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Insecticidal Activity of the CryIIA Protein from the NRD-12 Isolate of *Bacillus thuringiensis* subsp. *kurstaki* Expressed in *Escherichia coli* and *Bacillus thuringiensis* and in a Leaf-Colonizing Strain of *Bacillus cereus*†

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A 4.0-kb *Bam*HI-*Hin*dIII fragment encoding the *cryIIA* operon from the NRD-12 isolate of *Bacillus thuringiensis* subsp. *kurstaki* was cloned into *Escherichia coli*. The nucleotide sequence of the 2.2-kb *Acc*I-*Hin*dIII fragment containing the NRD-12 *cryIIA* gene was identical to the HD-1 and HD-263 *cryIIA* gene sequences. Expression of *cryIIA* and subsequent purification of CryIIA inclusion bodies resulted in a protein with insecticidal activity against *Heliothis virescens*, *Trichoplusia ni*, and *Culex quinquefasciatus* but not *Spodoptera exigua*. The 4.0-kb *Bam*HI-*Hin*dIII fragment encoding the *cryIIA* operon was inserted into the *B. thuringiensis-E. coli* shuttle vector pHT3101 (pMAU1). pMAU1 was used to transform an acrystalliferous HD-1 strain of *B. thuringiensis* subsp. *kurstaki* and a leaf-colonizing strain of *B. cereus* (BT-8) by using electroporation. Spore-crystal mixtures from both transformed strains were toxic to *H. virescens* and *T. ni* but not *Helicoverpa zea* or *S. exigua*.

Commercial products containing the HD-1 isolate of *Bacillus thuringiensis* subsp. *kurstaki* have been used successfully to control certain lepidopterous pests since the early 1970s. Much of the insecticidal activity of HD-1 is attributed to the three 130-kDa proteins (CryIA) associated with the bipyramidal crystal (7, 28). However, another class of insecticidal protein(s), P2, also is present in HD-1 (29). The predominant protein that constitutes the P2 cuboidal crystal (now designated CryIIA) has a molecular mass of approximately 65 kDa, and is toxic to both lepidopterans and dipterans. The genes encoding the CryIIA proteins have been cloned and sequenced from HD-1 and HD-263 of *B. thuringiensis* subsp. *kurstaki* (4, 27). Both genes and their deduced amino acid sequences are identical but share little nucleotide or amino acid homology with other Cry-type proteins (4, 7, 27).

The NRD-12 isolate of *B. thuringiensis* subsp. *kurstaki* is similar to HD-1 in that both isolates contain bipyramidal [consisting of CryIA(a), (b), and (c) proteins] and cuboidal crystals and exhibit similar toxicity of spore-crystal, purified crystal, or individual CryIA proteins against lepidopterans (13, 16, 17, 26). Only the *cryIA*(b) gene has been sequenced from NRD-12, and the deduced amino acid sequence of this protein differed from that of HD-1 CryIA(b) by eight amino acids (6). Therefore, the *cryIIA* gene sequence from NRD-12 will further establish the chemical and evolutionary relationships between HD-1 and NRD-12. Furthermore, extensive toxicity characterization of the CryIIA protein will help to determine whether this protein has the potential to control specific insect pests.

The stability and quantity of *B. thuringiensis* δ-endotoxin(s) on a leaf surface are often two of the limiting factors in obtaining maximum insect control. One possible mechanism for overcoming these problems is to have the δ-endotoxin(s) produced in colonizing microorganisms on the leaf surface. *B. thuringiensis* isolates from the phylloplane of various plants have been reported, but were not recovered from tomato leaves in an extensive survey of bacteria colonizing tomato leaves (1a, 19, 23). However, many strains of *Bacillus cereus* were isolated from tomato leaves, and one particular isolate, BT-8, was able to grow vegetatively on tomato leaves and achieve populations of 10^4 CFU per leaf over a period of up to 2 weeks. This strain was also antifungal against several important tomato pathogens including *Alternaria solani* (19). Because of the numerous reports of successful transformations of various *Bacillus* spp. with *B. thuringiensis* proteins (3, 10, 12), we attempted to insert the *cryIIA* gene into *B. cereus* BT-8 and determine whether this strain could express *B. thuringiensis* proteins that would kill insects. A bacterial species capable of surviving on leaf surfaces for several weeks with fungicidal and insecticidal activity should allow for reduced pesticide applications.

We report here the cloning, sequencing, and expression of the *cryIIA* gene from NRD-12 in *Escherichia coli*, *B. thuringiensis*, and *B. cereus* for determining the relationship of this *cryIIA* gene with that of other isolates and to determine the insecticidal activity of the encoded protein in various expression systems against economically important insect species.

MATERIALS AND METHODS

Bacterial strains, plasmids, and general methods. NRD-12 was originally obtained from the formulated material Javelin (Sandoz Corp., Wasco, Calif.), and HD-1 was isolated from Dipel 2X (Abbott Laboratories, North Chicago, Ill.) as described by Moar et al. (17). The acrystalliferous strain of HD-1 was obtained from P. Jarrett, Horticulture Research International, West Sussex, England. The BT-8 isolate of *B. cereus* was isolated from tomato leaves (19). The *E. coli* strains used were

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294 and XL1-Blue for cloning and sequencing and BL21 (λDE3) for protein expression. Commercially available plasmids used for cloning and sequencing were pBR322, pUC13, pBluescript KS, and pBluescript IIKS. The plasmid pT7-5 was provided by S. Tabor, Harvard Medical School, Boston, Mass. (25). The *E. coli-B. thuringiensis* shuttle vector pHT3101 was obtained from D. Lereclus, Institut Pasteur, Paris, France (10).

Protein purification and sequencing. NRD-12 was cultured on modified GYS medium until autolysis, and the inclusion bodies were isolated and purified (17). The NRD-12 cuboidal crystal was solubilized and separated by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (Millipore Corp., Bedford, Mass.). Proteins were sequenced with an Applied Biosystems (Foster City, Calif.) model 475A protein sequencer.

Plasmid library and DNA hybridization. Total plasmid DNA from NRD-12 and HD-1 was isolated (9, 15). An NRD-12 plasmid DNA library was constructed by digestion of plasmid DNA with *Bam*HI and *Hin*dIII, ligation into pBR322, and transformation into *E. coli* 294. An oligonucleotide (23-mer) corresponding to the first eight amino acids of the NRD-12 CryIIA crystal protein was designed against the corresponding nucleotide sequence of the HD-263 *cryIIA* gene (4) and labelled at the 5′ end with [γ-^{32}P]ATP by using polynucleotide kinase. This probe was used to screen the NRD-12 plasmid DNA library (20). Hybridizations were conducted at 25 to 30°C.

Plasmid profiles of HD-1 and NRD-12 were performed by running undigested and *Hin*dIII-digested total plasmid DNA on a 0.5% agarose gel. Southern blot hybridizations (20) of total plasmid DNA of HD-1 and NRD-12 were performed with the 2.2-kb *Acc*I-*Hin*dIII fragment as the template to generate multiple radiolabelled probes by utilizing 50 μCi of [α-^{32}P]dCTP and the oligolabelling kit of Pharmacia (Piscataway, N.J.). Hybridizations were performed at 68°C.

DNA sequencing. *E. coli* 294 colonies containing DNA hybridizing strongly to the 23-mer probe (pMTS1) were digested with *Acc*I and *Hin*dIII, filled in with deoxynucleoside triphosphates (dNTPs) and Klenow fragment, ligated into the *Sma*I site of pBluescript with T-4 DNA ligase, and transformed into *E. coli* XL1-Blue for sequencing (pMTS5). The 2.2-kb *Acc*I-*Hin*dIII fragment in pMTS5 was sequenced by using M-13 reverse, M-13 universal (U.S. Biochemical Corp., Cleveland, Ohio), and T-7 promoter (Promega, Madison, Wis.) primers and synthetic oligonucleotides. The synthetic oligonucleotides were selected from the published HD-1 *cryIIA* sequence (27). DNA was sequenced by using the dideoxy chain termination method (21), utilizing [α-^{35}S]dATP and the Sequenase DNA sequencing kit (U.S. Biochemical Corp.) (8).

Gene expression and inclusion body purification. To increase gene expression, the 2.2-kb filled-in *Acc*I-*Hin*dIII fragment in pBluescript was digested with *Xba*I and *Hin*dIII and ligated into the *Xba*I and *Hin*dIII sites of pT7-5 (pMTS6) and transformed into BL21 (λDE3). BL21 (λDE3) containing pMTS6 was grown in LB liquid medium with ampicillin at 50 μg/ml for 48 h. Refractile inclusion bodies were observed with a stereomicroscope after 24 h. Isolation and purification of insoluble inclusion bodies were performed as described by Masson et al. (13). The total protein concentration of CryIIA inclusion bodies was analyzed by using the Micro BCA Protein Assay (Pierce Chemical Co., Rockford, Ill.).

Protein and immunological analysis. Protein compositions of purified parasporal inclusion bodies, *E. coli* cells, and *E. coli* inclusion bodies were determined by SDS-PAGE. For Western blot (immunoblot) analysis, purified parasporal inclusion bodies and purified *E. coli* inclusion bodies were analyzed by SDS-PAGE. After completion, one duplicate was silver stained by using the Silver Stain-DPC kit (Integrated Separation Systems, Hyde Park, Mass.), and the other duplicate was transferred onto nitrocellulose (Micron Separations, Inc.) and incubated with an HD-1 P2 antibody supplied by G. Couche (Abbott Laboratories) (20).

Shuttle vector construction and *Bacillus* transformation. The 4.0-kb *Bam*HI-*Hin*dIII DNA fragment containing the *cryIIA* operon was excised from pMTS1 and ligated into the *Hin*dIII and *Bam*HI sites of pBluescriptII KS (pMAU1a). A 4.0-kb *Kpn*I-*Xba*I fragment containing the *cryIIA* operon was excised from pMAU1a and inserted into the *Kpn*I and *Xba*I sites of pHT3101 (pMAU1), which was confirmed by Southern blot hybridization using the 2.2-kb *Acc*I-*Hin*dIII fragment as a probe as described above. Transformation of *Bacillus* spp. with pMAU1 was performed with an electroporation apparatus from Bio-Rad Laboratories (Richmond, Calif.) by using the procedures described by Masson et al. (12). The resulting transformation mix was plated onto nutrient agar plates containing 50 μg of erythromycin per ml and incubated at 37°C for 3 days. Confirmation of pMAU1 transformation and expression was performed by observing cuboidal inclusions with a stereomicroscope. Additionally, purified inclusion bodies from these transformed bacilli were compared by using SDS-PAGE with the naturally occurring CryIIA protein of HD-1, the CryIIA protein expressed by the *cryIIA* gene in pMTS6, and the *cryIIA* operon in pT7-5 (pCL92; kindly provided by T. Cottrell, Auburn University) in *E. coli*.

Insect bioassays. Seven to nine concentrations of purified

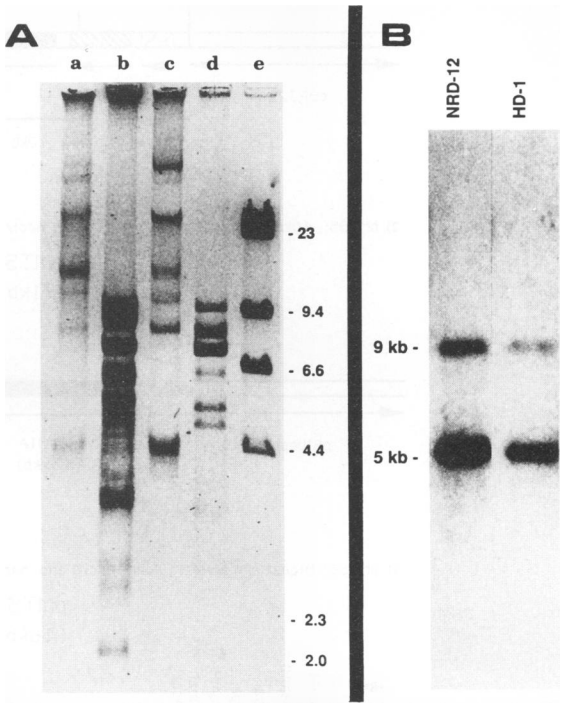

FIG. 1. (A) Undigested and *Hin*dIII-digested plasmid DNA of NRD-12 and HD-1. Lanes: a, undigested NRD-12; b, *Hin*dIII-digested NRD-12; c, undigested HD-1; d, *Hin*dIII-digested HD-1; e, *Hin*dIII-digested phage lambda DNA. (B) Hybridization of a ^{32}P-labelled 2.2-kb *Acc*I-*Hin*dIII fragment containing the NRD-12 *cryIIA* gene to *Hin*dIII-digested plasmid DNA from NRD-12 and HD-1. Hybridization was done at 68°C.

inclusion bodies were tested for each insect species. Toxin was added to 4 to 8 ml of artificial diet (18), and the mixture was poured into each well of a 16-well subset of a 128-well plastic tray for each concentration. One or two neonate *Spodoptera exigua* (Hübner), *Trichoplusia ni* (Hübner), or *Heliothis virescens* (F.) were placed into each well and incubated at 27 ± 1°C with a light-dark period of 16 and 8 h, respectively. Mortality was checked at 5 days for *T. ni* and *H. virescens* and at 7 days for *S. exigua*. There were 16 to 32 insects evaluated per concentration, and each treatment was replicated at least three times.

Mosquito bioassays using the purified inclusion bodies were performed with second-instar *Culex quinquefasciatus* (Say). Inclusion body preparations were suspended in water and placed into either 30-ml plastic cups or 24-well microtiter plates. Four to eight concentrations were used, and each concentration was replicated two to four times. Mortality was recorded at 48 h.

Lyophilized spore-crystal preparations of HD-1, acrystalliferous HD-1, BT-8, and HD-1 and BT-8 containing pMAU1 were prepared as described by Moar et al. (17). These preparations were bioassayed against *H. virescens*, *T. ni*, *Helicoverpa zea* (Boddie), and *S. exigua* as described above. All treatments were replicated four times.

Data were analyzed by using probit analysis (22) after correction for control mortality with Abbott's formula (1). Values from individual replicates were pooled. Control mortality was <10%.

RESULTS

Protein purification and sequencing. The first eight amino acids sequenced from the N terminus of native NRD-12 cuboidal crystal protein were identical to the CryIIA N-terminal amino acid sequence from HD-263 (NH_2-Met-Asn-Asn-Val-Leu-Asn-Ser-Gly) (4). Therefore, a synthetic oligonucleotide was designed from the first eight amino acids to use as a probe to identify the *cryIIA* gene from NRD-12, namely, 5′-ATG AAT AAT GTA TTG AAT AGT GG-3′.

Plasmid library and DNA hybridization. Although the plasmid profiles of NRD-12 and HD-1 were similar, they did not appear to be identical, especially after *Hin*dIII digestion (Fig. 1). This is not surprising because our HD-1 isolate has already been shown to be lacking the *cryIA*(b) gene which is located on a 5.3-kb *Hin*dIII fragment present in the HD-1-S-1971 standard (17, 28). This 2.2-kb *cryIIA* probe hybridized to both a 5.0-kb and a 9.0-kb DNA fragment in NRD-12 and HD-1 (Fig. 1). The *cryIIA* gene of HD-1 and HD-263 has been reported to be on a 5-kb *Hin*dIII-digested fragment (4, 27), and a 9-kb *Hin*dIII-digested fragment from HD-1 was reported to contain

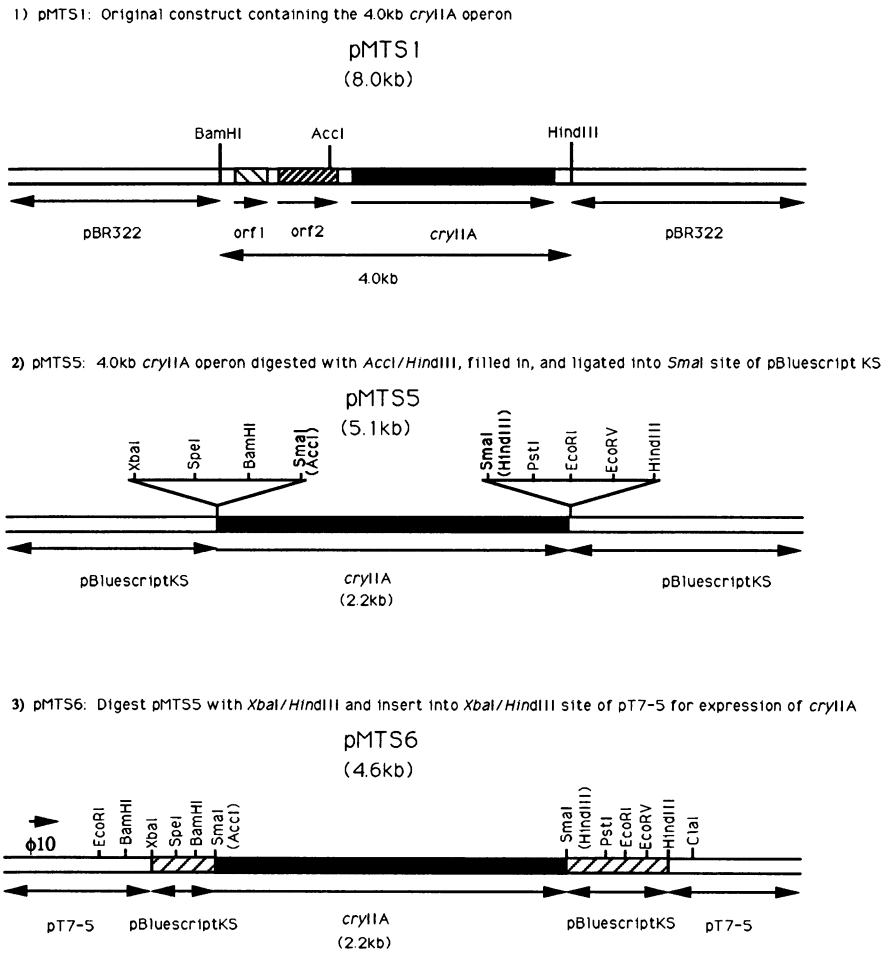

FIG. 2. Construction of pMTS plasmid series for cloning, sequencing, and expressing the *cryIIA* gene of *B. thuringiensis* subsp. *kurstaki* NRD-12.

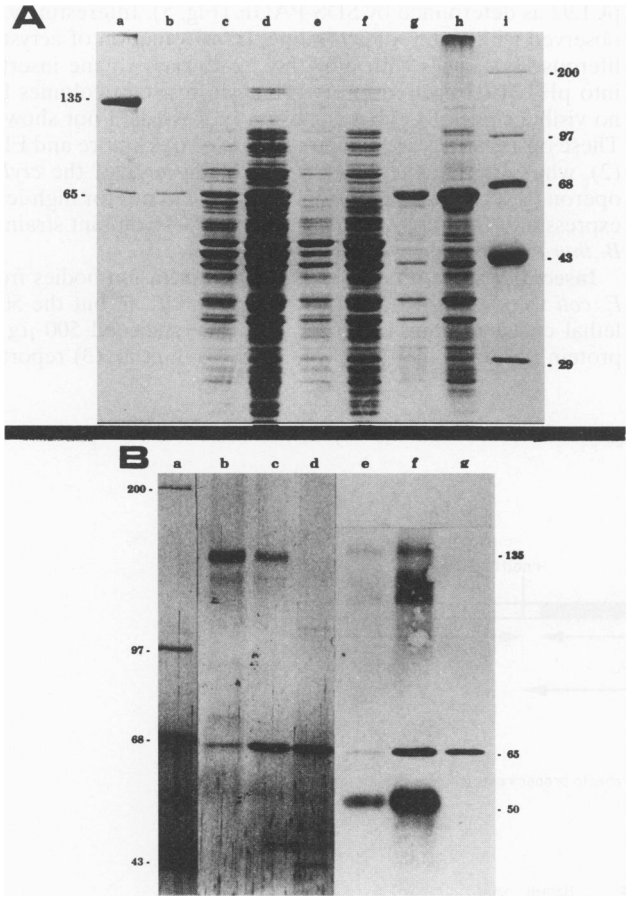

FIG. 3. (A) SDS-polyacrylamide gel of native and *E. coli*-expressed NRD-12 proteins stained with Coomassie brilliant blue. Lanes: a, NRD-12 P1 crystal protein; b, NRD-12 P2 crystal protein; c and d, *E. coli* BL21 (λDE3) harboring pMTS6 expressing NRD-12 CryIIA (low and high concentrations, respectively); e and f, *E. coli* BL21 (λDE3) harboring pT7-5 (low and high concentrations, respectively); g and h, low and high concentrations, respectively, of purified NRD-12 CryIIA protein inclusion bodies expressed from *E. coli* BL21 (λDE3) harboring pMTS6; i, molecular mass standards (in kilodaltons). (B) Electrophoretic analysis of native and *E. coli*-expressed NRD-12 P2 proteins. Lanes: a to d, SDS-polyacrylamide gel stained with silver; e to g, corresponding Western blot probed with HD-1 P2-specific antibody; a, molecular mass standards (in kilodaltons); b and e, NRD-12 P1 crystal protein; c and f, NRD-12 P2 crystal protein; d and g, purified NRD-12 CryIIA protein inclusion bodies expressed from *E. coli* BL21 (λDE3) harboring pMTS6.

a gene 89% homologous to *cryIIA* (*cryIIB*). The encoded protein CryIIB is 87% homologous to CryIIA that is lepidopteran active but not mosquitocidal (27).

A *Bam*HI-*Hin*dIII library was screened by using the *cryIIA* probe to identify the gene encoding the NRD-12 CryIIA protein because Widner and Whiteley (27) reported that a 4.0-kb *Bam*HI-*Hin*dIII fragment was located within a 5-kb *Hin*dIII fragment. Because Donovan et al. (4) reported only four clones hybridizing to their *cryIIA* probe from screening 1,000 colonies, colony hybridization was performed to screen for the NRD-12 *cryIIA* gene. Colony hybridization of the NRD-12 *cryIIA* *Bam*HI-*Hin*dIII library with [γ-^{32}P]ATP-labelled 23-mer (*cryIIA* probe) resulted in one strongly hybridizing colony (pMTS1) out of ca. 600 clones even after rinsing under stringent conditions at 50°C.

pMTS1 was digested with *Bam*HI, *Hin*dIII, and various other restriction endonucleases and analyzed on a 0.7% agarose gel. Analysis of the agarose gel showed that when pMTS1 was digested with either *Bam*HI or *Hin*dIII, a linearized approximately 8-kb fragment could be observed as was expected for a 4.0-kb insert and the 4.0-kb pBR322 vector. The DNA in the agarose gel was transferred onto nitrocellulose and incubated with the *cryIIA* probe. This Southern blot revealed that both the linearized *Bam*HI and *Hin*dIII fragments as well as the 4.0-kb *Bam*HI-*Hin*dIII fragment hybridized strongly to the *cryIIA* probe (data not shown). Digestion with *Acc*I and *Hin*dIII resulted in an approximately 2.2-kb *Acc*I-*Hin*dIII fragment that also hybridized strongly to the 23-mer probe (data not shown). Because Donovan et al. (4) demonstrated that the coding region of the *cryIIA* gene is located within this 2.2-kb fragment, this fragment was filled in with dNTPs and Klenow fragment, subcloned into the *Sma*I site of pBluescript KS (pMTS5) (Fig. 2), and sequenced.

DNA sequencing. Initially, pMTS5 was used to determine the nucleotide sequence of the *cryIIA* gene. Preliminary results of the nucleotide sequence of the *cryIIA* gene of NRD-12 showed that it was identical to that of HD-263 and HD-1 (4, 27). Therefore, DNA sequencing was expedited by synthesizing several small oligonucleotides (17 to 18 bases) selected from the published HD-1 *cryIIA* sequence (27). One available subclone containing a *Bgl*II-*Bam*HI insert ligated into pUC13 also was sequenced with M-13 reverse and M-13 universal primers.

The sequence of the NRD-12 *cryIIA* gene and its flanking regions are identical to that of HD-1 and HD-263 (data not shown) (4, 7, 27). This gene encodes a protein with a molecular mass of approximately 71 kDa.

Gene expression and inclusion body purification. For maximal protein expression, the 2.2-kb *Acc*I-*Hin*dIII fragment from NRD-12 was cut from pBluescript with *Bam*HI and *Xba*I, ligated into the *Bam*HI-*Xba*I site adjacent to the T7 promoter region of pT7-5 (pMTS6), and transformed into BL21 (λDE3) (Fig. 2). BL21 (λDE3) contains the T7 RNA polymerase gene, which recognizes the ϕ10 gene promoter present on pT7-5. Although this T7 polymerase gene is under control of the *lac* promoter, it still is expressed constitutively at low levels. Constitutive expression of the CryIIA protein was very high when this vector was used, and inclusion bodies containing the CryIIA protein could be seen under the light microscope within 24 h.

Protein and immunological analysis. When native NRD-12 CryIIA crystal proteins, *E. coli* cells expressing CryIIA, and purified CryIIA inclusion bodies were analyzed by SDS-PAGE, a distinct 65-kDa band corresponding to the CryIIA protein appeared in all lanes except for those lanes containing BL21 (λDE3) and pT7-5 only (Fig. 3).

To verify the expression of the CryIIA protein, a Western blot and N-terminal amino acid sequencing analysis were performed. The 65-kDa protein reacted very strongly to the HD-1 CryIIA antibody (Fig. 3). The HD-1 antibody also reacted strongly to an approximately 50-kDa protein in the native NRD-12 P2 protein lane and less strongly to the NRD-12 P1 protein lane (Fig. 3). Widner and Whiteley (27) reported that the gene encoding this protein also was contained in the 5.0-kb *Hin*dIII fragment from HD-1 (*orf2*), but this gene occurred primarily upstream of the *Acc*I site. However, this evidence again suggests that the NRD-12 and HD-1 isolates are very similar.

Sequencing the N terminus of the *E. coli*-expressed 65-kDa protein resulted in the first eight amino acids being identical to the amino acid sequence from the native NRD-12 P2 crystal

protein and the deduced amino acid sequence from the nucleotide sequence (data not shown).

Shuttle vector construction and *Bacillus* transformation. The 4.0-kb *cryIIA* operon was inserted into the *E. coli-B. thuringiensis* shuttle vector pHT3101 (pMAU1; Fig. 4), as confirmed by Southern blot analysis (data not shown). pMAU1 was inserted into competent *Bacillus* sp. cells by electroporation. Erythromycin-resistant colonies were selected for further observation. Colonies had growth characteristics similar to those of the wild-type parents, were erythromycin resistant, and contained inclusion bodies similar in shape to native CryIIA toxins. The proteins produced by HD-1 and BT-8 transformed with pMAU-1 were similar in molecular mass to the proteins produced by *E. coli* transformed with pMTS6 and pCL92 as determined by SDS-PAGE (Fig. 5). Interestingly, as observed with a stereomicroscope, transformation of acrystaliferous HD-1 cells with only the 2.2-kb *cryIIA* gene inserted into pHT3101 resulted in erythromycin-resistant colonies but no visible signs of CryIIA inclusion bodies (data not shown). These observations are similar to those of Crickmore and Ellar (2), who reported the requirement of the *orf2* of the *cryIIA* operon (directly upstream from the *cryIIA* gene) for high-level expression of the *cryIIA* gene in the IPS78/11 mutant strain of *B. thuringiensis* subsp. *israelensis*.

Insect bioassays. Purified NRD-12 P2 inclusion bodies from *E. coli* were quite toxic to *H. virescens* and *T. ni*, but the 50% lethal concentration (LC_{50}) for *S. exigua* exceeded 500 μg of protein per ml of diet (Table 1). Dankocsik et al. (3) reported

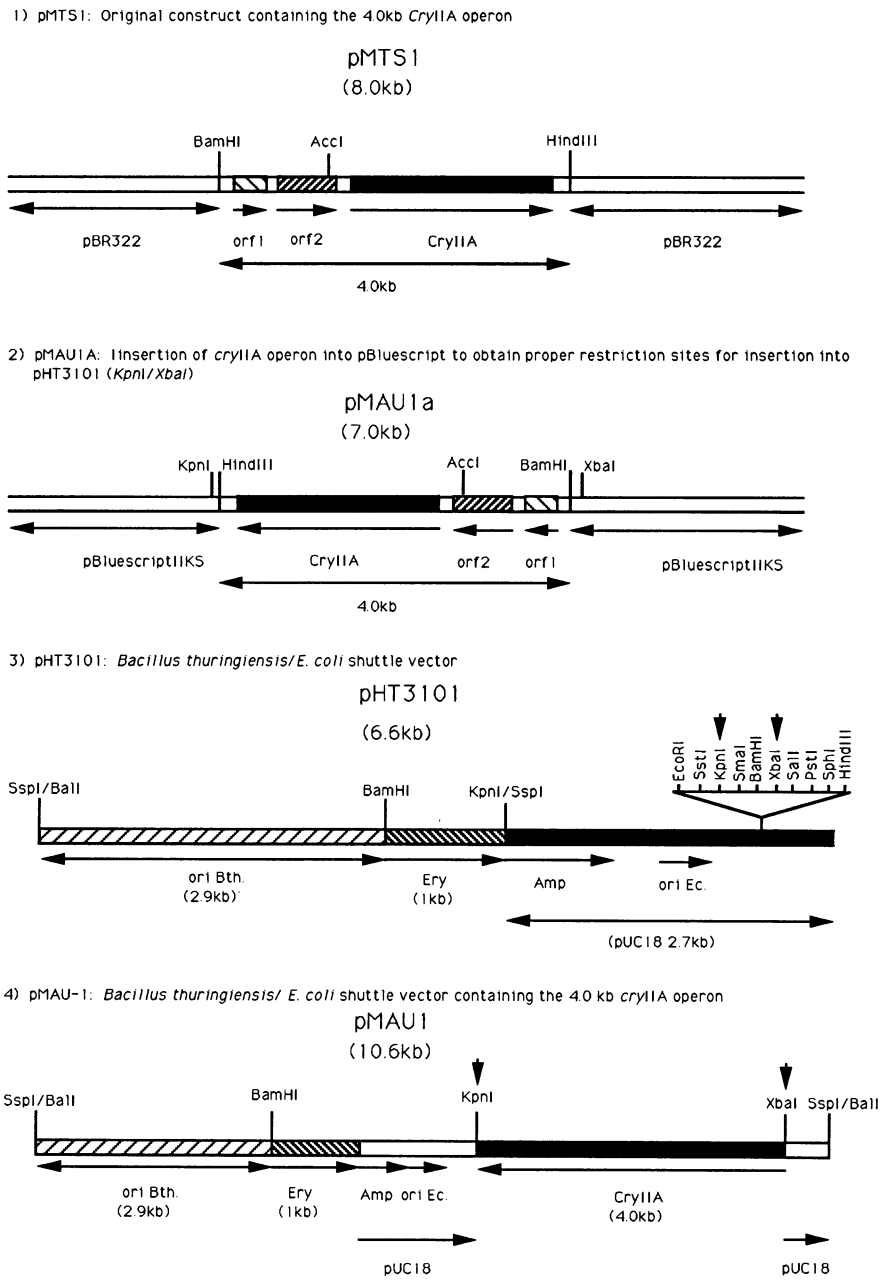

FIG. 4. Construction of pMAU1 for expressing the *cryIIA* gene of *B. thuringiensis* subsp. *kurstaki* NRD-12 in *Bacillus* spp.

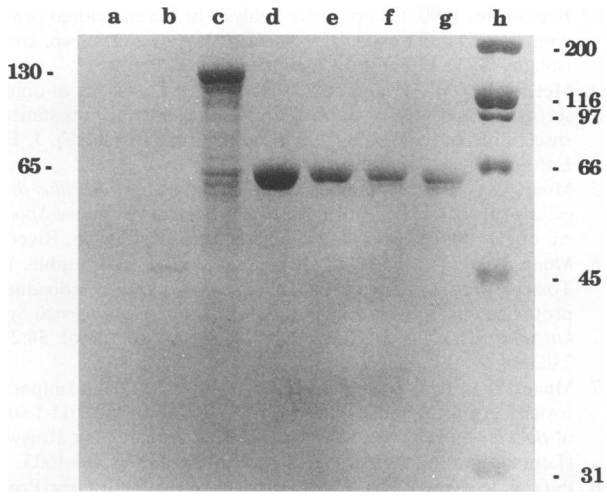

FIG. 5. SDS-polyacrylamide gel of native cultures and cultures expressing the NRD-12 CryIIA protein from *E. coli*, *B. thuringiensis* subsp. *kurstaki* HD-1, and *B. cereus* BT-8. Lanes: a, acrystalliferous HD-1; b, BT-8; c, HD-1; d, HD-1 containing pMAU1; e, BT-8 containing pMAU1; f, purified NRD-12 CryIIA protein inclusion bodies from *E. coli* expressing the 2.2-kb *cryIIA* gene; g, purified NRD-12 CryIIA protein inclusion bodies from *E. coli* expressing the 4.0-kb CryIIA operon; h, molecular mass standards (in kilodaltons).

similar trends in toxicity for *H. virescens* and *T. ni* by using *Bacillus megaterium* as a host for the HD-1 CryIIA protein. A comparison of the CryIIA toxicity data against *S. exigua* with native P2 crystal proteins from both HD-1 and NRD-12 (17) shows an approximately 10-fold reduction in activity for the *E. coli*-expressed CryIIA. Research is needed to determine whether a more toxic protein(s) is produced when this gene is expressed in *B. thuringiensis* containing the full plasmid complement. Donovan et al. (4) found that CryIIA-transformed *B. megaterium* was much more toxic to *H. virescens* and *Lymantria dispar* (L.) than the *E. coli*-expressed CryIIA, but because cells were used for the bioassay instead of purified protein, it is not clear whether this variation in toxicity was due to higher levels of CryIIA expression, changes in the protein structure, or other components associated with *B. megaterium*.

Bioassays using the purified NRD-12 CryIIA inclusion bodies were also conducted against second-instar *C. quinquefasciatus* and confirm that the NRD-12 CryIIA is also mosquitocidal, with an LC_{50} of 1.63 μg/ml. These results are in agreement with those of Widner and Whiteley (27).

Lyophilized spore-crystal preparations of HD-1, acrystalliferous HD-1, BT-8, and HD-1 and BT-8 containing pMAU1 were bioassayed against four insect species in diet incorporation tests. The acrystalliferous HD-1 isolate and BT-8 were nontoxic (≤12% mortality) to *H. virescens*, *T. ni*, *H. zea*, and *S. exigua* even at the highest concentration tested (100 μg of toxin per g of diet; Table 2). Isolates containing pMAU1 were toxic to *H. virescens* and *T. ni*, and there was no difference in toxicity against any insect between either isolate containing pMAU1. However, HD-1 was substantially more toxic against *H. virescens*, *T. ni*, and *H. zea* than either HD-1 containing pMAU1 or BT-8 containing pMAU1. These results agree with those of MacIntosh et al. (11) and Moar et al. (16), who demonstrated that CryIA(c) was toxic to *H. virescens* and *T. ni* at concentrations of less than 1 μg/g of diet. No preparation was even moderately toxic to *S. exigua*.

TABLE 1. Toxicity of *E. coli*-expressed CryIIA protein from *B. thuringiensis* subsp. *kurstaki* NRD-12 against *H. virescens*, *T. ni*, and *S. exigua*

Insect	n^a	Slope (SE)	LC_{50} (95% FL)[b]
H. virescens	1,070	2.39 (0.24)	0.56 (0.45–0.68)
T. ni	1,134	3.47 (0.20)	4.3 (4.0–4.63)
S. exigua	292	ND[c]	>500

[a] Total number of insects assayed.
[b] LC_{50} values are expressed in micrograms of total protein per gram of diet. Pooled data of three to four replicates are shown. FL, fiducial limits.
[c] ND, not determined.

TABLE 2. Toxicity of *Bacillus* sp. strains transformed with the *cryIIA* gene of *B. thuringiensis* subsp. *kurstaki* NRD-12 against *H. virescens*, *T. ni*, *H. zea*, and *S. exigua*[a]

Isolate	LC_{50} (95% FL)[b]	
	H. virescens	*T. ni*
HD-1 Cry(⁻) (*B. thuringiensis*)	>100	>100
BT-8 (*B. cereus*)	>100	>100
HD-1	1.15 (0.54–1.98)	3.15 (0.58–9.47)
HD-1/pMAU1	22.1 (14.3–38.1)	30.9 (20.9–53.5)
BT-8/pMAU1	32.1 (16.0–89)	44.3 (18.8–248)

[a] For *H. zea* and *S. exigua*, ≤20% mortality at the highest concentration tested (100 μg/g of diet) was observed, except for HD-1 against *H. zea*, which showed 63% mortality at a concentration of 100 μg/g of diet.
[b] LC_{50} values are expressed in micrograms of spore-crystal preparation per gram of diet. Pooled data of four replicates are shown. FL, fiducial limits.

DISCUSSION

The NRD-12 isolate contains a *cryIIA* gene identical to *cryIIA* genes in HD-1 and HD-263, further substantiating the relatedness between NRD-12 and HD-1. The CryIIA protein is toxic to both lepidopterans and dipterans. *H. virescens* was shown to be quite sensitive to CryIIA and, therefore, may be a candidate for targeting insect control efforts utilizing CryIIA. Additionally, because full-length CryIIA is approximately one-half the size of full-length CryIA proteins, its use in transgenic plants may be justified. CryIIA also may have some utility in *B. thuringiensis* resistance management because McGaughey and Johnson (14) reported no cross-resistance with certain isolates of *B. thuringiensis* against *Plodia interpunctella* (Hübner), which contained nonhomologous toxin proteins compared with HD-1. CryIIA is a nonhomologous Cry toxin compared with CryIA because CryIIA shares only 39% homology along a 100-amino-acid segment of CryIA(a) from HD-1 (4, 27). Tabashnik et al. (24) reported that the diamondback moth *Plutella xylostella* (L.), which was highly resistant to *B. thuringiensis* subsp. *kurstaki* and CryIA proteins, exhibited only minor cross-resistance to CryIIA. However, Gould et al. (5) reported substantial cross-resistance to CryIIA by *H. virescens*, which was selected for resistance to CryIA(c). Clearly, these results suggest that utilization of CryIIA for resistance management should proceed on an individual species basis.

We have demonstrated that CryIIA proteins can be expressed in both *B. thuringiensis* and *B. cereus*. Because many of the bacteria being evaluated for phylloplane use in the field against plant pathogens are *B. cereus* isolates, this research should help establish the range of possible transformations utilizing *cryIIA* and the pHT3101 shuttle vector. Although the overall toxicity of the *B. cereus* isolate BT-8 containing pMAU1

was low in relation to that of HD-1 and purified CryIIA, large populations of leaf-inhabiting BT-8 producing CryIIA may be sufficient to control certain leaf-feeding insects. Additionally, various constructs containing stronger promoters, *B. thuringiensis* δ-endotoxin genes encoding for proteins with enhanced activity against leaf-feeding insects such as *Spodoptera* spp., and a higher plasmid copy number could result in phylloplane-inhabiting bacteria with increased insecticidal activity.

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