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Characterization of Cry34/Cry35 Binary Insecticidal Proteins from Diverse *Bacillus thuringiensis* Strain Collections

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Bacillus thuringiensis **crystal proteins of the Cry34 and Cry35 classes function as binary toxins showing activity on the western corn rootworm,** ***Diabrotica virgifera virgifera*** **LeConte. We surveyed 6,499** ***B. thuringiensis*** **isolates by hybridization for sequences related to** ***cry35A*** **genes, identifying 78 strains. Proteins of the appropriate molecular mass (ca. 44 kDa) for Cry35 were observed in 42 of the strains. Full-length, or nearly full-length, sequences of 34** ***cry34*** **genes and 16** ***cry35*** **genes were also obtained from cloning, PCR analysis, and DNA sequencing. These included representatives of all known Cry34A, Cry34B, Cry35A, and Cry35B classes, as well as a novel Cry34A/Cry35A-like pair. Bioassay analysis indicated that** ***cry35*****-hybridizing strains not producing a ca. 14-kDa protein, indicative of Cry34, were not active on corn rootworms, and that the previously identified Cry34A/Cry35A pairs were more active than the Cry34B/Cry35B pairs. The** ***cry35*****-hybridizing** ***B. thuringiensis*** **strains were found in locales and materials typical for other** ***B. thuringiensis*** **strains. Comparison of the sequences with the geographic origins of the strains showed that identical, or nearly identical, sequences were found in strains from both Australasia and the Americas. Sequence similarity searches revealed that Cry34 proteins are similar to predicted proteins in** ***Photorhabdus luminescens*** **and** ***Dictyostelium discoidium*****, and that Cry35Ab1 contains a segment similar to beta-trefoil domains that may be a binding motif. The binary Cry34/Cry35** ***B. thuringiensis*** **crystal proteins thus appear closely related to each other, are environmentally ubiquitous, and share sequence similarities consistent with activity through membrane disruption in target organisms.**

Bacillus thuringiensis is a spore-forming bacterium that produces proteinaceous crystals during sporulation which are typically insecticidal, although crystals with activities on other invertebrates and crystals having no detected biological activity have also been reported (10, 19). Several *B. thuringiensis* insecticidal crystal proteins (ICPs) have been developed and commercialized as sprayable biopesticides and transgenic plant-incorporated protectants for agricultural applications. Because of this commercial value, numerous *B. thuringiensis* strain collections have been generated as sources of ICPs with improved or novel activities (29). Various isolates of this bacterium have been obtained worldwide by many investigators from a large number of habitats, including insectaries, many soil types, the phyloplane, grain products and grain processing facilities, aquatic habitats, and animal feces (13, 20, 25, 31).

The strains in any particular collection may be characterized in a number of ways, such as growth physiology, flagellar serotyping, profiling plasmid arrays or proteins, the use of monoclonal antibodies, and hybridization or PCR amplification based on sequences of known crystal protein genes (28, 29). There is only a poor correlation between any one of these characterization methods and the insecticidal activity of a particular strain for a number of reasons, including the presence of multiple genes per strain, variable gene families in a given serotype, differing expression levels of the genes present, and relative solubility in the insect midgut (11, 28). In surveys of several *B. thuringiensis* collections for a number of crystal protein genes, 40 to 50% of the strains either have lepidopteran activity or contain *cry1* genes (6, 25, 28, 29), which is perhaps a slightly higher level of representation than that of the approximately 40 *cry1* genes among the more than 130 distinct crystal protein sequences known (8). Studies of the frequency of non-*cry1* genes, or of strains having no hybridization with any of the tested probes (suggesting novel sequences), have not provided consistent results due to greater variability and lower frequency of such strains and genes in those studies (28).

The ca. 14-kDa Cry34A and ca. 44-kDa Cry35A proteins are insecticidal proteins from *B. thuringiensis* that function together in the intoxication and control of the western corn rootworm (WCR), *Diabrotica virgifera virgifera* LeConte (14, 18). Because both proteins are required for effective mortality of the insects, they have been referred to as binary insecticidal proteins. Soluble preparations of Cry34 and Cry35 proteins may be obtained using 40% NaBr or mildly acidic buffers, because the alkaline and reducing conditions that solubilize many other crystal proteins were ineffective (27; unpublished data). Synthetic genes encoding one binary pair, Cry34Ab1 and Cry35Ab1, have been engineered for expression in corn plants,

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TABLE 1. Wild-type *B. thuringiensis* strains used in this study

Isolate	NRRL reference	Cry34 accession no.	Cry34 designation	Cry35 accession no.	Cry34 designation	Reference or source
PS80JJ1	B-18679	AAG50341	Cry34Aa1	AAG50342	Cry35Aa1	14
PS149B1	B-21553	AAG41671	Cry34Ab1	AAG41672	Cry35Ab1	14
PS167H2	B-21554	AAG50118	Cry34Ac1	AAG50117	Cry35Ac1	14
PS69Q	B-30175	AY536899	Cry34Aa3	AY536895	Cry35Aa3	This study
PS185GG	B-30175	AY536897	Cry34Aa4	AY536892	Cry35Aa4	This study
KR1369	B-30200	AY536896	Cry34Ac3	AY536891	Cry35Ab3	This study
PS187G1	B-30185	AY552563		AY552583		This study
PS187Y2	B-30187	AY552565				This study
PS201G	B-30188	AY552566				This study
PS201HH2	B-30190	AY536898	Cry34Ba3	AY536893	Cry35Ba3	This study
PS242K10	B-30195	AY552575		AY552581		This study
KB54A1-6	B-30197	AY552577				This study
KR589	B-30198	AY552579		AY552580		This study
PS185L12	B-30179	AY552559				This study
PS185W3	B-30180	AY552560				This study
PS187L14	B-30186	AY552564		AY552582		This study
PS186FF	B-30182	AY552561				This study
PS131W2	B-30176	AY552554		AY552587		This study
PS158T3	B-30177	AY552556		AY552586		This study
PS158X10	B-30178	AY552557				This study
PS185FF	B-30182	AY552558		AY552585		This study
PS187F3	B-30184	AY552561		AY552584		This study
PS201L3	B-30189	AY536900	Cry34Ba2	AY536894	Cry35Ba2	This study
PS204C3	B-30191	AY552569				This study
PS204G4	B-18685	AY552570				This study
PS204I11	B-30192	AY552571				This study
PS204J7	B-30193	AY552572				This study
PS236B6	B-30194	AY552574				This study
PS246P42	B-30196	AY552576				This study
KR1209	B-30199	AY552578				This study
PS137A		AY552555				
PS201V2		AY552567				
PS203J1		AY552568				
PS207C3		AY552573				

and they provide high levels of protection to corn roots under typical growing conditions (26).

As an extension of our work on Cry34 and Cry35 proteins, we screened *B. thuringiensis* collections for strains having related sequences in the hopes of finding more active proteins or proteins sufficiently different in sequence that they might provide resistance management options due to novel molecular interactions in the insect. An additional aspect of this work was that we would also obtain information about the diversity and origin of sequences related to Cry34 and Cry35 and the frequency of *B. thuringiensis* strains having such genes in our collections.

MATERIALS AND METHODS

Bacterial isolates and culture conditions. The *B. thuringiensis* strains screened in this study were from the Dow AgroSciences strain collection. They included large and representative proportions of the collection described by Feitelson et al. (16), originating from a multicontinent geographic area, and two other collections, obtained independently, that were predominantly of Asian origin. A number of the Cry34- and Cry35-containing *B. thuringiensis* strains identified in this study as well as several recombinant *B. thuringiensis* strains expressing cloned *cry34* and *cry35* genes have been deposited with the Northern Regional Research Laboratory (NRRL), as noted in Tables 1 and 2.

Shake flask cultures of *B. thuringiensis* were grown to sporulation and lysis at 30°C for 72 h in NYS/CAA medium, pH 7.2, containing (per liter) 1.25 g of nutrient broth, 1.25 g of Bacto tryptone, 2 g of Casamino Acids, 0.5 g of yeast extract, and 10 ml of *B. thuringiensis* salt solution, which consisted of (per liter) 40.7 g of $MgCl_2 \cdot 6H_2O$, 20 g of $CaCl_2 \cdot 2H_2O$, 2 g of $MnCl_2 \cdot 4H_2O$, 0.04 g of $FeSO_4 \cdot 7H_2O$, 0.04 g of $ZnSO_4 \cdot H_2O$, and 0.04 g of $(NH_4)_2SO_4$, all in 14 mM HCl. Biomass containing sporulated cells and crystalline protein inclusions was harvested from the cultures by centrifugation at 10,000 × *g* for 20 min. Recombinant *B. thuringiensis* strains were prepared similarly in NYS/CAA medium supplemented with erythromycin (10 μg/ml).

ICP preparation and quantitation. For wild-type *B. thuringiensis* strains, whole-culture pellets were washed twice with the original culture volume of distilled water and collected by centrifugation as described above. The washed pellet was resuspended to 1/10th its original culture volume in extraction buffer (100 mM sodium citrate, pH 3.8), homogenized, and incubated for 2.5 h on a rocker platform at 4°C. Cell debris was removed by centrifugation at 26,000 × *g* for 30 min, and the supernatant containing soluble proteins was retained. For bioassays, the supernatant was dialyzed against 20 mM sodium citrate buffer, pH 5.5, to equilibrate the acidity of the sample with the insect diet. The dialyzed protein concentration was determined according to Bradford (5) using bovine serum albumin (BSA) as a standard. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (14) on 4 to 12% gradient Bis-Tris NuPAGE gels (Invitrogen) at 200 V for 50

TABLE 2. Recombinant *B. thuringiensis* strains used in this study

Isolate	Host (plasmid)	Binary toxin genes	Reference or source
Cry-B	Acrystalliferous cloning host	None	32
MR529	Cry-B(pHT370)	None	This study
MR539	Cry-B(pMYC2369)	None	This study
MR543	Cry-B(pMYC2426)	*cry34Aa1*/*cry35Aa1*	14
MR544	Cry-B(pMYC2427)	*cry34Ab1*/*cry35Ab1*	26
MR546	Cry-B(pMYC2429)	*cry34Ac1*/*cry35Ac1*	14
MR561	Cry-B(pMYC2476)	*cry34Ba2*/*cry35Ba2*	This study
MR562	Cry-B(pMYC2477)	*cry34Ba3*/*cry35Ba3*	This study

min. Proteins were visualized by staining with Gelcode Blue Stain G-250 (Pierce). For protein quantitation, laser-scanning gel densitometry was performed with a Molecular Dynamics Personal Densitometer SI with BSA as a standard.

For recombinant *B. thuringiensis* clones, whole-culture pellets were washed and resuspended in water. Proteins were visualized, and the concentration of binary ICPs was determined by densitometry as described above. Preparations were adjusted to 1 mg of binary ICP/ml and serially diluted for quantitative bioassays.

Bioassays. Methods for testing insecticidal activity of binary ICPs against western corn rootworm neonates in surface-applied bioassays were previously described (14).

Genomic DNA hybridization. Total genomic DNA from each *B. thuringiensis* isolate was prepared using the QIAGEN DNEasy kit. DNA in 96-well plates was denatured prior to blotting by diluting 10 μl of DNA solution and 10 μl of 4 M NaOH in 80 μl of sterile distilled water. Samples were incubated at 70°C for 1 h, after which 100 μl of 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (recipes for all DNA hybridization solutions are found in reference 21) was added to each well. Total genomic DNA from the Cry34Ab1/Cry35Ab1 source strain PS149B1 was included with each set of 94 samples as a positive hybridization control for *cry35Ab1*, and CryB total genomic DNA was included with each set of 94 samples as a negative hybridization control. Each complete set of 96 samples was applied to Magnacharge nylon membranes using two 48-well slot blot manifolds (Hoefer Scientific), followed by two washes with 10× SSC. Membranes were baked at 80°C for 1 h and kept dry until used. Membranes were prehybridized and hybridized in standard formamide solution, which contained 50% formamide, 5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.7]), 5× Denhardt's solution, 2% SDS, and 100 μg of single-stranded DNA/ml, at 42°C. Membranes were probed with an approximately 1.3-kbp DNA fragment that included the entire *cry35Ab1* coding sequence amplified by PCR from genomic DNA of PS149B1. The DNA probe was amplified by PCR, using as the forward primer 5′-ATGYTWGATACWAATA AAGTWTATGAAAT-3′ and as the reverse primer 5′-GTAGAAGCAGAAC AAGAAGGTATT-3′, corresponding to nucleotide coordinates 784 to 812 and 2106 to 2129, respectively, of GenBank accession no. AYO11120. The probe was radioactively labeled using the Prime-it II kit (Stratagene) and [^{32}P]dCTP, purified on Sephadex columns, denatured at 94°C, and added to fresh hybridization solution. Membranes were washed under two conditions: 2× SSC–0.1% SDS at 42°C (low stringency) and 0.2× SSC–0.1% SDS at 65°C (moderate to high stringency). Strains containing genes with homology to the *cry35Ab1* probe were identified by autoradiography.

PCR and DNA sequencing. Degenerate oligonucleotides for PCR were designed to amplify polynucleotides corresponding to open reading frame positions 1 to 341 of *cry34Ab1* and 1 to 1103 of *cry35Ab1* from *B. thuringiensis* strains identified by hybridization. The oligonucleotides are homologous to conserved sequence blocks identified by alignment of previously described *cry34A* and *cry35A* genes (14, 26). Forward primers for both genes were designed to begin at the ATG initiation codon. Reverse primers were designed from sequences as close to the 3′ end of each respective gene as possible. The sequence of the *cry34* forward primer was 5′-ATG TCA GCW CGY GAA GTW CAY ATT G-3′, while the sequence of the *cry34* reverse primer was 5′-GTY TGA ATH GTA TAH GTH ACA TG-3′. The sequence of the *cry35* forward primer was 5′-ATG TTA GAT ACW AAT AAA RTW TAT G-3′, and the sequence of the *cry35* reverse primer was 5′-GTW ATT TCT TCW ACT TCT TCA TAH GAA G-3′. PCR products were fractionated on 1% agarose gels and purified from the gel matrix using the QiaexII kit (QIAGEN). Purified DNA fragments were cloned using the TOPO TA kit (Invitrogen). Cloned PCR-derived fragments were then sequenced using Applied Biosystems automated sequencing systems and associated software.

Gene cloning and coexpression. The *cry34* and *cry35* genes from strains PS149B1, PS167H2, and PS80JJ1 were obtained previously (14), and the related genes from PS69Q, PS185GG, and PS201L3 were isolated using the same methods. The related genes from PS187G1, PS201HH2, and KR1369 were obtained using cosmid libraries constructed in the SuperCos1 vector (Stratagene), having inserts prepared by partial digestion with NdeII. XL1-Blue MR cells (Stratagene) were transfected with the packaged cosmids to obtain clones resistant to carbenicillin and kanamycin and were screened by hybridization with a *cry35*-specific probe generated from each strain produced as described above. Standard methods were used to subclone *cry34*/*cry35* operons into pHT370 (2) for expression of the Cry34 and Cry35 proteins in *B. thuringiensis*, as previously described (14). Acrystalliferous strain Cry-B was transformed with the pHT370-derived expression plasmids by electroporation as described for coexpression of binary ICPs from native *B. thuringiensis* promoters (14).

Sequence comparisons. Sequence similarity searches were performed using the PSI-BLAST (1) facility available at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/BLAST/) during January 2004. Searches were performed against the nonredundant databases using default parameters. Sequences of the Cry34 and Cry35 proteins were aligned using ClustalW, and unweighted paired groupwise mean averaging (UPGMA) dendrograms were generated as described previously (7). For this analysis, the less than full-length sequences resulting from cloned PCR fragments were trimmed to remove the common, conserved primer sequences, because the sequence hybridizing with the primer may not represent the amplified gene and amplification of certain combinations of redundant bases in the primers may have resulted in nonnative sequence. For Cry34 proteins, this trimming results in sequences about 75 to 81% of full length (missing the 8 N-terminally and 9 C-terminally located conserved residues and a 6- to 15-residue variable C-terminus segment). The trimmed Cry35 sequences (missing the 9 N-terminally and 7 C-terminally located conserved residues and a 15- to 17-residue variable C-terminus segment) are about 90% of full length.

Nucleotide sequence accession numbers. Nucleotide sequence accession numbers are listed in Table 1.

RESULTS

Distribution of binary insecticidal crystal genes and proteins in *B. thuringiensis* strain collections. Out of 6,499 *B. thuringiensis* strains from our collections, slot blot hybridization analysis of *B. thuringiensis* total genomic DNA using a full-length *cry35Ab1* gene probe was used to identify 78 strains that contained sequences putatively related to *cry35Ab1*. The *cry35Ab1* probe had previously been shown to hybridize to related *cry35* genes but not to other known classes of *cry* genes or to genomic *B. thuringiensis* DNA that were included as controls (data not shown).

These hybridizing strains were subsequently examined for the presence of proteins similar in size and solubility to Cry34 and Cry35 (14). Proteins were extracted from sporulated culture pellets by solubilization in mildly acidic buffer (pH 3.8) and analyzed by SDS-PAGE for the presence of ca. 14- and 44-kDa proteins. Under the culture conditions used in this study, 42 of the 78 hybridizing strains produced proteins of 40 to 44 kDa. Of these, 29 strains produced both 14- and 44-kDa proteins (Fig. 1, lanes 2 to 11, Fig. 2, lanes 2 to 17, and Fig. 3, lanes 2 and 9 to 12), while 11 strains produced proteins of 40 to 44 kDa in mass but lacked a copurifying 14-kDa protein (Fig. 1, lane 12; Fig. 3, lanes 3 to 8 and 13 to 16). Data for two additional strains are not shown. Further, several strains produced additional proteins, mostly greater than 70 kDa in mass, which were not examined further. Some prominent examples of these are found in lanes 7, 9, and 11 of Fig. 1, lanes 3 and 12 to 14 of Fig. 2, and lanes 9, 10, and 12 of Fig. 3. Strains producing Cry34B seem to consistently produce such proteins. The presence of additional crystal proteins has also been visualized by standard SDS-PAGE extraction and analysis of Cry34- and Cry35-producing strains by us (14, 27, and unpublished data) and by Baum et al. (3).

Protein preparations containing both 14- and 44-kDa protein species demonstrated various degrees of activity on WCR neonates, as summarized in Fig. 1 to 3. Preparations from strains that contained Cry34A-type ICPs as determined by DNA sequencing (see below) appeared generally more active toward WCR neonates than preparations from strains containing Cry34B-type ICPs. Further, no WCR activity was observed for those protein preparations containing a 40- to 44-kDa pro-

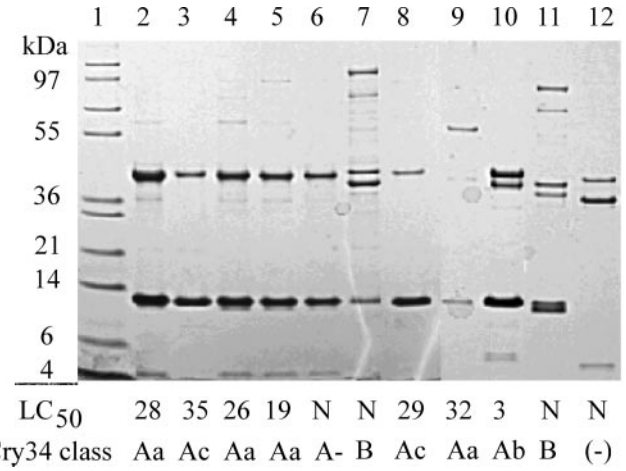

FIG. 1. Presence of binary ICPs in *B. thuringiensis* isolates identified by *cry35* nucleic acid hybridization. Lanes: 1, molecular mass markers; 2, PS69Q; 3, PS167H2; 4, PS185FF; 5, PS185GG; 6, PS187G1; 7, PS201L3; 8, PS242K10; 9, PS80JJ1; 10, PS149B1; 11, PS204G4; and 12, KB65A15-8. The 50% lethal concentration (LC_{50}) (in micrograms per squared centimeters) or N (indicating a dose response could not be calculated) is indicated below each lane. A− denotes sequences that would be a new tertiary rank, if complete. A (−) indicates the absence of a 14-kDa protein.

tein species but were lacking a 14-kDa protein (Fig. 1, lane 12; Fig. 3, lanes 3 to 8 and 13 to 16).

Operon cloning, expression, and insecticidal activity of Cry34B/Cry35B ICPs. Because the Cry34B/Cry35B subfamily is the most divergent among the binary ICPs described to date, we chose to examine representatives from this phylogenic group in more detail. Genes encoding two examples of Cry34B/Cry35B ICPs were cloned from strains PS201L3 and PS201HH2 and completely sequenced. Comparison of full-length holotype binary ICPs revealed that the deduced Cry34 protein sequences clustered with about 50% amino acid sequence identity, while the Cry35 proteins clustered with about 60% amino acid sequence identity (see Fig. 5 and 6).

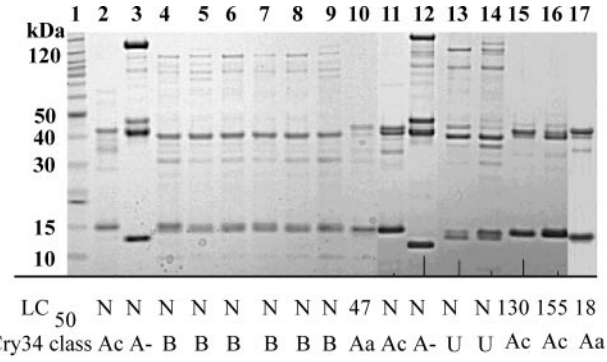

FIG. 2. Presence of binary ICPs in *B. thuringiensis* isolates identified by *cry35* nucleic acid hybridization. Lanes: 1, molecular mass markers; 2, PS158T3; 3, PS187G1; 4, PS201G; 5, PS201HH2; 6, PS204C3; 7, PS204J7; 8, PS204I11; 9, PS236B6; 10, PS246P42; 11, PS158X10; 12, PS187F3; 13, PS201H2; 14, PS203G2; 15, KR589; 16, KR1209; and 17, PS131W2. The 50% lethal concentration (LC_{50}) (in micrograms per squared centimeters) or N (indicating a dose response could not be calculated) is indicated below each lane. U indicates an undetermined *cry34* allele sequence. A− denotes sequences that would be a new tertiary rank, if complete.

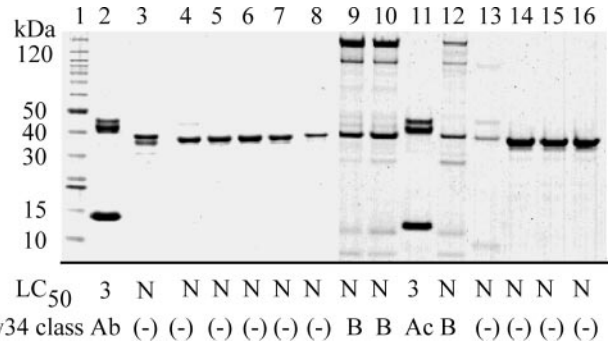

FIG. 3. Presence of binary ICPs in *B. thuringiensis* isolates identified by *cry35* nucleic acid hybridization. Lanes: 1, molecular mass markers; 2, PS149B1; 3, PS223L2; 4, KB10H-5; 5, KB59A54-4; 6, KB59A54-5; 7, KB59A58-4; 8, KB65A14-1; 9, PS201V2; 10, PS207C3; 11, KR1369; 12, PS137A; 13, PS147U2; 14, KB65A15-2; 15, KB65A15-3; and 16, KB65A15-7. The 50% lethal concentration (LC_{50}) (in micrograms per squared centimeters) or N (indicating a dose response could not be calculated) is indicated below each lane. A (−) indicates the absence of a 14-kDa protein.

To assess bioactivity of Cry34B/Cry35B ICPs, the PS201L3 and PS201HH2 operons encoding these proteins were subcloned into the shuttle vector pHT370 and transformed into acrystalliferous *B. thuringiensis* strain Cry-B to enable ICP expression from the native promoters. Recombinant *B. thuringiensis* strains MR561 and MR562, encoding Cry34Ba2/Cry35Ba2 and Cry34Ba3/Cry35Ba3, respectively, both produced crystalline inclusions comprised of ca. 14- and 44-kDa proteins when grown to sporulation (Fig. 4). Unfractionated protein preparations from recombinant strains expressing representative Cry34A/Cry35A and Cry34B/Cry35B binary ICPs were tested for activity against WCR neonates in surface-applied bioassays. As noted for the *B. thuringiensis* strains expressing Cry34B/Cry35B, the cloned, expressed Cry34B/Cry35B binary ICPs were much less active than Cry34A/Cry35A binary ICPs in comparative assays (Table 3).

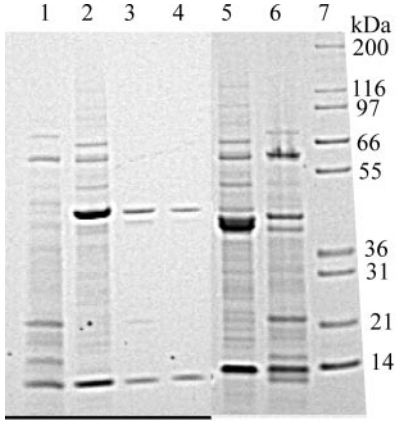

FIG. 4. SDS-PAGE analysis of coexpressed recombinant binary ICPs. Lanes: 1, MR529 negative control; 2, Cry34Aa1/Cry35Aa1; 3, Cry34Ac1/Cry35Ac1; 4,Cry34Ab1/Cry35Ab1; 5, Cry34Ba2/Cry35Ba2; 6, Cry34Ba3/Cry35Ba3; and 7, molecular mass markers.

TABLE 3. Activity of coexpressed recombinant binary ICPs

Bacterial strain	Binary ICP	LC_{50} (μg/cm^2)	% Mortality[a] (highest dosage, in μg/cm^2)
MR543	Cry34Aa1/Cry35Aa1	34 (21–130)[b]	
MR544	Cry34Ab1/Cry35Ab1	3 (2–5)	
MR546	Cry34Ac1/Cry35Ac1	7 (3–44)	
MR561	Cry34a2B/Cry35Ba2	NDR[c]	55 (110)
MR562	Cry34Ba3/Cry35Ba3	NDR	44 (170)
MR529 (negative control)	None	NDR	

[a] The effects of treatments for which the 50% lethal concentration (LC_{50}) could not be calculated are expressed in percent mortality at the top dose.
[b] 95% Confidence interval values are in parentheses.
[c] NDR, dose response could not be calculated.

Sequence diversity of binary ICP genes. We obtained sequence information from a number of the *cry34*- and *cry35*-hybridizing strains to assess the sequence diversity and geographic distribution of the various members of this binary toxin family. To determine the relatedness of binary ICP sequences among the hybridizing strains, nearly full-length genes corresponding to nucleotides 1 to 341 of *cry34Ab1* and 1 to 1103 of *cry35Ab1* were amplified by PCR. Sequences of 34 *cry34* genes were obtained from 29 of the 42 expressing strains (including the two expressing strains not shown in Fig. 1 to 3) and 5 nonexpressing strains. Note that the 11 expressing strains lacking a 14-kDa protein did not yield PCR products with either the *cry34* or *cry35* oligonucleotide primers used in this study, and two strains producing Cry34 proteins were not sequenced. We sequenced 16 *cry35* genes from strains having *cry34* sequences, including one nonexpressing strain. The nearly full-length PCR-generated sequences (identified by strain designations in Fig. 5 and 6) were N- and C-terminally truncated to remove sequences corresponding to the primers and were compared to the full-length sequences of several of the genes also obtained by standard genomic cloning. Analysis of the deduced polypeptide sequences was performed essentially as described previously (7) such that percent identity (or difference) is calculated based on the shorter sequence, allowing reasonable comparison of sequences of differing lengths. The analysis includes the sequences reported by Ellis et al. (14) and Rupar et al. (30), which have previous nomenclature assignments. The results revealed that the 34 Cry34 and 16 Cry35 sequences analyzed here form distinct families, as shown in Fig. 5 and 6. For the Cry34 (Fig. 5) sequences, nearly all classes of proteins have multiple identical sequences obtained from distinct bacterial isolates; for example, 8 instances for Cry34Aa and 11 instances for Cry34Ba. A similar pattern holds for Cry35 (Fig. 6), although in this case there are fewer identical sequences. For sequence novelty, aside from the more divergent Cry34B and Cry35B sequences, the truncated PCR-derived sequences of PS187G1 and PS187F3 are Cry34A-like and Cry35A-like sequences that would qualify for a new tertiary rank (7), being 89% identical to Cry34Aa and 87% identical to Cry35Aa. By contrast to the striking uniformity of the Cry34Aa and Cry35Ba sequences, there is more sequence variation of the Cry34Ab, Cry34Ac, Cry35Ab, and Cry35Ac sequences from roughly 93% or greater identity. Additionally, Cry34Ab1 and Cry35Ac1 appear to be the more divergent members of

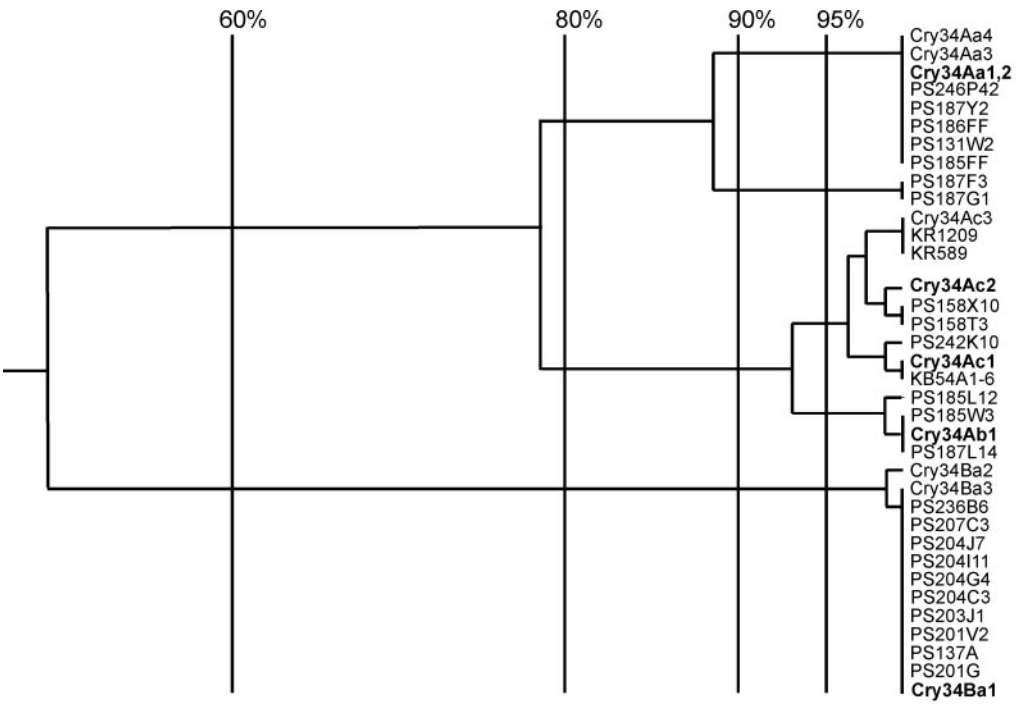

FIG. 5. Dendrogram showing the relatedness of Cry34 sequences. Vertical bars indicate the percent sequence identity on the dendrogram. Sequences with nomenclature assignments are full length (previously published assignments are in boldface); PCR-amplified subsequences are indicated by strain (see Materials and Methods for details).

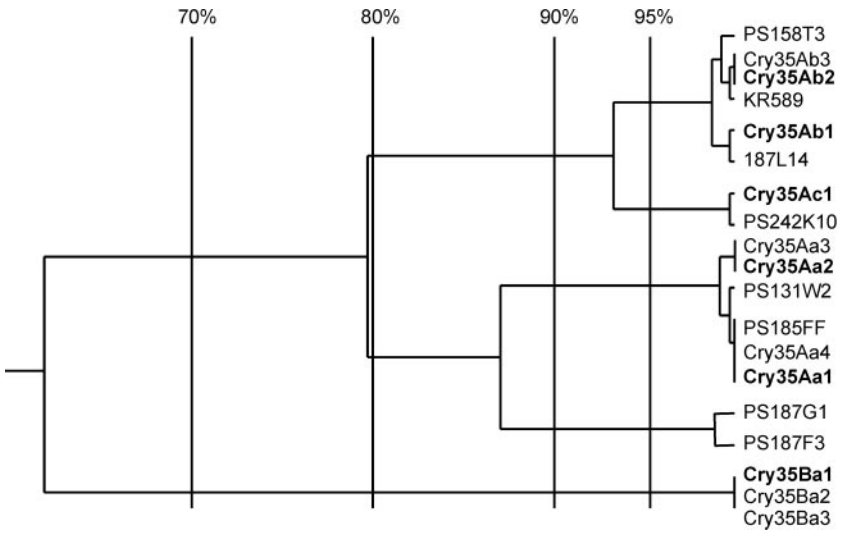

FIG. 6. Dendrogram showing the relatedness of Cry35 sequences. Vertical bars indicate the percent sequence identity on the dendrogram. Sequences with nomenclature assignments are full length (previously published assignments are in boldface); PCR-amplified subsequences are indicated by strain (see Materials and Methods for details).

these groups, so that in most cases *cry34Ac*-type genes are in operons with *cry35Ab*-type genes.

Geographic distribution of *cry34* and *cry35*. The hybridization screening reported here encompassed three *B. thuringiensis* collections obtained by different individuals from samples with differing geographic distributions. The rates of recovery of Cry35-hybridizing strains in the three collections were 0.5, 1.4, and 1.9%, for an overall rate of 1.2%. The strains containing the *cry34* and *cry35* genes were isolated from several continents (North and South America, Australia, and Asia). Table 4 summarizes the general distribution of strains containing the Cry34 sequences shown in Fig. 5. For Cry34Aa and Cry34Ba, the same sequence is found in *B. thuringiensis* strains isolated in multiple locations on essentially opposite sides of the world. A similar pattern may hold for the Cry34Ab and Cry34Ac sequences; however, there are fewer identical sequences.

The materials from which the strains containing *cry34* and/or *cry35* were isolated included dust, soil, leaf litter, nematodes (15), and insects. In most cases the materials were from an agricultural setting either inside or outside of structures. In two cases, however, the habitats were not associated with human activity. Therefore, *cry34* and *cry35* genes appear to have a broad habitat distribution in nature and are found in materials and environments that are typical for *B. thuringiensis* (31).

TABLE 4. Geographic distribution of different classes of Cry34 protein

Protein class	Sequence similarity (%)	Continent(s)	No. of geographically distinct areas[a]
Cry34Aa	100	Australia and Asia	2
		North America	2[b]
Cry34Ab/c	>93	Australia and Asia	4
		North America	1
		South America	1
Cry34Ba	>98	Australia and Asia	1
		North America	2

[a] Separate large geographic regions of at least several hundred miles or those separated by bodies of water.
[b] Includes information from Rupar et al. (30).

Flagellar serotyping was attempted, essentially by the method of de Barjac (9), on 15 of the *cry34*- and *cry35*-containing strains. Of these, 10 were not testable because they were not motile. Of the motile strains, three were serotype 4 (sotto or kenyae), one was subtyped to serovar 4a,4b (sotto), and one was serotype 5 (galleriae or canadensis). Except for one serotype 4 strain for which no sequence information is available, the motile strains were all Cry34Aa-expressing strains.

Sequence relatedness to other proteins. As noted previously, the Cry35 proteins are similar in sequence to the mosquitocidal *B. sphaericus* BinA and BinB proteins (14). Also included in this family is the Cry36 protein, which is a 58-kDa polypeptide homologous to Cry35 proteins over C-terminally located ca. 280 residues, and it is as closely related to the Bin proteins as it is to the Cry35 proteins (8, 10). A conserved domain search (22, 23) using Cry35Ab1 as a query also revealed that the N-terminal 146 residues of this protein contain two repeats of the tripartite beta-trefoil carbohydrate-binding domain (17), including two signature QxW motifs. Figure 7 shows the location and sequences of the homology of Cry35Ab1 to the beta-trefoil consensus.

Using Cry34Ab1 as a query sequence, the BLAST program (1) returns significant similarity values for other Cry34 proteins and additional non-*B. thuringiensis* proteins (Fig. 8). A hypothetical protein from *Dictyostelium discoidium* (GenBank accession no. AAO52140.1) is 1,107 residues in length and contains a conserved domain for the endonuclease/exonuclease/phosphatase family, which includes magnesium-dependent endonucleases and phosphatases involved in intracellular signaling from about residue 550 to 750. The Cry34 homology is in two blocks: residues 336 to 452 of AAO52140 align to

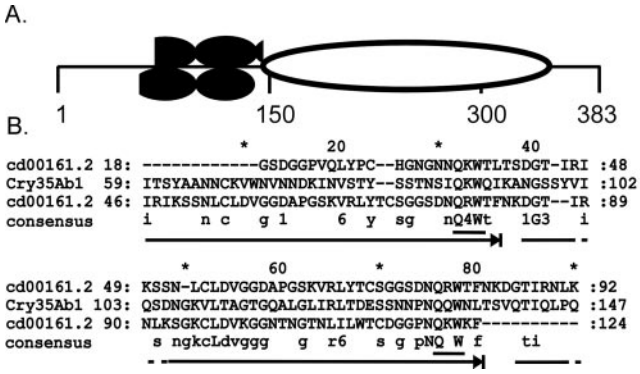

FIG. 7. Conserved domain search for Cry35Ab1. (A) The Cry35Ab1 protein sequence is indicated by a horizontal line, with blackened ovals indicating the position of two partial alignments of the tripartate beta-trefoil domain similarity (Conserved Domain Database [CDD] entry cd00161) and a white oval indicating a conserved domain based on the *B. sphaericus* 42-kDa mosquitocidal toxin (CDD entry pfam05431). (B) Multiple-sequence alignment showing two possible alignments of the cd00161 beta-trefoil consensus sequence against the Cry35Ab1 sequence. The QxW motifs of the beta-trefoil sequence are underlined below the consensus line. Lines with arrows denote the internal repeat sequences in the beta-trefoil domain. Consensus similarity groups are the following: 1-D,N; 3-S,T; 4-K,R; 6-L,I,V,M.

residues 3 to 118 of Cry34Aa1 at 27% identity, and residues 15 to 76 of AAO52140 align to residues 2 to 63 of Cry34Aa1 at 31% identity. The overall E value is $\sim 10^{-7}$. Two additional proteins related to Cry34 are from *Photorhabdus luminescens* (12) and may be detected using iterated PSI-BLAST (1). Both exhibit E values below the 0.005 significance cutoff. Plu1537 (GenBank accession no. NP_928828) is a 136-residue polypeptide that is 27% identical to Cry34Ab1. Plu2230 (GenBank accession no. NP_929487) is a 465-residue polypeptide that has a segment homologous to Cry34 near the N terminus and a more C-terminal segment again containing a phosphatase-like module.

DISCUSSION

Identification and characteristics of strains containing *cry34* and *cry35* genes. One objective of this study was to identify additional *B. thuringiensis* strains containing *cry34* and *cry35* genes in a broad survey of our *B. thuringiensis* collection. The use of a hybridization-based method allowed this to be done quickly and inexpensively. We anticipated that this method would allow the identification of genes having 45 to 50% sequence identity with the initial genes and without the strict sequence dependence of 3′ priming sites required for PCR-based analysis (28). The fact that the *cry34B* and *cry35B* genes were identified, having 50 to 60% sequence identity, and that some additional hybridizing strains clearly produced 44-kDa proteins but failed in PCR amplification with the primers described here, indicates that the general expectations of the screening strategy were met.

Of the strains identified by hybridization, less than half produced proteins of the expected size and extraction properties. It may be that somewhat different growth or extraction conditions would have allowed the identification of more strains producing proteins of the correct size; however, the presence of genes is known to not necessarily correlate with activity (28), and there are numerous reasons why Cry34/Cry35 proteins were not identified from some hybridizing strains. It is notable that we have *cry34* sequence information on five of the strains that did not produce readily identifiable 14-kDa proteins, reinforcing the greater frequency for detecting genes than expressed proteins. A number of *cry35*-hybridizing strains also produced 44-kDa proteins without the presence of a 14-kDa protein, and they failed in amplification attempts with either *cry34*-specific or *cry35*-specific PCR primers. Because these strains failed to give a clear dose response in our corn rootworm assays, they were not pursued further; however, they may contain yet more distant relatives of the *cry35* gene family. Compared to the previously described Cry34A/Cry35A binary toxins (14), corn rootworm bioassays also indicated that the Cry34B/Cry35B combination was much less active. This conclusion differs from that of Baum et al. (3), who found roughly similar activity for the Cry34A/Cry35A and Cry34B/Cry35B toxins. However, there were several differences in the assays, including weight reduction versus mortality-based scoring of the assays and the use of purified crystals versus either soluble or whole-culture materials, that may account for the different conclusions.

A number of the *cry35*-hybridizing strains producing 14- and/or 44-kDa proteins also produced proteins of other sizes. The identity of these proteins was not determined; however, we noted previously (14) that Cry34/Cry35Aa-producing strain PS80JJ1 also produces the Cry14Aa protein. A comparison of the hybridization screening results with a survey of the same collection for lepidopteran-active *cry9*-like genes showed that six strains were common to the two surveys. Further genotyping of these six strains by restriction analysis of gene-class-specific PCR products revealed the presence of some other common *Bacillus* insecticidal protein genes (*cry1*, *cry2*, *cry9*, and *vip3*; data not shown). While none of the identified strains was among the strains producing the 14- and/or 44-kDa proteins shown in Fig. 1 to 3, *cry34*/*cry35Ab*-like sequences were obtained from one of them: strain PS187L14 (Fig. 5 and 6). Thus, it is possible to find *B. thuringiensis* strains that contain genes for several well-known lepidopteran ICPs and corn rootworm binary ICPs.

It has been noted previously that a number of *B. thuringiensis* crystal protein genes having identical sequences have been found to have a worldwide distribution (7). In the present study, multiple, identical sequences were found for the Cry34 proteins, with Cry34Aa and Cry34Ba having numerous representatives. A similar trend seems likely for the smaller number of Cry35 sequences presented, although the proportion of identical sequences seems lower than that for Cry34, which may partly be due to the use of PCR in some cases and the larger in vivo mutational target of the *cry35* genes. As indicated in Table 4, the three major groupings of Cry34 sequences each contained representatives from strains obtained from widely separated geographies. The discovery of strains containing *cry34* and *cry35* at several locations in Australasia is in agreement with the conclusion of Martin and Travers (25) that this area is a rich source of *B. thuringiensis* strains and reinforces the notion made earlier (24) that the broad environmental distribution of *B. thuringiensis* strains was not related to specific insect targets, because western corn rootworm is presently

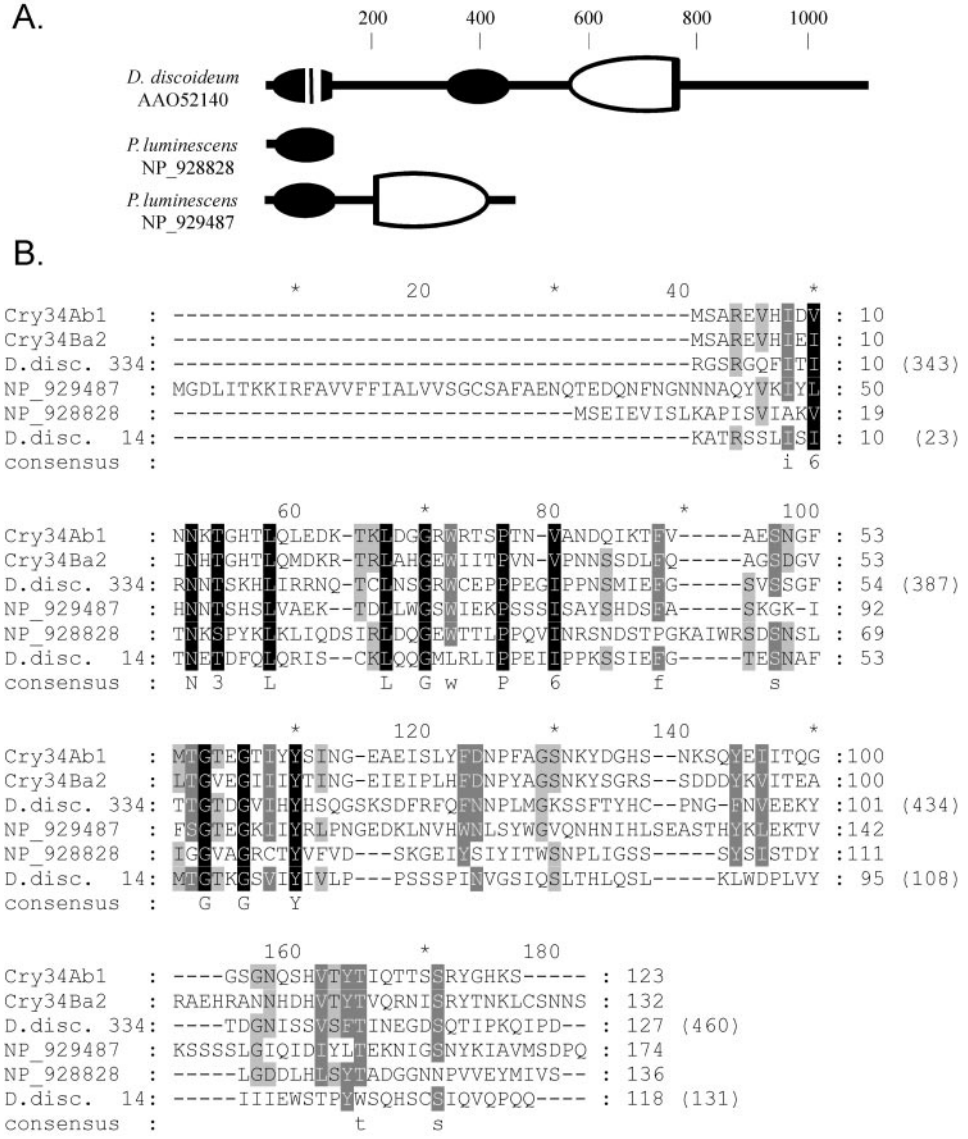

FIG. 8. Sequence homologies to Cry34. (A) Non-*Bacillus* sequences in GenBank showing homology to Cry34Ab1 in PSI-BLAST searches. Source organisms and protein accession numbers are indicated. The protein sequence is indicated by a horizontal line, with blackened ovals indicating the position of Cry34 similarity; significant gaps are indicated by segmentation, and white ovals indicating a phosphatase conserved domain (CDD entries COG5555, KOG1378, and KOG2679) for NP_929487 and a endonuclease/exonuclease/phosphatase domain (CDD entry pfam03372) for AAO52140. Truncated ovals indicate incomplete alignment of the conserved domain to the respective sequence. (B) Multiple-sequence alignment of unique Cry34 sequences and homologues. Black highlighting indicates conserved identity or similarity across all sequences, and dark and light gray shading indicates lesser degrees of conservation among the sequences. Consensus similarity groups are the following: 3-S,T and 6-L,I,V,M. The two different alignments with the *D. discoidium* sequence (accession no. AAO52140) are indicated by the start positions chosen for this alignment based on the PSI-BLAST alignment.

found only in North America and places in eastern Europe, where it was recently introduced. Taken together with the relatively typical materials from which the strains reported here were obtained, this indicates that *B. thuringiensis* strains containing genes related to *cry34* and *cry35* are not unusual in terms of geographic distribution or habitat.

Frequency of strains containing genes related to *cry34* and *cry35*. The overall rate of recovery of *cry35*-hybridizing strains was 1.2%, ranging from 0.5 to 1.9% for the three collections screened. The overall rate for strains from which Cry34 sequences were obtained was 0.52%, which is a more conservative indicator of the frequency of strains containing *cry34* (and *cry35*) in the collections. This compares with a roughly 40 to 50% frequency for *cry1* genes or common lepidopteran-active strains (6, 25, 28, 29).

As a point of comparison, PCR screening for a rarer yet familiar gene, *cry3A*, has also been reported in the literature. This gene and its corresponding beetle active protein have been registered in both sprayable *B. thuringiensis* and CellCap-killed recombinant insecticides as well as in transgenic potatoes. Ben-Dov et al. (4) found no *cry3*-containing strains, by PCR screening, among 215 environmental isolates (<0.5%). A

rate of 2.5% was reported for strains having the flat, square crystal morphology typical of Cry3Aa-containing strains of the 3,500 isolates in the International Entomopathogenic Bacillus Centre Collection at Institute Pasteur (19). Bravo et al. (6) found 6 *cry3A*-containing strains in a collection of 496 isolates (1.2%); however, they also screened for *cry3B* and *cry3C*, genes yielding an overall *cry3* rate of 5.6%. The latter figure would represent sequence diversity closer to the diversity of Cry34 and Cry35 sequences reported here.

Relatedness of Cry34 and Cry35 to other proteins. Sequence comparisons (8, 14) with other known *B. thuringiensis* insecticidal crystal proteins failed to reveal homology between Cry34 or Cry35 proteins with other previously described Cry, Cyt, or Vip insecticidal proteins. However, database searches revealed that Cry35 proteins are homologous, at 26 to 29% sequence identity, respectively, to the 42- and 51-kDa crystalline mosquitocidal proteins from *B. sphaericus* strain 2362 and share distinct blocks of conserved sequence homology with the *B. sphaericus* proteins (14). An extension of this search to conserved domain alignments (22, 23) using Cry35Ab1 as a query revealed segments of similarity to two different domains. Residues ~60 to ~147 are similar to the beta-trefoil, or QxW, domain typified by the binding subunit of ricin. This common carbohydrate-binding domain was formed by an apparent gene triplication, and the aligning segment of Cry35Ab1 represents two of the three triplicated elements (both the first or second two of the three elements align; Fig. 7A and B). Residues 146 through 348 are similar to a conserved domain P42, derived from the previously noted *B. sphaericus* 42- and 51-kDa proteins, other Cry35 proteins, and Cry36, a protein with some coleopteran activity that appears to be more related to the *B. sphaericus* proteins than to Cry35 (8, 30). Because of the close juxtaposition of the beta-trefoil and P42 conserved domain alignments, it seems more likely that a third beta-trefoil repeat would occur in the first 60 residues, despite the low sequence similarity, than in the P42 segment following residue 147.

Upon iterated PSI-BLAST searches of GenBank using Cry34 proteins as queries, some non-*Bacillus* proteins are also found with significant sequence similarity. One of the proteins is similar in size to the Cry34 proteins, but it is from the gram-negative insect pathogen *P. luminescens* (12). A second open reading frame in this organism encodes a protein of 465 residues having a segment of sequence similarity to the Cry34 proteins at its predicted N terminus and similarity to certain phosphatases near its predicted C terminus. A more surprising result comes from the eukaryotic organism *Dictyostelium discoidium*, where an 1,107-residue hypothetical protein encodes essentially complete and partial homologues of Cry34 and also a segment resembling magnesium-dependent endonucleases and phosphatases involved in intracellular signaling. The discovery of a homologue of a *B. thuringiensis* insect toxin in a gram-negative insect pathogen is somewhat unexpected, given the extensive study of other types of toxins in *P. luminescens*. However, the discovery of a homologue in *D. discoidium* and the presence of Cry34-like modules in putative proteins that also contain endonuclease and/or phosphatase modules is also intriguing. Because the only described role for Cry34-like polypeptides is as an insect toxin acting together with an unrelated protein, Cry35, it is interesting that Cry34-like modules are found covalently linked to additional functional domains. Whether the 136-amino-acid *P. luminescens* putative protein functions as an insect toxin will require experimental determination. However, the possible role of the Cry34-like modules in the longer *P. luminescens* and *D. discoidium* putative proteins is less clear. Because the *B. thuringiensis* binary toxins must interact with, and probably disrupt, membranes, it seems likely that the Cry34-like modules would perform some membrane anchoring or translocation function for the endonuclease and/or phosphatase modules in those proteins, whether they function as toxins, in cellular regulation, or in some other function.

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