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

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**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
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 x 10^5 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 fecal 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 enterotoxicity of NHE and CytK is 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, HBLa, was isolated from a strain of B. cereus that produces two distinct sets of HBL proteins (Beecher & Wong, 2000). The HBLa set exhibited biological activity similar to the prototypic HBL set, and the homologous proteins functioned interchangeably, suggesting that HBLa 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, HBLa, 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 pMD2 and 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::Δhblα was introduced into the B. thuringiensis triple mutant by conjugation as described by Janes & Stibitz (2006).
Screening for presence of enterotoxin genes. Gene sequences for Hbl (hblA, hblB, hblC), 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 and NHE 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 μl 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, hblD, 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 hblD-Da sequence was obtained from the PCR products generated with the following primer pairs using genomic DNA from the ΔhblD-Da 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 ΔhblH-Da and ΔhblH 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 ΔhblH-Da 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 ΔhblH-Da 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 BamHI (Promega) and ligated to either pMAD (ΔhblC, ΔhblD, ΔhblH) or pBKJ236 (ΔhblH-Da) that had been BamHI-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 (ΔhblH) of the series, pMAD::ΔhblH-Da 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.

### Table 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Source or reference</th>
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<tr>
<td><strong>B. thuringiensis</strong></td>
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<tr>
<td>VBTS 2477</td>
<td>B. thuringiensis subsp. kurstaki wild-type strain; cry1Aa cry1Ab cry1Ac cry2Aa cry2Ab</td>
<td>Valient Biosciences</td>
</tr>
<tr>
<td>AK7701</td>
<td>VBTS 2477 ΔhblH, single mutant</td>
<td>This study</td>
</tr>
<tr>
<td>AK7702</td>
<td>VBTS 2477 ΔhblH Δnhe, double mutant</td>
<td>This study</td>
</tr>
<tr>
<td>AK7703</td>
<td>VBTS 2477 ΔhblH Δnhe Δhbl, triple mutant</td>
<td>This study</td>
</tr>
<tr>
<td>AK7704</td>
<td>VBTS 2477 ΔhblH Δnhe Δhbl ΔhblΔnhe, quadruple mutant</td>
<td>This study</td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
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<tr>
<td>DH5α</td>
<td>General purpose strain</td>
<td>Hanahan (1983)</td>
</tr>
<tr>
<td>GM2929</td>
<td>dam-6 dam-13::Tn9, Cm'</td>
<td>E. coli Genetic Stock Center</td>
</tr>
<tr>
<td>SS1827</td>
<td>Helper strain for conjugation into B. thuringiensis, Amp&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Janes &amp; Stibitz (2006)</td>
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<tr>
<td><strong>Plasmids</strong></td>
<td></td>
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<tr>
<td>pBKJ236</td>
<td>Temperature-sensitive gene replacement vector, Ery&lt;sup&gt;+&lt;/sup&gt;, contains 18 bp recognition site for I-SceI</td>
<td>Janes &amp; Stibitz (2006)</td>
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</table>
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, or had undergone a successful gene replacement and to verify the absence of the plasmid.

The nhe and hbl operons were replaced with the Δnhe and Δhbl deletion constructs in an iterative manner to obtain the triple mutant.

We were unable to obtain the quadruple mutant using the pMAD::Δhbl2 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::Δhbl2 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 hbl2 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 (hbl2a4-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 electrophoresed 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/ hblLa Bam-R primers to identify clones that had retained the Δhbl2 locus. The quadruple mutant was grown in half-strength TSB at 37 °C and single colonies were pooled 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 L2 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 Oxoide *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 Oxoide 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], boilded 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 (LC50).

**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, hblCa, hblDa1, hblLa1, nheA, nheB, nheC and cytK) were present in strain VBTS 2477 (data not shown). The near full-length hblA operon from VBTS 2477 has 98 % identity to the *B. cereus* UW85 hblA operon at the nucleotide level. After hblA 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 hblA than to hbl. Therefore, this third set of HBL genes was denoted as hbl2. The original hblA detected originally was designated hblA1. Sequence analyses of the three nearly-full-length hbl operons in VBTS 2477 show that the hblA1 and hblA2 gene sequences are 96–97 % identical and the deduced protein sequences are 97–98 % identical. The hbl genes are 76–84 % identical to hblA1 and hblA2 genes, and the deduced proteins are 68–85 % identical.
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, hbla1, hbla2 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 D_hbla1, D_nhe, D_hbl and D_hbla2 (Fig. 1 and Fig. 2).

Biological activities of enterotoxin-deficient mutants
B. thuringiensis strain VBTS 2477, the single mutant (Δhbla1) and the double (Δhbla1Δnhe) mutant each exhibited a strong agglutination response (Table 2) when tested with the Oxoid BCET-RPLA kit, which detects the L2 component of HBL (Beecher & Wong, 1994a). The triple deletion mutant, in which hbl is deleted, exhibited a negative phenotype, indicating that expression of the L2 protein was abolished in this mutant. Since the hbla2 operon remained intact in the triple mutant, either L2(a2) is not expressed in strain VBTS 2477 or it does not react with the anti-L2 antibody in the RPLA kit. Haemolysis on sheep blood agar suggests that L2(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 L2a is antigenically distinct from L2. In the Tecra BDE assay, which detects NheA, both the wild-type and the single mutant (Δhbla1) 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. 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.](image1)

![Fig. 2. PCR analysis of B. thuringiensis VBTS 2477 and quadruple-enterotoxin-deletion mutant. PCR primers were used to amplify: hblCa (lanes 1–3; hblCa-F/hblAa-R), nhe (4–6; nheA-F/nheC-R) and (7–9; hblC-F/hblAa-R); hbla2 (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.](image2)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Oxoid RPLA*</th>
<th>Tecra BDE†</th>
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<td>4</td>
</tr>
<tr>
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<td>Δhbla2ΔnheΔhbla2</td>
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*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 hbla 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 hbla1/hbla2 and hbl homologues in *B. thuringiensis* strain VTBS 2477. Furthermore, the hbla1 and hbla2 genes in *B. thuringiensis* VTBS 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; Thaenthaneet 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 *hbla* variant. Furthermore, the Oxoid RPLA immunoassay did not detect the L2 component of HBLa2 in *B. thuringiensis* strain VBTS 2477, although it is probably expressed; haemolysis activity of the triple mutant, in which hblA2 is intact, is greater than in the quadruple mutant in which it is deleted. These data suggest that the L2,3 proteins are antigenically distinct from the prototypic L2 protein. Therefore, the Oxoid kit may be specific for the prototypic L2 protein only. In our work, we found hblA present in two strains of *B. cereus* and six strains of *B. thuringiensis*, including VTBS 2477 (data not shown), suggesting that hblA 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 VTBS 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* VTBS 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 hbla1 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 hbla1 deletion mutants without difficulty; however, hbla2 presented challenges. We attempted to use pMAD for construction of the hbla2 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 hbla2 deletion with the pBKJ236/pBKJ223 system. Our work illustrates an

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Protoxin in culture broth (mg ml⁻¹)</th>
<th>Proportion of crystal toxin as a protoxin of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>135 kDa (%)</td>
</tr>
<tr>
<td>VBTS 2477</td>
<td>8.4</td>
<td>63</td>
</tr>
<tr>
<td>AK7704, quadruple mutant</td>
<td>11.6</td>
<td>69</td>
</tr>
</tbody>
</table>

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.
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/oppsrdr1/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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3582


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