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Screening by Polymerase Chain Reaction of *Bacillus thuringiensis* Serotypes for the Presence of *cryV*-Like Insecticidal Protein Genes and Characterization of a *cryV* Gene Cloned from *B. thuringiensis* subsp. *kurstaki*

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Polymerase chain reaction screening using *cryV*-specific oligonucleotides, designed to amplify the 5′ half of *cryV*-type genes, revealed the presence of such genes in 7 of 21 *Bacillus thuringiensis* serotypes examined. Restriction analysis and hybridization studies indicated that these putative genes fall into at least three subclasses. The nucleotide sequence of the *cryV*-type gene cloned from *B. thuringiensis* subsp. *kurstaki* DSIR732 revealed an open reading frame coding for a protein of 719 amino acids, and lysates of *Escherichia coli* cells expressing the 81.2-kDa $CryV_{732}$ protein were toxic to *Epiphyas postvittana* (Lepidoptera: Tortricidae).

Bacillus thuringiensis, characterized by the production of parasporal crystals composed of proteins which exhibit highly specific insecticidal activity against the larvae of certain lepidoptera, coleoptera or diptera, has been used for many years as a successful biological insecticide. The insecticidal Cry proteins, encoded by *cry* genes, have been classified as CryI, -II, -III, or -IV, depending on the host specificity and the degree of amino acid homology (6). Reports of the occurrence of insect resistance to Cry proteins (10, 11, 17), which in at least one instance has been attributed to a reduction in the affinity of the proteolytically activated Cry toxin for binding to the membrane of the insect epithelial midgut cells (19), have led a number of research groups to undertake a search for novel *cry* genes. Increasing the diversity of *cry* genes available for either microbial insecticides or transgenic plants would facilitate the simultaneous use of genes encoding insecticidal proteins which bind to different membrane receptors on the insect midgut epithelial cell, and it should both increase the efficiency of pest control and delay the emergence of resistance. Recently, there have been reports from two research groups of a new class of *cry* genes (1, 18). In one instance, the nucleotide sequence of the 5′ 1.2 kb of the open reading frame (ORF) has been reported (1), whereas in the second instance, the entire nucleotide sequence has been reported, along with the fact that the 81-kDa product of the *cry* gene is toxic to larvae of both lepidoptera and coleoptera (18). As a consequence of this dual specificity, and in accordance with the classification criteria proposed by Höfte and Whiteley (6), the gene encoding the 81-kDa toxin has been designated *cryV*. As neither of the *cryV* genes mentioned above is publicly available, we were interested in cloning similar genes to assess their potential, in conjunction with other *cry* genes, to be used as part of a responsible management strategy for insect pest control.

Here we report the screening of a number of *B. thuringiensis* serotypes for the presence of *cryV*-type genes and the cloning and nucleotide sequence of a *cryV* gene from *B. thuringiensis* subsp. *kurstaki* DSIR732. We also report the expression of the *cryV* gene in *Escherichia coli* and the insecticidal activity of the 81-kDa *cryV* gene product.

Two oligonucleotides based on the sequences of the *cryV* genes cloned from *B. thuringiensis* subsp. *aizawai* EG6346 (1) and *B. thuringiensis* subsp. *kurstaki* JHCC4835 (18), and specific for *cryV* genes, were synthesized on a Milligen/Biosearch Cyclone Plus DNA synthesizer. The oligonucleotides, 13091/1 (5′-GCCGGAATTCAAGCTTATGAAACTAAAGAATCCAGA-3′) and 05091/2 (5′-GCCGTCTAGAGGATCCTTGTGTTGAGATA-3′), have homology to nucleotides 1 to 20 and 1142 to 1124, respectively, relative to the ATG initiation codons of the aforementioned sequences. A DNA template for the screening of 21 *B. thuringiensis* serotypes for the presence of *cryV*-type genes was prepared by resuspending cells from fresh CCY plates (15) in 100 μl of distilled H_2O, boiling for 2 min, and centrifuging for 5 min at 13,000 × *g*. Polymerase chain reactions (PCR) were carried out on 1 to 3 μl of the cell lysate with 0.5 μM primers 13091/1 and 05091/2, 80 μM deoxynucleoside triphosphates, 1× *Taq* polymerase buffer, and 0.5 U of *Taq* DNA polymerase in a 50-μl volume. PCR amplifications were carried out in an Ericomp thermal cycler with a denaturing step of 3 min at 94°C followed by 40 cycles of 1 min at 94°C, 1 min at 48°C, and 2 min at 72°C. Agarose gel electrophoresis of the resulting PCR products revealed that a fragment of approximately 1.2 kb, indicative of the presence of a *cryV*-type gene, was amplified from 7 of the 21 serotypes screened (Fig. 1A), *B. thuringiensis* subsp. *kurstaki* HD-1, *B. thuringiensis* subsp. *aizawai* HD-112, *B. thuringiensis* subsp. *kenyae* HD-136, *B. thuringiensis* subsp. *dendrolimus* HD-106, *B. thuringiensis* subsp. *tolworthi* HD-125, *B. thuringiensis* subsp. *galleriae* HD-8, and *B. thuringiensis* subsp. *kurstaki* DSIR732. No PCR products were observed with the remaining serotypes (*B. thuringiensis* subsp. *israelensis* HD-567, *B. thuringiensis* subsp. *alesti* HD-4, *B. thuringiensis* subsp. *kyushuensis* HD-541, *B. thuringiensis* subsp. *entomocidus* HD-9, *B. thuringiensis* subsp. *subtoxicus* HD-10, *B. thuringiensis* subsp. *dakota* HD-511, *B. thuringiensis* subsp.

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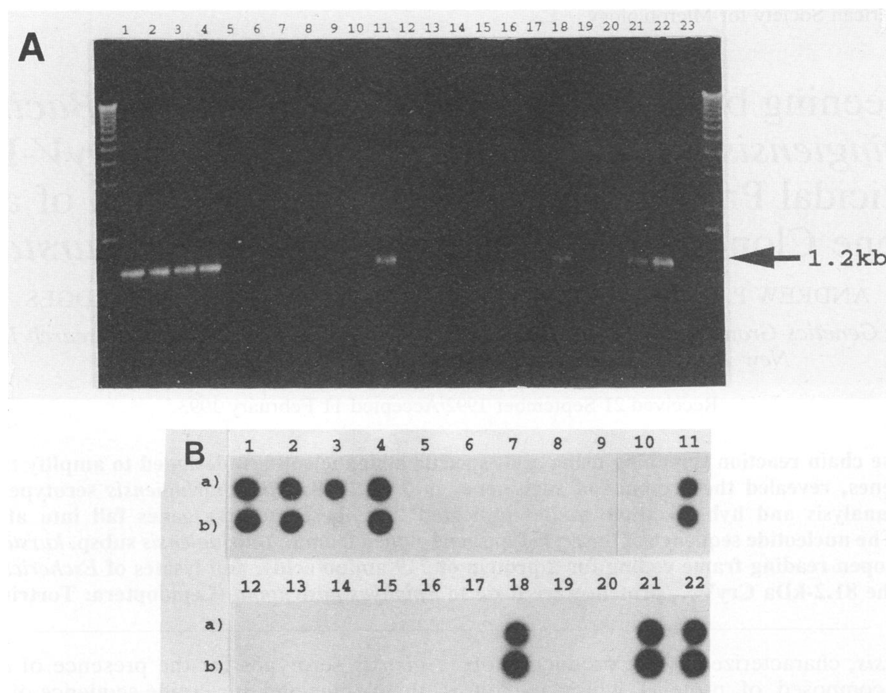

FIG. 1. (A) Agarose gel electrophoresis analysis of PCR products amplified from *B. thuringiensis* serotypes with primers specific for *cryV*-type genes. Lanes: 1, *B. thuringiensis* subsp. *kurstaki* HD-1; 2, *B. thuringiensis* subsp. *kenyae* HD-136; 3, *B. thuringiensis* subsp. *galleriae* HD-8; 4, *B. thuringiensis* subsp. *aizawai* HD-112; 5, *B. thuringiensis* subsp. *israelensis* HD-567; 6, *B. thuringiensis* subsp. *alesti* HD-4; 7, *B. thuringiensis* subsp. *kyushuensis* HD-541; 8, *B. thuringiensis* subsp. *entomocidus* HD-9; 9, *B. thuringiensis* subsp. *subtoxicus* HD-10; 10, *B. thuringiensis* subsp. *dakota* HD-511; 11, *B. thuringiensis* subsp. *dendrolimus* HD-106; 12, *B. thuringiensis* subsp. *toumanoffi* HD-201; 13, *B. thuringiensis* subsp. *wuhenensis* HD-525; 14, *B. thuringiensis* subsp. *finitimus* HD-19; 15, *B. thuringiensis* subsp. *indiana* HD-521; 16, *B. thuringiensis* subsp. *sotto* HD-6; 17, *B. thuringiensis* subsp. *kumamotoensis*; 18, *B. thuringiensis* subsp. *tolworthi* HD-125; 19, *B. thuringiensis* subsp. *ostriniae* HD-501; 20, *B. thuringiensis* subsp. *morrisoni* HD-12; 21, *B. thuringiensis* subsp. *kurstaki* DSIR732; 22, pPOM12 positive control; and 23, negative control (no template). Molecular mass markers (BRL 1-kb ladder) are present in the flanking lanes. The position of the 1.2-kb fragment indicative of the presence of a *cryV*-type gene is shown. (B) Dot blot analysis of PCR products. The PCR products produced by primers 13091/1 and 05091/2 on template DNA from *B. thuringiensis* serotypes were probed with the 5′ 1.2-kb fragment of the cloned $cryV_{732}$ gene. Lanes 1 to 22 are as indicated above. Results of low-stringency washes (rows a) and high-stringency washes (rows b) are shown.

toumanoffi HD-201, *B. thuringiensis* subsp. *wuhenensis* HD-525, *B. thuringiensis* subsp. *finitimus* HD-19, *B. thuringiensis* subsp. *indiana* HD-521, *B. thuringiensis* subsp. *sotto* HD-6, *B. thuringiensis* subsp. *kumamotoensis*, *B. thuringiensis* subsp. *ostriniae* HD-501, and *B. thuringiensis* subsp. *morrisoni* HD-12).

Restriction analysis of the 1.2-kb PCR products amplified from the seven serotypes indicated that they fall into three subclasses when digested with *Kpn*I: those which gave 0.6-, 0.33-, and 0.24-kb fragments (*B. thuringiensis* subsp. *kurstaki* HD-1, *B. thuringiensis* subsp. *aizawai* HD-112, *B. thuringiensis* subsp. *tolworthi* HD-125, *B. thuringiensis* subsp. *kenyae* HD-136, and *B. thuringiensis* subsp. *kurstaki* DSIR732), those which gave 0.93- and 0.24-kb fragments (*B. thuringiensis* subsp. *dendrolimus* HD-106), and those in which the 1.2-kb fragment was not cleaved by *Kpn*I (*B. thuringiensis* subsp. *galleriae* HD-8). The *cryV* gene cloned from *B. thuringiensis* subsp. *kurstaki* JHCC4835 (18) would also fall into the first subclass of *cryV* genes; however, the *cryV* gene fragment cloned from *B. thuringiensis* subsp. *aizawai* EG6346 (1) would be expected to produce *Kpn*I fragments of 0.57 and 0.6 kb and may represent a fourth subclass of *cryV* genes.

The 1.2-kb 5′ end of the *cryV* gene amplified from *B. thuringiensis* subsp. *kurstaki* DSIR732, and herein referred to as $cryV_{732}$, was radioactively labelled with a BRL random primer labeling kit incorporating [α-^{32}P]dATP and used in dot blot hybridizations to assess the degree of homology between the 1.2-kb amplified fragments of the seven serotypes. Dot blot hybridizations were carried out by denaturing the DNA in 0.5 M NaOH, neutralizing with 3 M sodium acetate (pH 4.8), transferring the DNA to Amersham Hybond N$^+$ nylon membranes by using the Bio-Rad Bio-Dot apparatus, and treating the membranes with 0.4 M NaOH to fix the DNA to them. Prehybridization and hybridization were carried out in a Techne Hybridiser HB-1 unit according to the manufacturer's recommendations. At low-stringency washes (65°C in 0.1% sodium dodecyl sulfate (SDS)–1× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]), the $cryV_{732}$ probe hybridized to the 1.2-kb PCR products amplified from each of the seven serotypes mentioned above (Fig. 1B). At high-stringency washes (65°C in 0.1% SDS–0.1× SSC), the probe hybridized to the PCR products obtained from six of the seven serotypes but did not hybridize to the PCR product amplified from *B. thuringiensis* subsp. *galleriae* HD-8 (Fig. 1B). These results indicated that in the 5′ ends of the putative *cryV* genes amplified from six of the seven serotypes, there was a significantly high degree of homology, which under the stringency conditions used was expected to be 95% or more. The exception to this high

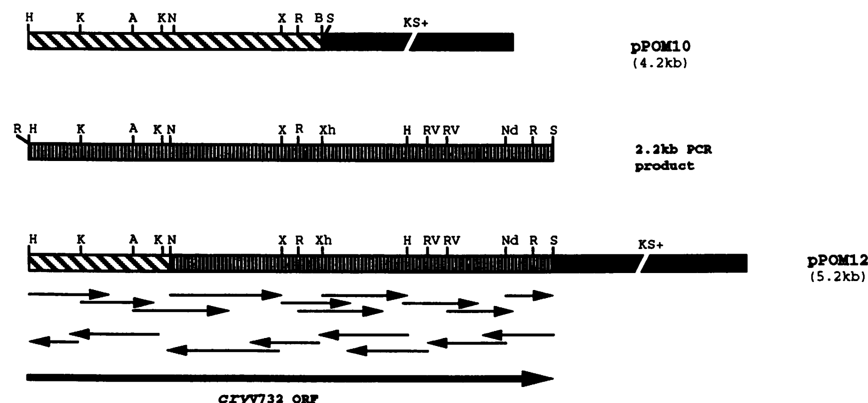

FIG. 2. Cloning and sequencing strategy of the *cryV*$_{732}$ gene. The Bluescript KS$^+$ sequences (2.95 kb) (filled boxes), the 1.2-kb PCR product encoding the 5′ end of the *cryV*$_{732}$ gene (hatched boxes), and the 2.2-kb *cryV*$_{732}$ PCR product (shaded boxes) are shown. pPOM12 carries the full-length *cryV*$_{732}$ clone, the 0.6-kb 5′ end originating from the 1.2-kb PCR product, and the 1.56-kb 3′ end originating from the 2.2-kb PCR product. The extents of the nucleotide sequences obtained from various deletions and subclones of pPOM12 (small arrows) and the direction of the *cryV*$_{732}$ ORF (large arrow) are indicated. The restriction sites within the cloned DNA are indicated as follows: A, *Acc*I; B, *Bam*HI; H, *Hin*dIII; K, *Kpn*I; N, *Nsi*I; Nd; *Nde*I; R, *Eco*RI; RV, *Eco*RV; S, *Sst*I; X, *Xba*I; Xh, *Xho*I. Restriction sites of the KS$^+$ polylinker are not represented.

degree of homology was in the 5′ end of the putative *cryV* gene from *B. thuringiensis* subsp. *galleriae* HD-8, which in hybridizing under conditions of approximately 80% homology but not hybridizing under conditions of 95% homology indicates that the putative *cryV* gene of *B. thuringiensis* subsp. *galleriae* HD-8 has a greater degree of heterogeneity than the putative *cryV* genes of the other serotypes examined.

The 1.2-kb PCR fragment amplified from *B. thuringiensis* subsp. *kurstaki* DSIR732 was cloned as a *Hin*dIII-*Bam*HI fragment into Bluescript KS$^+$ (Stratagene) by standard recombinant DNA techniques (12), generating pPOM10 (Fig. 2). Preliminary determination of the nucleotide sequence of this 1.2-kb clone and alignment with the *cryV* gene sequences previously reported (1, 18) revealed that the fragment did encode the 5′ end of a *cryV*-type gene. To obtain a full-length *cryV*-type clone from *B. thuringiensis* subsp. *kurstaki* DSIR732, PCR were carried out on total DNA with primers 13091/1 and 05121/2 (5′-GCCGGGATCCTCTAGAG CTCTACATGTTACGCTCAATAT-3′), which are homologous to the 5′ and 3′ ends of the *B. thuringiensis* JHCC4835 *cryV* ORF, respectively. Analysis of the PCR products revealed a 2.2-kb fragment, indicative of a *cryV*-type gene. Assembly of the full-length clone of the *cryV*$_{732}$ gene was achieved by subcloning the 1.56-kb *Nsi*I-*Sst*I fragment of the 2.2-kb PCR product, encoding the 3′ end of the *cryV* gene, into the *Nsi*I-*Sst*I sites of pPOM10, carrying the 5′ end of the *cryV* gene, and generating pPOM12 (Fig. 2). Dideoxy chain-termination sequencing (13) of both strands of the *cryV*$_{732}$ clone was carried out as described elsewhere (5) on double-stranded templates of various deletion derivatives and subclones of pPOM10 and pPOM12 (Fig. 2). The nucleotide sequence revealed an ORF of 2,157 nucleotides coding for a protein of 719 amino acids with an estimated molecular mass of 81,215 Da. Alignment of the nucleotide sequence of the *cryV* gene reported by Tailor et al. (18) with the *cryV*$_{732}$ gene revealed only two nucleotide differences, a T-to-G transition at nucleotide position 697 and a T-to-C transition at nucleotide position 1398, relative to the ATG translational start codon. The former nucleotide difference results in a tyrosine-to-aspartate transition at amino acid residue 233; the latter nucleotide difference is silent. The *cryV*$_{732}$ sequence also has 97% homology at the DNA level to the 5′ end of the *cryV* gene cloned from *B. thuringiensis* subsp. *aizawai* EG6346 reported by Chambers et al. (1).

The amino acid sequence of the CryV$_{732}$ protein has a number of features characteristic of most *B. thuringiensis* Cry proteins (6): five highly conserved amino acid blocks (C1 to C5), a hydrophobic domain upstream of the C1 block between amino acids 54 and 93, and a potential tryptic cleavage site between amino acids 637 and 638, which is within the C5 block and is conceivably the corresponding cleavage point for activation of the CryV$_{732}$ protoxin by insect gut proteases, releasing the activated toxin of approximately 65 kDa.

The observed homology between the *cryV*$_{732}$ gene and the previously reported *cryV* genes (1, 18) suggested that the *cryV*$_{732}$ gene may share additional characteristics with other *cryV* genes. Both previously reported *cryV* genes are located approximately 500 bp 3′ of a *cryI*-type gene, are prevented from expression as the distal part of an operon by an intergenic transcriptional terminator, and lack an upstream promoter-like sequence (1, 7, 18). The consequences of these features are that the *cryV* genes are either cryptic or very weakly expressed. Analysis of the crystals produced by *B. thuringiensis* subsp. *kurstaki* DSIR732 revealed only two major proteins of 133 and 65 kDa, the former being the product of a *cryIA*(c) gene (4). This suggests that the *cryV*$_{732}$ gene is also either cryptic or weakly expressed.

Alignment of the CryV$_{732}$ amino acid sequence with those of CryI through CryIV revealed that, overall, CryV$_{732}$ is most closely related to CryIB, having 62% perfect homology throughout the entire sequence, as opposed to 35 to 47% homology to CryIA through -G proteins, 22% homology to CryIIA and -B proteins, 34 to 40% homology to CryIIIA and -B proteins, and 23 to 33% homology to CryIVA through -D proteins. More detailed analysis of the CryV$_{732}$ and CryIB amino acid sequence alignments showed that the N-terminal 34 amino acids have negligible homology, whereas the level of homology from amino acids 35 to 547 is 66%. Between amino acids 548 and 645, the CryV$_{732}$ protein is, in fact, more closely related to CryIA(b) than to CryIB (71% homol-

ogy as opposed to 43%), whereas the C-terminal region of the $CryV_{732}$ protein (amino acids 646 to 719) reverts to being more homologous to CryIB (77%). As *cry* genes are generally localized on large conjugative plasmids and therefore have the potential to be mobilized between strains and because *cry* genes are often flanked by inverted-repeat transposon-like elements, it is conceivable that recombination events between *cryIB*- and *cryIA*(b)-like genes could account for the apparent hybrid-like structure of the $cryV_{732}$ gene. Such recombination events have previously been suggested to account for the apparent hybrid structure of the *cryIE* gene (20).

Although the region of $CryV_{732}$ from amino acids 35 to 547 shows 66% homology to the CryIB amino acid sequence, the internal region of this segment from amino acids 401 to 445 shows only 28% homology. This heterogeneic region lies between the C2 and C3 conserved amino acid blocks, an area which has been implicated as a region which determines the specificity of the Cry proteins against insect genera (3, 14). X-ray crystallography studies of the CryIIIA protein indicate the presence of three distinct structural domains, of which domain II has been proposed as the region involved in receptor binding (8). The corresponding region of the $CryV_{732}$ protein, which would constitute domain II, lies between amino acid residues 282 and 500 and therefore includes the region of heterogeneity between the $CryV_{732}$ and CryIB proteins. This variability in domain II may account for the fact that the CryV protein has toxicity to both lepidoptera and coleoptera (18), whereas the CryIB protein has toxicity to lepidoptera only (6).

To achieve expression of the $cryV_{732}$ gene in *E. coli*, PCR amplifications were carried out on pPOM12 DNA with primers 17012/1 (5′-GCCGCTGCAGCCCGGGAGGAAAC AGACCATGAAACTAAAGAATCCAGA-3′) and 17012/2 (5′-GCCGGTTAACCTGCAGCTACATGTTACGCTCAATAT GG-3′), which are homologous to the 5′ and 3′ ends of the $cryV_{732}$ ORF, respectively. Primer 17012/1 introduced an *E. coli* ribosome binding site 5′ of the $cryV_{732}$ translational initiation codon. The modified $cryV_{732}$ gene was cloned as a *Sma*I-*Hpa*I fragment into the *Sma*I site of Bluescript KS^+, generating pPOM13, and was cloned in such an orientation that the T7 promoter sequence present in KS^+ was located upstream of the ribosome binding site and the $cryV_{732}$ ORF. pPOM13 was transformed into *E. coli* BL21(DE3) (16), which carries the T7 RNA polymerase gene under the transcriptional control of the inducible *lacUV5* promoter. BL21(DE3) harboring pPOM13 was grown in L broth (12) to an A_{650} of 0.5, and the expression of the T7 RNA polymerase was induced by the addition of isopropylthio-β-galactosidase (0.4 mM). The preparation of BL21(DE3) lysates and subsequent Western blotting (immunoblotting) of the lysates with antiserum raised against the P1 proteins of *B. thuringiensis* subsp. *kurstaki* HD-1 were carried out as described elsewhere (4). Immunoblotting indicated that in the absence of the inducer, the 81-kDa protein was produced at low levels, probably as a consequence of leaky expression of the T7 RNA polymerase (Fig. 3). Extracts of protein samples taken after induction showed a significant increase in the level of the 81-kDa protein produced, in addition to the presence of 65-, 48-, and 43-kDa proteins, assumed to be proteolytic degradation products of the 81-kDa protein and indicating a lack of stability of the $CryV_{732}$ protein in *E. coli*. Control lanes containing extracts of untransformed BL21(DE3) showed no evidence of the 81-kDa protein or its proteolytic degradation products.

Lysates of BL21(DE3) harboring pPOM13, which had

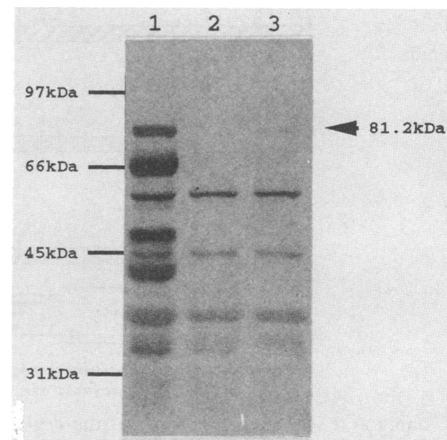

FIG. 3. Western immunoblot analysis of recombinant $CryV_{732}$ protein. Lanes: 1, lysate of BL21(DE3) harboring pPOM13, 3 h after induction of *cryV* expression; 2, BL21(DE3) control lysate; 3, lysate of BL21(DE3) harboring pPOM13, prior to induction of *cryV* expression. The positions of the molecular mass standards and the position of the recombinant 81.2-kDa $CryV_{732}$ protein are indicated.

been induced to give high-level expression of the $CryV_{732}$ protein, were used to assess the toxicity of the recombinant $CryV_{732}$ protein against a member of each of the Lepidoptera, Coleoptera, and Diptera orders of insects. Dilutions of lysates were incorporated into artificial insect diet (9) and aliquoted into the wells of microtiter trays, and a single first-instar larva of either *Epiphyas postvittana* or *Tenebrio molitor* was placed into each well, with 10 insects per lysate dilution. The trays were incubated at 22°C with high humidity for 7 to 10 days prior to assessment of larval mortality. Confidence limits and 50% lethal concentrations were determined by probit analysis (2). Activity against *Culex pervigilans* was assessed by incorporation of dilutions of *E. coli* lysates into distilled water, into which three third-instar larvae were introduced. Mortality was assessed after 24 and 48 h of incubation at 25°C. Lysates of BL21(DE3) transformed with pPOM13 showed toxicity to *E. postvittana* (Lepidoptera: Tortricidae), a major horticultural insect pest in New Zealand, with a 50% lethal concentration of 67 μg (95% confidence limit, 164 to 40 μg) of total protein extract per ml of diet, whereas no toxicity to *E. postvittana* was observed with lysates of untransformed BL21(DE3). Lysates tested at 2 mg of total protein per ml of diet against *T. molitor* (Coleoptera: Tenebrionidae) and 2 mg of total protein per ml of distilled water against *C. pervigilans* (Diptera: Cuclidae) showed no larval mortality, feeding inhibition, or growth rate reduction, indicating that the 81-kDa $CryV_{732}$ protein was nontoxic to these insects. Although Tailor et al. (18) reported that the CryV protein is toxic to both lepidoptera and coleoptera, they also reported that the 81-kDa protein is not toxic to all coleoptera but appears to be specific to the genus *Diabrotica*. As the $CryV_{732}$ protein and that encoded by the *cryV* gene cloned from *B. thuringiensis* JHCC4835 (18) differ by only one amino acid and the difference occurs in the C2 conserved amino acid block, which is thought to be involved in toxicity and not specificity (8, 21), it seems likely that the $CryV_{732}$ protein may also be toxic to *Diabrotica* spp. The absence of *Diabrotica* insects in New Zealand has prevented us from being able to demonstrate the toxicity of the $CryV_{732}$ protein to such insects.

The results presented here indicate that *cryV* or *cryV*-like

genes may be relatively common in *B. thuringiensis* serotypes. It is conceivable that they are in fact even more common than our results suggest if, for example, there is a degree of heterogeneity in the region of one or both of the primers which were used in the PCR screening. The apparent widespread occurrence of *cryV*-like genes and our demonstration of the heterogeneity in this class of gene coupled with the insecticidal activity of the *cryV* gene products suggest that these genes may prove to be useful additions to the *cry* genes available for use in a responsible management strategy for insect pest control.

Nucleotide sequence accession number. The GenBank/EMBL accession number for the nucleotide sequence of the $cryV_{732}$ gene is M98544.

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