 enterotoxin genes (*hblC*, *hblD*, *hblA*, $hblC_a$, $hblD_a$, $hblA_a$, *nheA*, *nheB*, *nheC* and *cytK*) were present in strain VBTS 2477 (data not shown). The near full-length hbl_a operon from VBTS 2477 has 98 % identity to the *B. cereus* UW85 hbl_a operon at the nucleotide level. After hbl_a was deleted, a third *hblD* homologue was revealed in VBTS 2477. Further analysis showed that this gene was part of a third *hbl* operon (Fig. 1), which exhibits higher sequence similarity to hbl_a than to *hbl*. Therefore, this third set of HBL genes was denoted as hbl_{a2}, and the hbl_a detected originally was designated hbl_{a1}. Sequence analyses of the three nearly-full-length *hbl* operons in VBTS 2477 show that the hbl_{a1} and hbl_{a2} gene sequences are 96–97 % identical and the deduced protein sequences are 97–98 % identical. The *hbl* genes are 76–84 % identical to hbl_{a1} and hbl_{a2} genes, and the deduced proteins are 68–85 % identical.

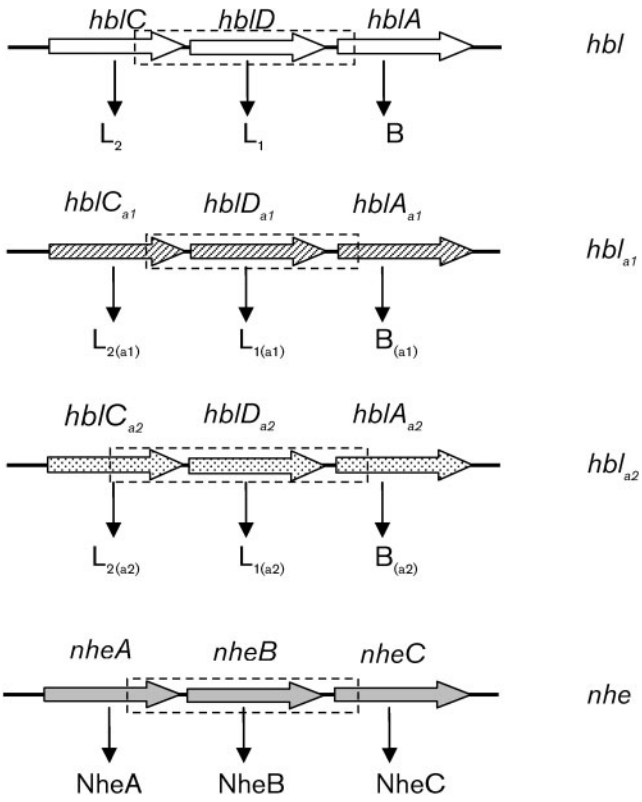

Fig. 1. *hbl* and *nhe* operons in *B. thuringiensis* VBTS 2477. The dotted rectangles indicate the deletion that was introduced into each operon. Vertical arrows indicate the protein product of the gene.

Sequence analysis of the *cytK* gene in strain VBTS 2477 revealed that it is the less toxic variant, *cytK-2* (Fagerlund *et al.*, 2004). The CytK-2 protein is 89 % identical to CytK-1 at the amino acid level and exhibits only about 20 % of the toxicity of CytK-1 toward human intestinal cells (Fagerlund *et al.*, 2004), making its role in virulence uncertain.

Deletion constructs and gene replacement

SOEing PCR was used to generate deletion constructs of *hbl*, hbl_{a1}, hbl_{a2} and *nhe* that contained a portion of the first gene spliced to a portion of the last enterotoxin gene of the operon (Supplementary Table S1). The native genes in *B. thuringiensis* VBTS 2477 were successively replaced with the deletion constructs by an iterative process in the order Δhbl_{a1}, Δnhe, Δhbl and Δhbl_{a2} (Fig. 1 and Fig. 2).

Biological activities of enterotoxin-deficient mutants

B. thuringiensis strain VBTS 2477, the single mutant (Δhbl_{a1}) and the double (Δhbl_{a1} Δnhe) mutant each exhibited a strong agglutination response (Table 2) when tested with the Oxoid BCET-RPLA kit, which detects the L_2 component of HBL (Beecher & Wong, 1994a). The triple deletion mutant, in which *hbl* is deleted, exhibited a negative phenotype, indicating that expression of the L_2 protein was abolished in this mutant. Since the hbl_{a2} operon remained intact in the triple mutant, either $L_{2(a2)}$ is not expressed in strain VBTS 2477 or it does not react with the anti-L_2 antibody in the RPLA kit. Haemolysis on sheep blood agar suggests that $L_{2(a2)}$ is expressed in VBTS 2477 since the haemolytic activity of the quadruple mutant is diminished compared with the triple mutant (data not shown). Therefore, it is likely that L_{2a} is antigenically distinct from L_2. In the Tecra BDE assay, which detects NheA, both the wild-type and the single mutant (Δhbl_{a1}) exhibited positive reactions (Table 2). The double mutant, in which *nhe* had been deleted, exhibited a negative reaction, as did the triple and quadruple mutants.

SDS-PAGE analysis indicated that VBTS 2477 and the quadruple mutant produce similar quantities of the

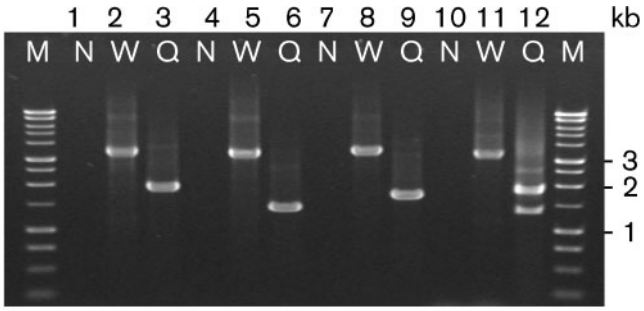

Fig. 2. PCR analysis of *B. thuringiensis* VBTS 2477 and quadruple-enterotoxin-deletion mutant. PCR primers were used to amplify: hbl_{a1} (lanes 1–3; hblCa-F/hblAa-R), *nhe* (4–6; nheA-F/nheC-R) and (7–9; hblC-F/hblA-R); hbl_{a2} (10–12; hblCa_Bam-F/hblAa_Bam-R). M, molecular mass standards (Promega 1 kb ladder); N, negative control (no DNA); W, wild-type strain; Q, quadruple mutant.

Table 2. Detection of enterotoxins by commercial immunoassays

Strain	Genotype	Oxoid RPLA*	Tecra BDE†
VBTS 2477	Wild-type	1024	4
AK7701	Δhbl_{a1}	1024	4
AK7702	Δhbl_{a1} Δnhe	1024	1
AK7703	Δhbl_{a1} Δnhe Δhbl	Negative	1
AK7704	Δhbl_{a1} Δnhe Δhbl Δhbl_{a2}	Negative	1

*RPLA assay results are reported as the reciprocal of the highest dilution (in a series of twofold dilutions) that produced a positive agglutination. Identical results were obtained in two independent experiments.

†BDE assay results are reported according to the manufacturer's instructions where scores of 3, 4 or 5 are positive and 1 or 2 are negative. Identical results were obtained in two independent experiments.

insecticidal crystal protoxins (Table 3). The wild-type and quadruple mutant had similar insecticidal activity against three lepidopteran species: cabbage looper, diamondback moth and beet armyworm (Table 4).

DISCUSSION

In this study we identified and deleted genes encoding four presumptive enterotoxins in a strain of *B. thuringiensis*. This is the first report, to our knowledge, of a strain of *B. cereus* or *B. thuringiensis* containing three homologues of HBL. This work provides genetic evidence that the enterotoxins are not required for insecticidal activity in the tested species.

We demonstrate that the enterotoxins are characterized by both conservation and heterogeneity. Heterogeneity in the HBL components was first noted by Schoeni & Wong (1999) in Western blot analyses, which led to the isolation of two distinct sets of HBL components from *B. cereus* (Beecher & Wong, 2000). Recently, sequence analysis revealed an hbl_a operon in *Bacillus weihenstephanensis* KBAB4 and *B. cereus* 03BB108, which also contains the *hbl* operon (Stenfors Arnesen *et al.*, 2008). The two homologues in strain 03BB108 have 78–82 % identity to each other (Stenfors Arnesen *et al.*, 2008), which is comparable to the similarity observed among the hbl_{a1}/hbl_{a2} and *hbl* homologues in *B. thuringiensis* strain VBTS 2477. Furthermore, the hbl_{a1} and hbl_{a2} genes in *B. thuringiensis* VBTS 2477 are 91–94 % identical to those in *B. weihenstephanensis* KBAB4 and 84–96 % identical to those in *B. cereus* 03BB108.

Many strains of *B. cereus* and *B. thuringiensis* isolated from diverse origins contain some combination of genes encoding NHE, HBL and CytK (Gaviria Rivera *et al.*, 2000; Hansen & Hendriksen, 2001; Guinebretière *et al.*, 2002; Ehling-Schulz *et al.*, 2005; Thaenthanee *et al.*, 2005; Ehling-Schulz *et al.*, 2006; Moravek *et al.*, 2006; Swiecicka *et al.*, 2006; Ngamwongsatit *et al.*, 2008). The *nhe* operon is nearly universal among *B. cereus* and *B. thuringiensis* strains, whereas the *hbl* operon and *cytK* gene are less common. However, past studies may underestimate the prevalence of *hbl* homologues because the genes are detected most often by PCR and the primers used would probably not amplify the hbl_a variant. Furthermore, the Oxoid RPLA immunoassay did not detect the L_2 component of HBL_{a2} in *B. thuringiensis* strain VBTS 2477, although it is probably expressed; haemolysis activity of the triple mutant, in which hbl_{a2} is intact, is greater than in the quadruple mutant in which it is deleted. These data suggest that the L_{2a} proteins are antigenically distinct from the prototypic L_2 protein. Therefore, the Oxoid kit may be specific for the prototypic L_2 protein only. In our work, we found hbl_a present in two strains of *B. cereus* and six strains of *B. thuringiensis*, including VBTS 2477 (data not shown), suggesting that hbl_a is a common variant of the *hbl* family.

The broad distribution and presence of multiple copies and variants of HBL in *B. cereus* and *B. thuringiensis* strains suggests that they play an ecological role in the life of the bacterium. Kyei-Poku *et al.* (2007) showed that the *nhe* operon and the *hblA* gene are expressed in spruce budworm larvae, and therefore speculated that the enterotoxins might have a role in insect pathogenicity. Salamitou *et al.* (2000) showed that a mutant of *B. thuringiensis* disrupted in the *plcR* gene, which encodes a regulator of extracellular virulence factors including the enterotoxins, exhibits reduced mortality toward *Galleria mellonella*, suggesting that genes regulated by *plcR* contribute to entomopathogenicity in this insect. However, deletion of the *nhe* and three *hbl* operons from strain VBTS 2477 did not affect its toxicity to the three lepidopteran pests we tested.

We did not encounter difficulty in introducing a deletion that spanned all three genes of the *nhe* operon in *B. thuringiensis* VBTS 2477, as has been reported by others (Ramarao & Lereclus, 2006; Fagerlund *et al.*, 2008; Stenfors Arnesen *et al.*, 2008). However, we did initially have difficulty obtaining deletions in the *hbl* and hbl_{a1} operons. We first attempted to construct these mutants by introducing the deletion constructs on a suicide vector that does not replicate in *B. thuringiensis*. The constructs integrated, but we did not find any double recombinants containing the deletions. In contrast, when we reintroduced the deletion constructs on a temperature-sensitive vector (pMAD), we obtained the *hbl* and hbl_{a1} deletion mutants without difficulty; however, hbl_{a2} presented challenges. We attempted to use pMAD for construction of the hbl_{a2} deletion mutant as well, and obtained integrants, but only found double recombinants that had reverted to wild-type at a low frequency. In contrast, we obtained the hbl_{a2} deletion with the pBKJ236/pBKJ223 system. Our work illustrates an

Table 3. Crystal toxin accumulation in culture broths from 7.5 l fermenters

Protein quantified by gel analysis software (Bio-Rad Quantity One 4.1.1) of SDS-PAGE gels stained with Colloidal Blue (Invitrogen). Values represent one replication.

Strain	Protoxin in culture broth (mg ml^{-1})	Proportion of crystal toxin as a protoxin of:	
		135 kDa (%)	60 kDa (%)
VBTS 2477	8.4	63	37
AK7704, quadruple mutant	11.6	69	31

Table 4. Insecticidal activity against lepidopteran larvae

B. thuringiensis culture broths from 7.5 l fermenters were fed to 4-day-old *T. ni*, 2-day-old *S. exigua* and 4-day-old *P. xylostella* larvae. Larval mortality was assessed after 3 days. CI, Confidence interval.

Strain	Insecticidal activity, LC_{50} [μg broth (ml diet)$^{-1}$] against:*		
	T. ni (95 % CI)	*S. exigua* (95 % CI)	*P. xylostella* (95 % CI)
VBTS 2477	168 (158–178)	653 (538–773)	11.5 (7.48–18.1)
AK7704, quadruple mutant	145 (131–160)	632 (545–730)	11.1 (9.91–12.8)

*Values represent the mean of three replicates for *T. ni*, two replicates for *S. exigua* and *P. xylostella*. For each replicate 30 larvae of *T. ni* and *S. exigua*, and 40 larvae of *P. xylostella* were tested.

unexplained dimension of the members of the *B. cereus* group, which have genetic proclivities that defy simple explanations. It is therefore fortuitous that a number of gene-replacement vectors are now available to deal with loci that are recalcitrant to recombination with one.

The potential for spores of a *B. thuringiensis* bioinsecticide to enter the human food supply and cause food poisoning has been a point of debate. The US Environmental Protection Agency states in the 1998 Reregistration Eligibility Decision on *Bacillus thuringiensis* that enterotoxin levels produced by commercial strains of *B. thuringiensis* are low compared with a clinical isolate of *B. cereus*, and because there are no reports of food poisoning caused by *B. thuringiensis*, it is unlikely to contribute to the prevalence of the diarrhoeal syndrome (US Environmental Protection Agency, 1998; http://www.epa.gov/oppsrrd1/REDs/0247.pdf). Recent studies, however, have shown that levels of enterotoxin production vary among strains of *B. cereus* and *B. thuringiensis* (Hansen & Hendriksen, 2001; Guinebretière *et al.*, 2002; Moravek *et al.*, 2006; Fagerlund *et al.*, 2007), and the pathogenic potential of a particular strain probably results from many factors, including the number of bacterial cells ingested, the number and type of enterotoxins present, the levels of enterotoxin expression, the type of food in which they are ingested, the degree of stomach acidity, the concentration of bile and the composition of the gut microbiota (Clavel *et al.*, 2004, 2007).

The levels of *B. thuringiensis* recovered from fresh produce range from fewer than 100 c.f.u. g^{-1} from cabbage products (Hendriksen & Hansen, 2006) to $>10^4$ c.f.u. g^{-1} on cucumbers and cherry tomatoes (Frederiksen *et al.*, 2006). The infective dose of *B. cereus* diarrhoeal syndrome is 10^5–10^8 total cells or spores (Stenfors Arnesen *et al.*, 2008). Frederiksen *et al.* (2006) postulated that it is therefore conceivable that a human could consume an infective dose from such produce. A recent report analysed *B. cereus*-group strains isolated from 39 food poisoning outbreaks and determined that in four of the outbreaks only *B. thuringiensis* isolates were recovered from food samples (McIntyre *et al.*, 2008), suggesting that *B. thuringiensis* can be responsible for food poisoning when *B. cereus* is implicated, because isolates are not routinely examined for the presence of crystal toxin genes or proteins (Granum, 2002; European Food Safety Authority, 2005). Although food poisoning generally, and that caused by *B. cereus* in particular, is vastly underreported, evidence suggests that if *B. thuringiensis* is a cause of food poisoning, it is probably not one of the more common causes. The CDC report 'Surveillance for Foodborne Disease Outbreaks – United States, 2006' indicates that *B. cereus* accounted for 1 % as a cause of foodborne disease outbreaks, a drop from 2 % for 2001–2005, and few of these were associated with foods derived from crops that are typically treated with *B. thuringiensis* as an insecticide (CDC, 2009).

For many years, *B. thuringiensis* bioinsecticides have been considered safe and effective despite the presence of enterotoxin genes. This work has improved upon this strain of *B. thuringiensis* subsp. *kurstaki* by removing any potential for enterotoxin production without negatively affecting the insecticidal activity of the strain. Furthermore, removal of the enterotoxin genes allows for differentiation from other *B. thuringiensis* and *B. cereus* strains.

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REFERENCES

Agata, N., Ohta, M., Mori, M. & Isobe, M. (1995). A novel dodecadepsipeptide, cereulide, is an emetic toxin of *Bacillus cereus*. *FEMS Microbiol Lett* **129**, 17–20.

Arnaud, M., Chastanet, A. & Débarbouillé, M. (2004). New vector for efficient allelic replacement in naturally nontransformable, low-GC-content, Gram-positive bacteria. *Appl Environ Microbiol* **70**, 6887–6891.

Beecher, D. J. & MacMillan, J. D. (1991). Characterization of the components of hemolysin BL from *Bacillus cereus*. *Infect Immun* **59**, 1778–1784.

Beecher, D. J. & Wong, A. C. (1994a). Identification and analysis of the antigens detected by two commercial *Bacillus cereus* diarrheal enterotoxin immunoassay kits. *Appl Environ Microbiol* **60**, 4614–4616.

Beecher, D. J. & Wong, A. C. (1994b). Improved purification and characterization of hemolysin BL, a hemolytic dermonecrotic vascular permeability factor from *Bacillus cereus*. *Infect Immun* **62**, 980–986.

Beecher, D. J. & Wong, A. C. (2000). Tripartite haemolysin BL: isolation and characterization of two distinct homologous sets of components from a single *Bacillus cereus* isolate. *Microbiology* **146**, 1371–1380.

Beecher, D. J., Pulido, J. S., Barney, N. P. & Wong, A. C. L. (1995). Extracellular virulence factors in *Bacillus cereus* endophthalmitis: methods and implication of involvement of hemolysin BL. *Infect Immun* **63**, 632–639.

Bergdoll, M. S. (1988). Ileal loop fluid accumulation test for diarrheal toxins. *Methods Enzymol* **165**, 306–323.

Bizzarri, M. F. & Bishop, A. H. (2007). Recovery of *Bacillus thuringiensis* in vegetative form from the phylloplane of clover (*Trifolium hybridum*) during a growing season. *J Invertebr Pathol* **94**, 38–47.

Carlson, C. R., Caugant, D. A. & Kolstø, A. B. (1994). Genotypic diversity among *Bacillus cereus* and *Bacillus thuringiensis* strains. *Appl Environ Microbiol* **60**, 1719–1725.

CDC (2009). Surveillance for foodborne disease outbreaks – United States, 2006. *MMWR* **58**, 609–615.

Clavel, T., Carlin, F., Lairon, D., Nguyen-The, C. & Schmitt, P. (2004). Survival of *Bacillus cereus* spores and vegetative cells in acid media simulating human stomach. *J Appl Microbiol* **97**, 214–219.

Clavel, T., Carlin, F., Daragaignaratz, C., Lairon, D., Nguyen-The, C. & Schmitt, P. (2007). Effects of porcine bile on survival of *Bacillus cereus* vegetative cells and Haemolysin BL enterotoxin production in reconstituted human small intestine media. *J Appl Microbiol* **103**, 1568–1575.

Damgaard, P. H. (1995). Diarrhoeal enterotoxin production by strains of *Bacillus thuringiensis* isolated from commercial *Bacillus thuringiensis*-based insecticides. *FEMS Immunol Med Microbiol* **12**, 245–250.

Ehling-Schulz, M., Svensson, B., Guinebretier, M. H., Lindbäck, T., Andersson, M., Schulz, A., Fricker, M., Christiansson, A., Granum, P. E. & other authors (2005). Emetic toxin formation of *Bacillus cereus* is restricted to a single evolutionary lineage of closely related strains. *Microbiology* **151**, 183–197.

Ehling-Schulz, M., Guinebretier, M. H., Monthán, A., Berge, O., Fricker, M. & Svensson, B. (2006). Toxin gene profiling of enterotoxic and emetic *Bacillus cereus*. *FEMS Microbiol Lett* **260**, 232–240.

European Food Safety Authority (2005). Opinion of the scientific panel on biological hazards on *Bacillus cereus* and other *Bacillus* spp. in foodstuffs. http://www.efsa.europa.eu/cs/BlobServer/Scientific_Opinion/biohaz_ej175_op_bacillus_enfinal1.pdf?ssbinary=true.

Fagerlund, A., Ween, A., Lund, T., Hardy, S. P. & Granum, P. E. (2004). Genetic and functional analysis of the *cytK* family of genes in *Bacillus cereus*. *Microbiology* **150**, 2689–2697.

Fagerlund, A., Brillard, J., Fürst, R., Guinebretière, M. H. & Granum, P. E. (2007). Toxin production in a rare and genetically remote cluster of strains of the *Bacillus cereus* group. *BMC Microbiol* **7**, 43.

Fagerlund, A., Lindbäck, T., Storset, A. K., Granum, P. E. & Hardy, S. P. (2008). *Bacillus cereus* Nhe is a pore-forming toxin with functional properties similar to the ClyA (HlyE, SheA) family of haemolysins, able to induce osmotic lysis in epithelia. *Microbiology* **154**, 693–704.

Fisher, R. & Rosner, L. (1959). Toxicology of the microbial insecticide, Thuricide. *J Agric Food Chem* **7**, 686–689.

Frederiksen, K., Rosenquist, H., Jørgensen, K. & Wilcks, A. (2006). Occurrence of natural *Bacillus thuringiensis* contaminants and residues of *Bacillus thuringiensis* insecticides on fresh fruits and vegetables. *Appl Environ Microbiol* **72**, 3435–3440.

Gaviria Rivera, A. M., Granum, P. E. & Priest, F. G. (2000). Common occurrence of enterotoxin genes and enterotoxicity in *Bacillus thuringiensis*. *FEMS Microbiol Lett* **190**, 151–155.

Granum, P. E. (2002). *Bacillus cereus* and food poisoning. In *Applications and Systematics of Bacillus and Relatives*, pp. 37–46. Edited by R. Berkeley, M. Heyndrickx, N. Logan & P. De Vos. Oxford: Blackwell Science Ltd.

Granum, P. E., O'Sullivan, K. & Lund, T. (1999). The sequence of the non-haemolytic enterotoxin operon from *Bacillus cereus*. *FEMS Microbiol Lett* **177**, 225–229.

Guinebretière, M. H., Broussolle, V. & Nguyen-The, C. (2002). Enterotoxigenic profiles of food-poisoning and food-borne *Bacillus cereus* strains. *J Clin Microbiol* **40**, 3053–3056.

Hanahan, D. (1983). Studies on transformation of *Escherichia coli* with plasmids. *J Mol Biol* **166**, 557–580.

Hansen, B. M. & Hendriksen, N. B. (2001). Detection of enterotoxin *Bacillus cereus* and *Bacillus thuringiensis* strains by PCR analysis. *Appl Environ Microbiol* **67**, 185–189.

Hardy, S. P., Lund, T. & Granum, P. E. (2001). CytK toxin of *Bacillus cereus* forms pores in planar lipid bilayers and is cytotoxic to intestinal epithelia. *FEMS Microbiol Lett* **197**, 47–51.

Heinrichs, J. H., Beecher, D. J., MacMillan, J. D. & Zilinskas, B. A. (1993). Molecular cloning and characterization of the *hblA* gene encoding the B component of hemolysin BL from *Bacillus cereus*. *J Bacteriol* **175**, 6760–6766.

Helgason, E., Økstad, O. A., Caugant, D. A., Johansen, H. A., Fouet, A., Mock, M., Hegna, I. & Kolstø, A. B. (2000). *Bacillus anthracis*, *Bacillus cereus*, and *Bacillus thuringiensis* – one species on the basis of genetic evidence. *Appl Environ Microbiol* **66**, 2627–2630.

Hendriksen, N. B. & Hansen, B. M. (2006). Detection of *Bacillus thuringiensis kurstaki* HD1 on cabbage for human consumption. *FEMS Microbiol Lett* **257**, 106–111.

Hill, K. K., Ticknor, L. O., Okinaka, R. T., Asay, M., Blair, H., Bliss, K. A., Laker, M., Pardington, P. E., Richardson, A. P. & other authors (2004). Fluorescent amplified fragment length polymorphism analysis of *Bacillus anthracis*, *Bacillus cereus*, and *Bacillus thuringiensis* isolates. *Appl Environ Microbiol* **70**, 1068–1080.

Horton, R. M., Hunt, H. D., Ho, S. N., Pullen, J. K. & Pease, L. R. (1989). Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. *Gene* **77**, 61–68.

Janes, B. K. & Stibitz, S. (2006). Routine markerless gene replacement in *Bacillus anthracis*. *Infect Immun* **74**, 1949–1953.

Jara, S., Maduell, P. & Orduz, S. (2006). Diversity of *Bacillus thuringiensis* strains in maize and bean phylloplane and their respective soils in Colombia. *J Appl Microbiol* **101**, 117–124.

Jensen, G. B., Larsen, P., Jacobsen, B. L., Madsen, B., Smidt, L. & Andrup, L. (2002). *Bacillus thuringiensis* in fecal samples from greenhouse workers after exposure to *B. thuringiensis*-based pesticides. *Appl Environ Microbiol* **68**, 4900–4905.

Jensen, G. B., Hansen, B. M., Eilenberg, J. & Mahillon, J. (2003). The hidden lifestyles of *Bacillus cereus* and relatives. *Environ Microbiol* **5**, 631–640.

Kotiranta, A., Lounatmaa, K. & Haapasalo, M. (2000). Epidemiology and pathogenesis of *Bacillus cereus* infections. *Microbes Infect* **2**, 189–198.

Kyei-Poku, G., Gauthier, D., Pang, A. & van Frankenhuyzen, K. (2007). Detection of *Bacillus cereus* virulence factors in commercial products of *Bacillus thuringiensis* and expression of diarrheal enterotoxins in a target insect. *Can J Microbiol* **53**, 1283–1290.

Lindbäck, T., Fagerlund, A., Rødland, M. S. & Granum, P. E. (2004). Characterization of the *Bacillus cereus* Nhe enterotoxin. *Microbiology* **150**, 3959–3967.

Lund, T. & Granum, P. E. (1996). Characterisation of a non-haemolytic enterotoxin complex from *Bacillus cereus* isolated after a foodborne outbreak. *FEMS Microbiol Lett* **141**, 151–156.

Lund, T. & Granum, P. E. (1997). Comparison of biological effect of the two different enterotoxin complexes isolated from three different strains of *Bacillus cereus*. *Microbiology* **143**, 3329–3336.

Lund, T., De Buyser, M. L. & Granum, P. E. (2000). A new cytotoxin from *Bacillus cereus* that may cause necrotic enteritis. *Mol Microbiol* **38**, 254–261.

Margulis, L., Jorgensen, J. Z., Dolan, S., Kolchinsky, R. & Rainey, F. A. (1998). The *Arthromitus* stage of *Bacillus cereus*: intestinal symbionts of animals. *Proc Natl Acad Sci U S A* **95**, 1236–1241.

Martin, P. A. & Travers, R. S. (1989). Worldwide abundance and distribution of *Bacillus thuringiensis* isolates. *Appl Environ Microbiol* **55**, 2437–2442.

McIntyre, L., Bernard, K., Beniac, D., Isaac-Renton, J. L. & Naseby, D. C. (2008). Identification of *Bacillus cereus* group species, associated with food poisoning outbreaks in British Columbia, Canada. *Appl Environ Microbiol* **74**, 7451–7453.

Moravek, M., Dietrich, R., Buerk, C., Broussolle, V., Guinebretière, M. H., Granum, P. E., Nguyen-The, C. & Märtlbauer, E. (2006). Determination of the toxic potential of *Bacillus cereus* isolates by quantitative enterotoxin analyses. *FEMS Microbiol Lett* **257**, 293–298.

Ngamwongsatit, P., Buasri, W., Pianariyanon, P., Pulsrikarn, C., Ohba, M., Assavanig, A. & Panbangred, W. (2008). Broad distribution of enterotoxin genes (*hblCDA*, *nheABC*, *cytK*, and *entFM*) among *Bacillus thuringiensis* and *Bacillus cereus* as shown by novel primers. *Int J Food Microbiol* **121**, 352–356.

Raffel, S. J., Stabb, E. V., Milner, J. L. & Handelsman, J. (1996). Genotypic and phenotypic analysis of zwittermicin A-producing strains of *Bacillus cereus*. *Microbiology* **142**, 3425–3436.

Ramarao, N. & Lereclus, D. (2006). Adhesion and cytotoxicity of *Bacillus cereus* and *Bacillus thuringiensis* to epithelial cells are FlhA and PlcR dependent, respectively. *Microbes Infect* **8**, 1483–1491.

Rasko, D. A., Altherr, M. R., Han, C. S. & Ravel, J. (2005). Genomics of the *Bacillus cereus* group of organisms. *FEMS Microbiol Rev* **29**, 303–329.

Rosenquist, H., Smidt, L., Andersen, S. R., Jensen, G. B. & Wilcks, A. (2005). Occurrence and significance of *Bacillus cereus* and *Bacillus thuringiensis* in ready-to-eat food. *FEMS Microbiol Lett* **250**, 129–136.

Ryan, P. A., MacMillan, J. D. & Zilinskas, B. A. (1997). Molecular cloning and characterization of the genes encoding the L_1 and L_2 components of hemolysin BL from *Bacillus cereus*. *J Bacteriol* **179**, 2551–2556.

Salamitou, S., Ramisse, F., Brehélin, M., Bourguet, D., Gilois, N., Gominet, M., Hernandez, E. & Lereclus, D. (2000). The *plcR* regulon is involved in the opportunistic properties of *Bacillus thuringiensis* and *Bacillus cereus* in mice and insects. *Microbiology* **146**, 2825–2832.

Schoeni, J. L. & Wong, A. C. (1999). Heterogeneity observed in the components of hemolysin BL, an enterotoxin produced by *Bacillus cereus*. *Int J Food Microbiol* **53**, 159–167.

Silo-Suh, L. A., Lethbridge, B. J., Raffel, S. J., He, H., Clardy, J. & Handelsman, J. (1994). Biological activities of two fungistatic antibiotics produced by *Bacillus cereus* UW85. *Appl Environ Microbiol* **60**, 2023–2030.

Smith, R. A. & Couche, G. A. (1991). The phylloplane as a source of *Bacillus thuringiensis* variants. *Appl Environ Microbiol* **57**, 311–315.

Stenfors Arnesen, L. P., Fagerlund, A. & Granum, P. E. (2008). From soil to gut: *Bacillus cereus* and its food poisoning toxins. *FEMS Microbiol Rev* **32**, 579–606.

Swiecicka, I. & Mahillon, J. (2006). Diversity of commensal *Bacillus cereus sensu lato* isolated from the common sow bug (*Porcellio scaber*, Isopoda). *FEMS Microbiol Ecol* **56**, 132–140.

Swiecicka, I., Van der Auwera, G. A. & Mahillon, J. (2006). Hemolytic and nonhemolytic enterotoxin genes are broadly distributed among *Bacillus thuringiensis* isolated from wild mammals. *Microb Ecol* **52**, 544–551.

Thaenthanee, S., Wong, A. C. & Panbangred, W. (2005). Phenotypic and genotypic comparisons reveal a broad distribution and heterogeneity of hemolysin BL genes among *Bacillus cereus* isolates. *Int J Food Microbiol* **105**, 203–212.

U.S. Environmental Protection Agency (1998). Reregistration Eligibility Decision Document: *Bacillus thuringiensis*; EPA-738-R-98–004; U.S. Environmental Protection Agency, Office of Pesticide Programs, US Government Printing Office: Washington, DC, March 1998. (http://www.epa.gov/oppsrrd1/REDs/0247.pdf)

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